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Textbook of Assisted Reproductive Techniques

Sixth Edition

Volume 1: Laboratory Perspectives

Edited by
DAVID K. GARDNER
ARIEL WEISSMAN
COLIN M. HOWLES
ZEEV SHOHAM

CRC Press
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Textbook of Assisted Reproductive Techniques

Established as the definitive reference for the IVF clinic, this Sixth Edition has been extensively revised, with the addition of several important new contributions on laboratory topics, including KPIs for the IVF laboratory, quality control in the cloud, artificial intelligence, AI in gamete and embryo selection, demystifying vitrification, microfluidics, gene editing, disaster management, enhanced imaging of early embryo development, and artificial gametes. As previously, methods, protocols, and techniques of choice are presented by IVF pioneers and eminent international experts.

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PREFACE

The first edition of the *Textbook of Assisted Reproductive Techniques* was published in 2001. As the textbook now enters its sixth edition, some 45 years since the birth of Louise Brown, the world's first test tube baby in the United Kingdom, it is remarkable to reflect upon the changes in assisted human conception that have been documented in each successive edition of the textbook.

Over the past 20 years, we have witnessed the widespread implementation of single blastocyst transfer, and the ability to undertake trophectoderm biopsy and genetic analysis now using next-generation sequencing to accurately determine chromosomal copy number, and to provide precise genetic diagnosis for patients as needed. This shift in practice of transferring only one high-quality embryo has brought us closer to the mantra of "one embryo, one baby." Cryopreservation, historically performed using slow-rate controlled freezing, has now been superseded by vitrification for both oocytes and embryos, with oocyte cryopreservation becoming a realistic treatment for fertility preservation, especially for oncology patients and younger women wishing to preserve their fertility. Improvements in laboratory culture techniques and incubation devices, including time-lapse imaging, have also contributed to the adoption of single embryo transfers without reducing the chance of a live birth. Excitingly, more technologies are now available for sperm assessment, and the knowledge underpinning *in vitro* maturation has facilitated the development of potential new approaches for IVF.

As for ovarian stimulation protocols, there has been, over the past 20 years of this textbook series, a major shift in practice. The clinical acceptance of the GnRH antagonist protocol, first registered in 1999, took more than 10 years to be widely adopted. With the possibility of using a GnRH agonist to trigger follicular maturation, the protocol has become the preferred choice, facilitating the concept of an "OHSS-free clinic." A plethora of new pharmaceutical FSH agents have been introduced into practice that have resulted in increased patient convenience and drug delivery precision (due to the use of pen devices) rather than increased live birth rates. This is a further reflection of the complexity of the overall IVF treatment process—in particular, the pivotal role that the embryology laboratory continues to play in improving cycle success.

Sadly, however, over the duration of this textbook's life span, we have lost several authors—all dear friends and colleagues—whom we miss and to whom we are grateful for their enormous contributions to our field during their lifetimes:

- Marinko Biljan, Quebec
- Isaac Blickstein, Rehovot
- Jean Cohen, Paris
- Howard W Jones Jr, Norfolk
- Michelle Lane, Adelaide
- Ragaa Mansour, Egypt
- Queenie V Neri, New York
- Lynette Scott, Boston
- Carl Wood, Melbourne
- Yury Velinsky, Chicago

Finally, we lost one of the pioneering fathers of this field, Bob Edwards, a giant in our field on whose shoulders we have all been fortunate to stand.

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UPDATED GUIDELINES FOR SETTING UP AN ASSISTED REPRODUCTIVE TECHNOLOGY LABORATORY

Jacques Cohen, Mina Alikani, and Antonia Gilligan

There are many ways to set up and operate assisted reproductive technology (ART) laboratories; one set-up may have little in common with another but prove to be equally successful. This is important to remember as one ventures into establishing a new clinic or open-laboratory ART system. Facilities for ART range from a makeshift *in vitro* fertilization (IVF) laboratory with a minimum of equipment to a fully equipped laboratory specifically designed for ART, sometimes with additional space dedicated to clinical training and research. There have been major changes in IVF and ART laboratories during the last decade with the introduction of robotics of embryo culture and cryopreservation of all embryos. More such changes are expected soon. The change to robotics of micromanipulation procedures such as intracytoplasmic sperm injection (ICSI), cryopreservation, cryo-storage, and gamete and embryo handling is requiring a change to highly technical and ever more specialized space. Some spaces may reduce in size because of automation and miniaturization. Other spaces such as cryo-storage are likely to increase due to the growing number of samples along with the potential use of relatively large robots for automated tracking during cryo-storage. This chapter does not cover gamete retrieval laboratories, which may incorporate retrieval and transport of gametes and embryos to other locations. Although such models can be successful, compelling evidence showing that they produce optimal results is still lacking [1, 2]. IVF, ICSI, and oocyte cryopreservation can be applied to transported oocytes, and in certain situations "transport IVF" is a welcome alternative for those patients whose reproductive options have been limited by restrictive governmental regulations [2, 3]. This chapter discusses the more typical purpose-built, all-inclusive laboratories that are adjacent or in close proximity to oocyte retrieval, cryo-storage, and embryo transfer facilities, with an emphasis on the special problems of construction. For choices of culture system, culture medium, supplementation, viability assays, handling and processing of gametes and embryos including freezing and vitrification, and cryo-storage, the reader is referred to other relevant chapters in this textbook. An international expert meeting on the construction, technical, and operational requirements for ART laboratory air quality established 50 consensus points regarding site suitability, design criteria for new construction, and laboratory commissioning [4]. This consensus meeting has provided standards for existing laboratories and guidelines for constructing new laboratories. The Cairo consensus meeting also proposed guidelines regarding current laboratory practice such as how to decrease volatile organic compounds in incubators and in the ambient space.

Personnel and experience

Although the environment, physical plant, and equipment require special consideration in the design of an integrated gamete and embryo culture facility, the staff will carry out the procedures and therefore is essential to the success of the entire operation. Successful clinical practice, in general, and ART, in particular, are almost entirely dependent on the skill and experience level of medical and laboratory personnel. Some planners look for a facility and location first, but staffing and caseload must be considered early on in the process. For the laboratory staff, enthusiasm is another key factor to success, especially because there are still few formal teaching and skills examination programs in place for a specialty in ART. Most clinical embryologists are trained using an apprenticeship program, but such institutions are rare and there are no internationally accepted guidelines. Non-apprentice hands-on training facilities are now offered in several locations in the USA, with one-on-one training options ranging from weeks to several months. This has been a huge step forward in assisting clinics to reduce or facilitate traditional in-house training. Good clinical outcome requires a cautious and rational assessment of individual abilities, so laboratory staff, directors, and embryologists must consider their experience in the context of what will be required of them [5, 6].

This chapter aims to provide information necessary for experienced practitioners to set up a new laboratory. Setting up a new laboratory or thoroughly renovating an existing facility is very much an art, as is the practice of ART itself. We do not recommend that new laboratories and ART clinics are built by administrators, engineers, or architects without considerable input from experienced embryologists, technical staff, and clinicians. Another consideration is to have input from existing laboratory staff rather than let outside consultants make all the decisions.

Programs should develop a system of tracking individual performances for crucial clinical and laboratory procedures such as embryo transfer efficiency, ICSI, and biopsy proficiency, among others. This is easily achievable using a conventional performance tracking system or digital record-keeping. Certain regulatory bodies such as the College of American Pathologists (CAP) in the USA and the Human Fertilisation and Embryology Authority (HFEA) in the UK provide guidelines and licensing for embryologists, sometimes even for subspecialties such as the performance of ICSI, the practice of embryo biopsy, and directing IVF and andrology laboratories. So far, such licensing has done little more than increase workload because licensing does not necessarily guarantee skill (or success) and the licenses may not be valid across borders.

Tradition also plays its role. For example, in some Asian countries, embryology directors are usually medical professionals. Thus, academic titles are often seen as being more important than actual qualifications. What then qualifies someone to be a laboratory director or an embryologist? The answer is not simple. In general, current licensing authorities, including the American Board of Bioanalysis (ABB), consider individuals trained in general pathology or reproductive medicine and holding an MD degree along with individuals holding a PhD degree qualified to

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be laboratory directors if they meet some other requirements. However, pathologists do not necessarily have experience in gamete and embryo cell culture, and some reproductive medicine specialists, such as urologists and immunologists, may have never worked with gametes and embryos. It is possible for a medical practitioner to direct a laboratory in certain countries without ever having practiced gamete and embryo handling! *Eppur si muove* ("And yet it moves"), as Galileo said when condemned by the Roman inquisition for the heresy of accepting Copernican astronomy. Once there are rules, even silly ones, it can be hard to change them.

Empirical and statistical requirements for staff

There is considerable disagreement about what should be the required experience for embryologists. Hands-on experience in all facets of clinical embryology is an absolute condition when starting a new program. Even highly experienced experimental embryologists and animal scientists should be directly supervised by experienced clinical personnel. The period during which close supervision must continue depends on the types of skills required, the daily caseload, and time spent performing procedures. Clearly, performing 100 cases over a one-year period is a very different circumstance than performing the same number over six weeks; the period of supervision then should be adjusted accordingly. Experience is not just dependent on caseload but also on egg yield, as workload is proportional to type and extent of follicular stimulation protocols.

The optimal ratio of laboratory staff to the expected number of procedures is debatable, and, unfortunately, economics play an all too important role here. However, with the incorporation of new technologies and treatment modalities in routine care, the complexity of IVF laboratory operations has increased substantially over the past decade, in turn requiring more careful consideration of staffing levels [6]. According to some calculations, whereas a "traditional" IVF cycle required roughly 9 personnel hours, a contemporary cycle can require up to 20 hours for completion. Thus, the number of embryologists required for safe and efficient operation of the laboratory has also increased. Based on a comprehensive analysis of laboratory tasks and their complexity, an Interactive Personnel Calculator was introduced nearly 10 years ago to help laboratory directors and administrators determine staffing needs [6]. This calculator may benefit from updating since the overall level of activity is determined by a clinic's quality assurance program, culture methodology, the average number of oocytes retrieved, and the incidence of cryo-storage of both gametes and embryos. ART laboratories have transitioned to cryo-storage facilities over the years, but automation and continuous digital tracking of samples are still not implemented in spite of evident advantages and the availability of at least one FDA-approved robotic system (TMRW, USA). Overall, it is safe to say that the ratio of laboratory staff to caseload should be high enough so that embryologists can not only safely perform procedures but also dedicate time to quality control and continued education and training to maintain the high standards required for success. Staff burnout is not just a function of the quantity of procedures but also of the quality of staff interactions and high-intensity aspects of assisted reproduction. The challenge of keeping these standards within national health systems or in the face of insurance mandates that must provide a wide range of services on a minimal budget is real but should not be insurmountable. Patients usually do not benefit from such constraints, as a comparison of results in different health service systems in Western countries would suggest. There are limitations to such comparisons, but live births per embryo and cumulative data from fresh and cryopreservation cycles are considered objective assessments [7].

The job description for the embryologist ideally includes all embryology and andrology tasks, except for medical and surgical procedures. Embryologists are often involved in other important tasks as well, including patient management, follicular monitoring, genetic counselling, marketing, running diagnostic laboratories, and administration. It should be realized that these tasks may detract from their main responsibilities. First and foremost, the duty of an embryologist is to safely perform gamete and embryo handling and culture procedures. Second, but equally important, the embryologist should maintain quality control standards, both by performing routine checks and tests and by maintaining detailed logs of incidents, changes, unexpected events, and corrective measures. Across all these duties, the following seven positions can be clearly defined: director, supervisor, senior embryologist, embryologist, trainee, assistant, and technician. There may also be positions for others to do preimplantation genetic testing and research; to validate new procedures; and for quality control supervision, technical supervision, and administrative work. Obviously, not all of these separate positions are necessary for smaller centres, and tasks can be combined.

Although a seemingly unimportant detail, one of the most important jobs in the IVF laboratory at Bourn Hall Clinic in Cambridge, UK, during the first few years of operation was that of a professional witness and embryology assistant. This position was the brainchild of Jean Purdy, the third partner with Patrick Steptoe and Robert Edwards, who was involved in the work that led to the birth of Louise Brown. The embryology assistant effectively enforced and oversaw the integrity of the chain of custody of gametes and embryos during handling, particularly when large numbers of patients were being treated simultaneously. The "witness" also ensured that embryologists performed only those procedures for which they were qualified. Interestingly, recent literature suggests that this crucial concept has not been universally and fully understood or adopted by all IVF laboratories. In one group of laboratories [8], "limited and consequently virtually ineffective" witnessing processes were only abandoned in favor of a more robust witnessing program after implementation of a failure mode and effects analysis (FMEA) showed a high risk of error in gamete and embryo identification. The authors stated that, "Only after FMEA optimization has the witness embryologist been formally recognized as a committed role, specifically trained for witnessing shift work." Hopefully, this and other similar studies [9] that show the effectiveness of a witnessing system will encourage more laboratories to re-examine their practices and allocate adequate resources to ensuring the safety and efficiency of all procedures performed by the laboratory.

Facility, design, and budget

In the early days of IVF some clinics were built in remote areas, based on the premise that environmental factors such as stress could affect the patient and thereby the outcome of treatment. Today's laboratories are commonly placed in city centres and large metropolitan areas in order to service large populations locally. It is important that patients understand that there have been millions of others like them before and that, in general,

IVF is a routine, though complex, medical procedure. It is clear that the choice of a laboratory site is of great importance for a new program. The recent development of better assays for determining the baseline quality of the environment facilitates site selection. There is now awareness that some buildings or building sites could be intrinsically harmful to cell tissue culture [10–13]. The direct effect of poor air quality and the presence of volatile organic compounds (VOCs) on IVF outcomes has been demonstrated by recent studies of countermeasures such as novel filtration systems and an understanding of the partitioning of compounds across liquid phases such as water and oil [4, 12, 14]. A laboratory design should be based on the anticipated caseload and any subspecialty. Local building and practice permits must be assessed prior to engaging and completing a design. There are five basic types of design:

1. Laboratories using only transport IVF
2. Laboratories adjacent to clinical outpatient facilities that are only used part of the time
3. Full-time clinics with intra-facility egg transport using portable warming chambers
4. Fully integrated laboratories with clinical areas
5. Moveable temporary laboratories

Before developing the basic design for a new laboratory, environmental factors must be considered. Although air quality in modern laboratories can be controlled to a degree, it can never be fully protected from the exterior environment and adjoining building spaces. Designers should first determine if the building or the surrounding site is scheduled to undergo renovations, demolition, or major changes of any kind in the foreseeable future. City planning should also be reviewed. Historical environmental data and trends, future construction, and the ability of maintenance staff to maintain and service the IVF laboratory need to be determined. Activity related to any type of construction can have a significant negative impact on any proposed laboratory. Prevalent wind direction, industrial hazards, and general pollution reports such as ozone measurements should also be determined. Even when these factors are all deemed acceptable, basic air sampling and determination of VOC concentrations is necessary inside and outside the proposed building area. IVF laboratory VOC concentrations have traditionally involved determination in parts per billion (ppb) when evaluating individual compounds such as deleterious aldehydes. This has required determination by laborious methods such as gas chromatography and mass spectrometry, but more recently total VOC counts have become a way of assessing laboratory conditions, allowing the introduction of affordable handheld units that measure in parts per million (ppm). At least one easy to use cloud-based handheld unit exists (Graywolf Sensing Solutions, USA) that can determine common VOCs in ppb in real time, although it is relatively expensive. The outcome of initial space tests will determine which design requirements are needed to remove VOCs from the laboratory area. In most cases, an over pressured laboratory (at least 0.10–0.20 inches of water) that uses a high number [7–14] of fresh air changes per hour is the best solution, because it also provides for proper medical hygiene. The Cairo consensus [4] has set important standards for this crucial element of laboratory design and maintenance. It was recommended that total VOCs be maintained less than 500 $\mu g/m^3$ (~400–800 ppb total VOC, depending on molecular species); less than 5 $\mu g/m^3$ aldehydes. Experience has shown that aldehydes can be very toxic to IVF conditions even at low levels. Few handheld devices can quantify this group of chemicals accurately.

The laboratory walls and ceiling should have the absolute minimum number of penetrations. This generally requires a solid ceiling, sealed lighting, and airtight utility connections. Contrary to many vendors' representations, commercial suspended ceilings using double-sided tape and clips are not ideal. Doors will require seals and sweeps and should be lockable. Ducts and equipment must be laid out in such a way that routine and emergency maintenance and repair work can be performed outside the laboratory with minimal disruption to the laboratory. Air handling is not optimal when using an open plenum design. In the ideal case, 100% outside air with chemical and physical filtration will be used with sealed supply and return ducts.

While providing cleaner air, 100% outside air sourcing will maximize the life of a chemical filter and will provide a lower concentration of VOCs in the IVF laboratory's air. In climates where temperatures routinely exceed 32°C with 85%-plus relative humidity, 100% outside air could result in an unacceptable level of humidity (>60%), which could allow mould growth. In these cases, the use of limited return air from the lab is acceptable. A 50% outside air system with 15–30 total air changes per hour to maintain over pressure works well and the relative humidity becomes very controllable. To place this in perspective, traditional medical operating room design calls for 10%–15% outside air.

The air supply equipment may supplement outside air with recirculated air, with processing to control the known levels of VOCs. On rare occasions, laboratories will require full-time air recirculation, although most may actually find the outside air to be perfectly clean at least most of the time. Outside air is often erroneously judged to be polluted without proper chemical analysis, whereas inside air is usually considered "cleaner" because it may "smell" better. In most laboratory locations, conditions are actually the reverse, and designers should not "follow their instincts" in these matters. Humidity must also be completely controlled according to climate and seasonal variation. The system must be capable of supplying the space with air with a temperature as high as 30°C–35°C at less than 40% relative humidity. Air inlets and outlets should be carefully spaced to avoid drafts that can change local "spot" temperatures or expose certain equipment to relatively poor air or changes in air quality. Laminar flow hoods and micromanipulation workstations should not be located too close to air supply fixtures to avoid disruption of the sterile field and to minimize cooling on the microscope stage. Semi-enclosed workstations based on Class 2 cabinets or neonatal isolette incubators can be considered to optimize the work environment and bridge the gap between the incubator and the workstation. A detailed layout and assessment of all laboratory furniture and equipment is therefore essential prior to construction and has many other benefits.

Selection of an experienced and subspecialized (and flexible) architect and a mechanical engineer for the project is essential. Confirm what their past experience has been in building biologically clean rooms. The use of "environmentally friendly" or "green" products has been suggested by some designers. The reliance on "natural" products does not ensure a clean laboratory. In one case, wood casework with a green label was found to be a major source of formaldehyde. Floor coverings using recycled vinyl and rubber were selected for their low environmental impact, without considering the significant release of trapped gases by the material.

Supervision of the construction is also critical. Skilled tradespersons using past training and experience may not follow all the architect's instructions. The general contractor and builders must be briefed on why these novel construction techniques are being used. They must understand that the use of untested methods and products can compromise the project (and the payment of their fees!). Contractual agreement is recommended. An initial operations and materials discussion with construction crews is highly recommended along with frequent inspections by a senior embryologist and informed architect.

Just as the organization and flow of traffic in a world-class restaurant result in a special ambience where more than just the food is the attraction, appropriate modular placement of equipment ensures safety and comfort in the over pressured IVF laboratory. Placement of stacks of incubators, gamete handling areas (laminar flow units or isolettes), and micromanipulation stations should minimize distances that dishes and tubes need be moved. Ideally, an embryologist should be able to finish one complete procedure without moving more than three meters in any direction; not only is this efficient, but also it minimizes accidents in a busy laboratory. Design and implementation of a work area incorporating product, gas and liquid nitrogen supplies, and a workstation, refrigerator, and incubators is feasible even without the embryologists having to walk between storage cabinets and equipment. Such a modular design can be duplicated multiple times within a larger air handling area allowing the handling of large numbers of gametes and embryos. For logistical reasons, sperm preparation and cryopreservation may be placed in adjacent areas. The number of modules can easily be determined by the expected number of cases and procedure types, the average number of eggs collected, and the number of embryologists expected to work simultaneously. Each person should be provided with sufficient workspace to perform all procedures without delay. Additional areas can contain simple gamete handling stations or areas for concentrating incubators. Cryopreservation and storage facilities are often located in separate spaces; if separated, these areas should always be adjacent to the main laboratory. Storage spaces could be separated further using closets or rooms with negative pressure. Embryology laboratories have undergone tremendous changes recently, with the advent of successful cryopreservation through vitrification. Vitrification allows replacement in natural cycles on a routine basis. What used to be a small room is now becoming a sizable cryo-storage facility, even though offsite storage location is recommended for samples with unclear future dispositions. The need for a separate area for ancillary activities such as medium preparation is clearly diminishing now that commercial manufacturers provide all the basic needs of an IVF laboratory. Administration should probably be performed in separate offices on a different air handling system from the main laboratories, though prospective studies regarding the effect of actual paperwork on outcomes do not exist.

Last but not least, it is preferable to prepare semen in a separate laboratory altogether, adjacent to one or more collection rooms. The semen laboratory should have ample space for microscopes, freezing, and sterile zoning. Proper separation of patient samples during processing is essential, and some elemental design features accommodating this may be considered before the first procedures are carried out. Some thought should go into planning the semen collection area. This small room should be at the end of a hallway preferably with its own exit; it should be soundproof, not too large, with a sink, and under negative pressure if possible. Clear instructions on how to collect semen for ART should be provided in the room. The room should also be adjacent to the semen preparation laboratory, preferably with a double-door pass-through for samples. This pass-through should have a signalling device so the patient can inform the embryologist that the sample is ready; it also permits male patients to leave the area without having to carry a specimen container.

Equipment and storage

A detailed list of equipment should be prepared and checked against the planned location of each item; it can later be used as the basis of maintenance logs. It is important to consider the inclusion of crucial equipment and spare instruments in the laboratory design to allow for unexpected malfunction. Similarly, two or more spare incubators should not be seen as excessive; at least one spare follicle aspiration pump and micromanipulation station (equipped with a laser) should also be included. The use of a spare liquid nitrogen-primed Dewar is now mandatory in some countries. There are many other instruments and equipment pieces the malfunction of which would jeopardize patient care, although some spares need not be kept on hand as manufacturers may have them available; however, such details need to be repeatedly checked as suppliers' stocks continue to change. It may also be useful to team up with other programs or an embryology research laboratory locally so that a crucial piece of equipment can be exchanged in case of unexpected failure.

Some serious thought is needed when contemplating the number and type of incubators (for a comprehensive review, see [15]). The ratio of incubators to patient procedures depends on incubator size and capacity and it varies considerably from program to program. It is clear that the number and type of incubator, along with the length and number of incubator door openings, affect results. In principle, the number of cases per incubator should be kept to a minimum. The smaller box incubators should not handle more than two to three cases. In benchtop incubators, the use of one dish slot per patient is not recommended. Dishes for one patient should be kept in one compartment, preferably with its own lid door. Several other incubators can be used for general purposes during micromanipulation and for other generic uses to limit further the number of incubator openings. Strict guidelines must be implemented and adhered to when maintaining distinct spaces for separating culture dishes or tubes of different patients. Tracking of incubators and even shelves or compartment spaces within each incubator is recommended so their performance can be evaluated on an ongoing basis. Separate compartments within an incubator may be helpful and can be supplied by certain manufacturers. Servicing and cleaning of equipment such as incubators may have to be done when the laboratory is not performing procedures. Placement of incubators and other pieces of equipment on castors may be helpful in programs where downtime is rare. Pieces of equipment can then be serviced outside the laboratory. New incubators and equipment pieces that come in contact with gametes and embryos must be "burned in" or "off-gassed." Protocols vary per equipment type and manufacturer.

When there are several options available to the laboratory designer, supply and evacuation routes should be planned in advance. One of the most susceptible aspects of ART is cryopreservation. In case of an emergency such as a fire or power failure, it may be necessary to relocate the liquid nitrogen-filled Dewars without using an elevator, or to relocate the frozen samples using a temporary container. This may seem an extreme consideration, especially in the larger laboratories that stockpile

thousands of samples, but plans should be made. It may be possible to keep a separate storage closet or space near the building exit where long-term samples, which usually provide the bulk of the storage, can be kept; this would require repeated checking of a facility that is not part of the laboratory. Liquid nitrogen tank alarms with remote notification capability should be installed on all Dewars holding gametes and embryos. The route of delivery of liquid nitrogen and other gas cylinders must be relatively easy, without stairways between the laboratory and the delivery truck, and should be sensibly planned. Note that the flooring of this route is usually destroyed within months because of liquid nitrogen spills and wear caused by delivery containers, so the possibility of an alternative delivery corridor should be considered for these units.

Liquid nitrogen containers and medical gas cylinders are preferentially placed immediately adjacent to the laboratory in a closet or small, ventilated room with outside access. Pipes and tubes enter the laboratory from this room, and cylinders can be delivered to this room without compromising the laboratory area in any way. Providing liquid nitrogen and even liquid oxygen vapor to triple gas incubators is nowadays a preferred option since vapor is cleaner than compressed gas. This allows liquid nitrogen vapor to be pumped into the cryopreservation laboratory using a manifold system and minimal piping. Lines should be properly installed and insulated to ensure that they do not leak or allow condensation and conserve energy at the same time. Medical gases can be directed into the laboratory using pre-washed vinyl/Teflon-lined tubing such as fluorinated ethylene propylene, which has high humidity, temperature, and UV radiation stability. Lines should be properly marked every meter indicating the incubators supplied in order to facilitate later maintenance. Alternatively, solid manifolds made from stainless steel with suitable compression fittings can be used. Avoid the soldered or brazed copper lines used in domestic plumbing applications wherever possible; copper lining can be used but should be cleaned and purged for a prolonged period prior to use in the laboratory. Copper line connections should not be soldered as this could cause continuous contamination. This recommendation may conflict with existing building codes, but non-contaminating alternatives can be found. A number of spare lines or conduits hidden behind walls and ceilings should be installed as well, in case of later renovation or facility expansion.

Large programs should consider the use of exterior bulk tanks for carbon dioxide and liquid nitrogen. This removes the issue of tanks for incubators or cryopreservation. These tanks are located where delivery trucks can hook onto and deliver directly to the tank. Pressurized gas lines or cryogenic lines then run the carbon dioxide or liquid nitrogen to the IVF laboratory for use.

Placement of bulky and difficult pieces of equipment should be considered when designing doorways and electrical panels. Architects should be fully informed of all equipment specifications to avoid the truly classic door width and height mistakes. Emergency generators should always be installed, even where power supplies are usually reliable. The requirements can be determined by an electrical engineer. Thankfully, these units can be removed from the laboratory but must be placed in well-ventilated areas that are not prone to flooding. Additional battery "uninterruptible power systems" may be considered as well, but may be of limited capability and costly. Buildings should also be checked for placement of the main power inlets and distribution centres, especially because sharing power lines with other departments or companies may not be advisable. Circuit breakers should be easily accessible to embryologists or building maintenance staff. General knowledge of the mechanical and electrical engineering of the building and the laboratory specifically will always be advantageous. Leaving all the building mechanics and facilities to other individuals is often counterproductive. Embryologists need to be involved with facilities management and be updated with construction decisions inside and outside the building in a timely manner.

Ample storage spaces should always be planned for IVF laboratories. In the absence of dedicated storage space, laboratory space ends up being used instead, filling all cabinets and negating any advantages of the original design. The dedicated storage area should be used to stock all materials in sufficient quantity to maintain a steady supply. A further reason to include storage areas in laboratory design—sufficient on its own to justify the space—is that new supplies, including sterile disposable items, release multiple compounds for prolonged periods. This "outgassing" has been determined to be a major cause of air pollution in a number of laboratories in which supplies were stored inside the lab. Separate storage space therefore provides the best chance of good air quality, especially when it is supplied by separate air handling system and under negative pressure. It should be large enough to handle bulky items and mobile shelving for boxes. One should be careful to avoid the natural inclination to save extra trips by bringing too many items into the laboratory, or the gains made by careful design may be lost. As a possible makeshift solution, storage cabinetry in the laboratory can be designed with separate negative pressure air handling to minimize release of VOCs from off-gassing package materials.

Microscopes and visualization of cells

Though dissecting microscopes are crucial for the general handling of gametes and embryos, many people still consider inverted microscopes to be a luxury even though they are in regular use with micromanipulation systems. Proper visualization of embryos is key to successful embryo selection for transfer or freezing; if the equipment is first class, visualization can be done quickly and accurately [16]. Even so, appropriately detailed assessment still depends on the use of an oil overlay system to prevent damage by prolonged exposure. Each workstation and microscope should be equipped with a still camera and/or video camera and monitor. Still photos can be placed in the patient file, and video footage permits speedy review of embryonic features with colleagues after the gametes are safely returned to the incubator; this is also helpful for training new embryologists. Recordings can be uploaded onto a patient health information, secure cloud service or kept on secure servers in the facility. Interference optics such as Hoffman and Nomarski are preferable because they permit the best measure of detail and depth. Novel visualization of internal elements such as spindles using polarized microscopy requires additional equipment but can be incorporated into routine operation [18]. Ideally, the captured photos should be digitally stored for recall in the clinic's medical database.

Development of new time-lapse microscopy technologies has made continuous and uninterrupted monitoring of embryo development a reality. This is an invaluable teaching and learning tool. However, equipment costs are high and, for many laboratories, prohibitive. Equipment for time-lapse technology can be sizable and may require separate consideration in terms of lab design and bench space.

Construction, renovation, and building materials

Construction and renovation can introduce a variety of compounds into the environment of the ART laboratory, either temporarily or permanently. Either can have major adverse effects on the outcome of operations [10–12, 18, 19]. The impact of the exterior environment on IVF success has been demonstrated. Pollutants can have a significant negative effect on success in an IVF laboratory [10, 20]. These effects can range from delayed or abnormal embryonic development, reduced or failed fertilization, and reduced implantation rates to pregnancy loss and failure of a treatment cycle. Many of the damaging materials are organic chemicals that are released or outgassed by paint, adhesives from flooring, cabinets, and general building materials, along with laboratory equipment and procedures. It is important to realize that the actual construction phase of the laboratory can cause permanent problems. Furthermore, any subsequent renovation activity in adjacent areas can also cause similar or even greater problems. Neighbouring tenants can be informed of the sensitivity of gametes and embryos in culture. At the very least, changes undertaken in adjacent areas should be supervised by IVF laboratory personnel to minimize potential damage. However, new construction immediately outside the building is considerably more problematic. City works such as street construction are very hard to predict and nearly impossible to control. A good relationship with the neighbours should be maintained and a working relationship with building owners and city planners should be established so that the IVF laboratory is kept informed of upcoming changes.

For the construction of a new laboratory or if changes are to be made to areas adjacent to the IVF facility, the following guidelines should be followed. First, the area to be demolished and reconstructed needs to be physically isolated from the IVF laboratory (if this is not the new IVF laboratory itself). The degree of isolation should be equivalent to an asbestos or lead abatement project. The isolation should be done through (i) physical barriers consisting of poly-sheeting supported by studding where needed; (ii) limited access to the construction area and the use of an access passageway with two doors in series; (iii) removal of all construction waste via an exterior opening or proper containment of waste before using an interior exit; (iv) negative air pressure in the construction area exhausting to the exterior, far removed from the laboratory's air intake and properly located with regard to the prevailing winds and exterior airflow; (v) extra interior fans during any painting or the use of adhesives to maximize removal of noxious fumes; and (vi) compiling and logging of a Safety Data Sheet (SDS; previously MSDS) for all paints, solvents, and adhesives in use.

Follow-up investigations with manufacturers and their representatives may be helpful because specifications of equipment may be changed without notice. The negative pressurization of the laboratory space requires continuous visual confirmation via a ball and tube pressure indicator or simply paper strips. Periodic sampling for particulates, aldehydes, and organics could be done outside the demolition and construction site, provided this is economically feasible. Alternatively, tracer gas studies can be done to verify containment. The general contractor of the demolition and construction should be briefed in detail on the need to protect the IVF facility and techniques to accomplish this. When possible, the actual members of the construction crew themselves should be selected and briefed in detail. Large filter units using filter pellets of carbon and permanganate can be placed strategically. Uptake of organics can be assayed, but the frequency of routine filter changes should be increased during periods of construction activity.

Selection of building materials

Many materials release significant amounts of VOCs; a typical list includes paints, adhesives, glues, sealants, and caulking, which release alkanes, aromatics, alcohols, aldehydes, ketones, and other classes of organic materials. This section outlines steps to be taken to reduce these outgassing chemicals. Any and all interior painting throughout the facility should only be done on prepared surfaces with water-based paint formulated for low-VOC potential. During any painting, auxiliary ventilation should be provided using large industrial construction fans, with exhaust vented to the exterior. Paints that can significantly influence air quality should be emission tested (some suppliers already have these test results available). SDSs are generally available for construction materials. Suppliers should be encouraged to conduct product testing for emission potential. The variety of materials and applications complicates the testing process, but several procedures have been developed to identify and quantify the compounds released by building materials and furnishings. Interior paints must be water-based, low-volatile paints with acrylic, vinyl acrylic, alkyd, or acrylic latex polymers. Paints meeting this specification can also contain certain inorganic materials. Low-volatile paints may still contain low concentrations of certain organics. No interior paint should contain formaldehyde, acetaldehyde, isocyanates, reactive amines, phenols, and other water-soluble volatile organics. Adhesive glues, sealants, and caulking materials present some of the same problems as paints. None of these materials used in the interior should contain formaldehyde, benzaldehyde, phenol, and similar substances. Although water-based versions of these are generally not available, their composition varies widely. Silicone materials are preferred whenever possible, particularly for sealants and caulking work. A complete list of guidelines for material use during the construction of a tissue culture laboratory is available elsewhere [21].

"Burning in" of the finished facility

New IVF laboratories and new facilities around existing laboratories have often been plagued by complaints of occupants who experience discomfort from the chemicals released by new construction and furnishings. The ambient levels of many of these materials can be reduced by "burning in" the facility. A typical burn-in consists of increasing the temperature of the new area by 10°C–20°C and increasing the ventilation rate; even higher temperatures are acceptable. The combination of elevated temperature and higher air exchange aids in the removal of the volatile organics. Upon completion of the construction, the air handling system should be properly configured for the burn-in of the newly constructed area. As previously stated, the system must be capable of supplying the space with air at a temperature of 30°C–35°C, at less than 40% relative humidity. The burn-in period can range from 10 to 28 days, and the IVF laboratory should be kept closed during this time. If these temperatures cannot be reached by the base system, use auxiliary electrical heating to reach the minimum temperature. During burn-in, all lighting and some auxiliary equipment should be turned on and left running continuously. Naturally, ventilation is critical if

redistribution of irritants is to be avoided; the whole purpose is to purge the air repeatedly. Auxiliary equipment should of course be monitored during the burn-in.

The same burn-in principle applies to newly purchased incubators or other laboratory equipment. Removal of volatile organics is especially important in the critical micro-environment of the incubator. Whenever possible, it is advantageous to purchase incubators months in advance of their intended initial use and to operate them at an elevated temperature in a clean, protected location. An existing embryology laboratory is not a good space for the burn-in of a new incubator.

Most of the equipment available for use in an ART laboratory has not been designed or manufactured to be VOC-free. Special attention must be invested in new laboratory equipment to eliminate or reduce VOC levels by as much as possible before first use.

Most manufacturers do not address the issues of VOC outgassing in product manuals, even if the equipment has been expressly designed for the IVF field. Unpacking, cleaning, and operating equipment prior to final installation in a lab for outgassing the "new car smell" is always recommended.

Incubators should be unpacked, inspected, cleaned, outgassed, operated, recleaned, calibrated, and tested well in advance. The process can take several months to accomplish, but is generally a very essential task that is rewarded with the most suitable culture system that the selected incubator model can provide. When possible, operating incubators at elevated temperatures above the typical culture temperature will hasten the release or burn-off of VOCs. Extended operation at between 40°C and 45°C works well to burn off VOCs if this is within the manufacturer's recommended temperature range. Incubator model VOC loads can vary greatly. Accurate VOC testing may be expensive and time-consuming, but it is recommended to test a specific incubator model to determine the new unit's typical VOC characteristics and how much time outgassing may require.

Handheld VOC testing devices are available and can be used to help monitor the decline of total VOCs, but cannot match the level of accuracy of an environmental organic chemist's testing. Handheld VOC meter technology generally is not sensitive enough to monitor low-molecular-weight classes of VOCs. They are reasonably affordable, easily used, and can provide a means of monitoring VOC reduction to help determine if the outgassing time may be sufficient to observe a reduction of VOCs.

New incubators are generally tested with a mouse embryo assay (MEA), replicating a culture system as part of a new incubator commissioning process. Most laboratories today use some variation of an oil culture system. The oil can serve as an excellent filter against potential VOCs but may not protect a culture system from the full range of VOC exposure, particularly low-molecular-weight compounds such as aldehydes. Incubator MEA commissioning should include both an oil and an open exposed media test to help evaluate the success of preparing the incubator. The dual MEA approach works well for humidified incubator systems, but may not be applicable if a dry, non-humidified culture system is used. Most dry, non-humidified culture systems are designed to recirculate chamber air and incorporate a VOC filtration strategy. Open culture generally cannot be used with non-humidified incubators. The manufacturer's recommendations should be followed. Non-humidified incubators may require extended off-gassing and should be tested prior to use to confirm that they do not have a VOC issue. Chemical VOC filters should be replaced after burn-off prior to any MEA testing.

Laminar flow hoods and isolettes are also important potential VOC sources that should not be overlooked. They should be given ample time to operate and outgas as they can contribute to a lab's VOC contamination load. High-efficiency particulate air (HEPA) and chemical filters should be selected for low-VOC manufacturing traits and also may require off-gassing. Care must be taken when outgassing laminar flow hoods and isolettes as they require a HEPA-filtered environment or replacement of their filters when transferred to an IVF lab.

After the burn-in is complete, commissioning of the IVF suite should be conducted to verify that the laboratory meets the design specifications. The ventilation and isolation of the laboratory should be verified by a series of tests using basic airflow measurements and tracer gas studies. The particulate levels should be determined to verify that the HEPA system is functional. Particulate sampling can be performed using US Federal Standard 209E. Microbial sampling for aerobic bacteria and fungi is often done in new facilities using an Andersen sampler followed by microbiological culturing and identification. The levels of VOC contamination should be determined. Possible methods are included in the US Environmental Protection Agency protocols using gas chromatography/mass spectroscopy and high-performance liquid chromatography that is sensitive at the microgram per cubic meter level [22–25].

Maintenance, planning, and sterilization

Even the best systems and designs will eventually fail unless they are carefully maintained. The heating, ventilation, and air conditioning (HVAC) will require filter changes, coil cleaning, replacement of drive belts, and chemical purification media. The most prevalent failure concerns the initial particulate filter. These are inexpensive filters designed to keep out large dust particles, plant debris, and insects, among other things. If such filters are not replaced promptly and regularly, they will fail, allowing the HVAC unit to become contaminated. The HEPA filters and chemical media also require inspection and periodic replacement. Maintenance staff should report their findings to the IVF laboratory.

The IVF laboratory must have a cleaning facility for surgical instruments. Ongoing use of an autoclave is not a problem as long as the released steam is rapidly exhausted to the outside. This keeps the relative humidity in the facility to controllable limits. Autoclaves should not be placed on the IVF laboratory's HVAC system, but rather in a room that is built using tight construction and is exhausted directly outside of the building. The use of cold sterilizing agents is not advised. Aldehydes such as glutaraldehyde and *ortho*-phthalaldehyde from the autoclave can be transported inside the IVF laboratory.

Insurance issues

ARTs have become common practice worldwide and are regulated by a combination of legislation, regulations, or committee-generated practice standards. The rapid evolution and progress of ART reveal new legal issues that require consideration. Even the patient population is changing, as it becomes more acceptable for single persons and same sex/homosexual couples to seek and receive treatment. Donation of gametes, embryos, and gamete components; enforcement of age limits for treatment; selective fetal reduction; pre-implantation genetic diagnosis; surrogacy; and many other practices in ART present practitioners and

society at large with challenges, which are often defined by social norms, religion, and law and are specific to each country.

Furthermore, financial and emotional stresses often burden patients seeking treatment in countries where medicine is not socialized, and infertility treatment is not covered by insurance. This translates into an increasing number of ART lawsuits related to failed treatments in spite of generally improved success rates. Laboratory personnel and the laboratory owner should therefore obtain an insurance policy of a sufficiently high level and quality commencing prior to the first day of operations. Litigation-prone issues need special consideration, and include:

- Cancellation of a treatment cycle prior to egg retrieval
- Failure to become pregnant
- Patient identification errors
- Cryo-storage mishaps

These issues occur even if experienced practitioners consider themselves at low risk of exposure. Prior to engaging in the practice of ART, protocols must be established to identify potential problem areas and establish countermeasures.

Conclusions

It may be surprising how many professionals continue to pursue the establishment of new ART clinics at a time when competition is fierce, financial benefits are small, and existing ART services may appear to be approaching saturation in many areas and countries. Appearances can be misleading, however, and ART centres of excellence that deserve the trust and confidence of patients and serve as models for other practices are always needed.

This chapter provides some guidance for those who aspire to establish such outstanding, well thought out and planned ART practices. Although it cannot safeguard practitioners against adverse events, it introduces concepts in the proper design, construction, and operation of ART facilities that are of fundamental importance to treatment success; these guidelines have been painstakingly compiled through decades of practical experience and research. The approach is best adopted as a whole rather than dissected into its components and adopted in part or selectively. Keep in mind that resisting the urge to cut corners in the wrong places avoids future headaches and positions you and your patients on the path to success.

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2

QUALITY CONTROL

Maintaining Stability in the Laboratory

Ronny Janssens, Neelke De Munck, and Johan Guns

Introduction

It has now been almost 40 years since *in vitro* fertilization (IVF) was developed by Edwards and Steptoe. Over these decades, practice in medically assisted reproduction (MAR) has evolved from a new, experimental procedure into a well-established routine treatment of infertility driven by the development of new procedures such as intracytoplasmic sperm injection (ICSI), extended culture, pre-implantation genetic testing (PGT), vitrification, ongoing research, the development of better and safer products and culture media, more stringent quality control programs by commercial companies, and a better understanding of possible factors that might have an impact on the outcome of the procedure. Although success rates have improved over time, it is hard to define which laboratory practices contribute to this success [1]. In a survey of US high-performing centers, factors that were identified as being vital to excellent outcomes were experience of physicians, embryologists, and staff members, along with consistency of approach, attention to detail, and good communication.

Together with the evolution from research toward worldwide routine application, we have seen increasing regulatory requirements and the development of professional standards for embryology laboratories. In the beginning of this century, both US and European authorities issued regulations to ensure the quality and safety of human tissues and cells, and now the European Union Tissues & Cells Directive 2004/23/EC (EUTCD) [2] has been implemented in all EU member states.

Although the legislation differs between the US and Europe and the interpretation and translation into national legislation of the EUTCD in the EU member states is different from country to country, there is a common requirement to implement a quality management system (QMS) in any ART laboratory.

All is well, until disaster strikes you. Remember Captain Smith, a very experienced captain on the helm of the Titanic when it sunk in 1912. Sometimes things do not go as expected and disasters or errors occur. All embryologists are or will be confronted with Murphy's Law: if anything can go wrong, it will go wrong. It is our challenge and professional duty to beat Murphy's Law and be better than Captain Smith and here, quality management can help.

Although sometimes seen as a burden, quality management supports a successful clinic. It is a tool to avoid unwanted and uncontrolled fluctuations in a process and ensures the consistency of approach and attention to detail so that stable results can be achieved over time. Essential elements of quality management (and relevant standards for quality management) leading to standardization are risk management, validation, standard operating procedures, communication, and training.

Risk management

Treatment is influenced by internal and external factors that create uncertainty in achieving the desired outcome. The effect of this uncertainty is "risk." Prospective risk management [3] is an instrument dealing with the possibility that some future event(s) might cause harm [4]. It includes strategies and techniques for recognizing and confronting any such threat and provides a disciplined environment for proactive decision-making (or beating Murphy's Law). Risk management is now an essential element of accreditation or certification standards and is even mandatory for some regulatory authorities such as the UK Human Fertilisation and Embryology Authority (HFEA) [5] and European Directorate for the Quality of Medicines & Healthcare (EDQM) [6]. In a risk assessment procedure, you identify what the risks are, what would be the cause, what would be the consequence, and what controls could be in place to minimize risk. There are many risk assessment techniques [7], but the two most commonly used are "failure mode and effects analysis (proactive)" and "fault tree analysis (retrospective)." It is good practice to perform a proactive risk assessment before introducing or changing a procedure. The Euro Good Tissue & Cell Practices (Euro GTP II) provides practical tools to evaluate and quantify risks [8]. Once risks are identified, they can be controlled or treated so that the likelihood or the consequence (impact) of an event is reduced. A good example of proper risk treatment in the IVF laboratory is the installation of a real-time equipment monitoring system (EMS). The EMS increases the detection of equipment malfunctioning and reduces the consequence by warning in time so that loss of valuable biological material can be prevented. Although there is an important investment cost to installing a real-time EMS, it has been demonstrated that, even for a small laboratory, an automated system can represent not just increased functionality, but it also saves money within three years [9]. Monitoring and alarming are essential tools for quality control and maintaining stability in the laboratory and are also required by EU directive 2006/86/EC [10], ISO 15189:2012 [11], and the HFEA code of practice [5].

Validation

The ISO definition of validation is "confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled." IVF is a process (a set of interrelated or interacting activities that transform inputs into outputs). A basic objective of validation is to ensure that each step and each variable of the process is identified and controlled and process variability is reduced so that the finished product meets customer requirements (e.g., consistent high pregnancy rates).

The US Food and Drug Administration (FDA) [12] and EDQM [6] published guidelines that outline the general principles for process validation. Quality, risks, safety, and efficacy should be considered from the design phase of a process. Certainly in IVF, the quality of the "end product" cannot be measured, so each contributing factor (infrastructure, equipment, and utilities) and all the steps of the process need to be known and controlled.

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Ideally, prospective validation is preferred, but certainly in existing IVF clinics this is not always possible. According to EDQM, establishments performing very simple, minimal manipulation of a limited range of tissues and cells in accordance with published methods, or following long-established practices using the same materials and equipment, may rely on ongoing quality control and periodic reviews. Such establishments should still document their validation policy, explaining their approach on the basis of risk assessment, and should perform a retrospective verification of their critical processes to confirm that the method has the intended (clinical) outcome. The qualification of premises, equipment, suppliers, software, materials, consumables, reagents, and personnel should be ensured and should result in written reports. During the validation, in-process controls should be defined in order to monitor the process.

Process validation is needed before the introduction (process design) of a new method into routine use, whenever the conditions change for which a specific method has been validated (other instruments, changes in environment, etc.), and whenever the method is changed [12, 13].

During routine use, periodic verification of critical parameters and, when technically feasible by using modern technology, continuous process verification are necessary to ensure that the process remains in a state of control. Examples of laboratory processes that need to be validated are cleaning and decontamination procedures, sperm processing, IVF/ICSI, egg collection, embryo culture, cryopreservation, and embryo replacement.

In addition, equipment needs to be qualified in order to provide a high degree of assurance that it will consistently meet its predetermined specifications with minimal variation. Equipment qualification is broken down into three phases: installation qualification (IQ), operational qualification (OQ), and performance qualification (PQ). IQ is the first step and ensures that the equipment is correctly installed according to the manufacturer's specifications. As an example, a new incubator needs to be installed on a solid, vibration-free surface, the room temperature should be within a defined range, and the instrument should be connected to CO_2 and the main power. During the next step, OQ, the equipment is calibrated and tests are performed in order to document a baseline of the critical parameters of the equipment. For an incubator, this is defining set points for CO_2, temperature, and oxygen and a verification of these parameters with independent, calibrated measuring equipment. The PQ phase then tests the ability of the incubator to perform over long periods within an acceptable tolerance range. The equipment, utility, and system should then be maintained, monitored, and calibrated according to a regular schedule by responsible personnel with appropriate qualifications and training. Parameters of calibration and equipment qualification should be traceable to international standards. Calibrated equipment should be labeled, coded, or identified so that the calibration status and recalibration due date are clear. If equipment is not used for a certain period of time, then the calibration status needs to be verified before use.

Documentation

Good documentation is an essential part of any QMS. The process validation and equipment qualification and laboratory standard operating procedures need to be correctly and completely documented. These documents should be approved by the laboratory director or a delegated manager, regularly reviewed, and updated. Before any new or changed procedure may be introduced into routine, staff should be trained; the training should be specific and focused on the role of the employee.

Change control

The core principle of quality management is about change; change for continuous improvement or the plan, do, check, act cycle. Whenever processes or procedures are changed, the impact of the change should be justified and documented in order to prove that the change does not adversely affect the process.

Change control is a systematic approach that is used to ensure that any intended modification to the process, equipment, instruments, facility, and so on, is introduced in a coordinated manner and to reduce the possibility that unwanted or unnecessary changes will be introduced into the culture system [14].

Unplanned deviation from these approved processes or documents with potential impacts on quality, safety, or efficacy should be registered as non-conformity in the QMS.

The key principle of change control is to understand and document what was done, why, when, where, by whom, how, and what were the results.

Changes potentially requiring revalidation or clinical testing prior to implementation (decision based on risk analysis) are changes of facilities and installations, which may influence the process (cleanrooms or heating, ventilation, and air conditioning [HVAC]), changes in materials (puncture needles or transfer catheters) or reagents (culture media), changes in the process itself (implementation of new technology or findings based on current knowledge), changes in equipment, or support system changes (cleaning, supply, or information technology). All changes that have the potential to impact quality, safety, and efficacy should be justified, documented, approved (or rejected), communicated, and made known to laboratory staff and implemented in practice.

A change request procedure should be incorporated into the QMS.

Quality control and quality assurance

There is only one thing that is truly important in an IVF laboratory: everything. This was the conclusion of an international expert meeting to establish consensus guidelines on IVF culture conditions [15]. In this report, more than 50 consensus guideline points were established on different topics: embryo culture—basic principles and interactions; temperature in the IVF laboratory; humidity in culture; carbon dioxide control and medium pH; oxygen tension for embryo culture; workstations—design and engineering; incubators—maintaining the culture environment; micromanipulation—maintaining a steady physicochemical environment; handling practices; assessment practices; culture media—buffering and pH, general composition and protein supplementation, sequential or single step media for human embryo culture; use and management—cold chain and storage; test equipment—calibration and certification; and laboratory equipment and real-time monitoring.

Having established in detail for your protocols which aspects of the process are important to delivering the required quality (by proper validation), it is necessary that in-process controls are properly monitored. Regular monitoring of key performance indicators [16] provides good evidence of a clinic's performance, but, unfortunately, a real decline in pregnancy rates may only be detected very late. It is therefore crucial to establish strict quality control procedures, routines, and controls to ensure that

procedures and pieces of equipment operate appropriately and the process remains "in control."

This section focuses on the instruments and techniques used to document environmental and process parameters such as temperature and gas concentrations, and discusses the quality control and quality assurance of laboratory personnel, infrastructure, equipment, culture media, and contact materials.

Infrastructure and environment

Cleanrooms and air quality

The relation between environmental toxicants and fertilization and embryo development has been reported by several authors [17, 18]. More recently, positive pressure in the lab, high-efficiency particulate air (HEPA) filtration of laboratory air, filtration for volatile organic compounds (VOCs), and use of chemical active compounds are identified as factors that are common in high-performing IVF programs [1]. Most modern IVF centers are now located in cleanrooms.

HVAC functioning of a cleanroom should be monitored by a building monitoring system (BMS). The air quality requirement can vary from country to country, but most modern IVF laboratories are housed in cleanrooms classified ISO 8 to ISO 7 or EU GMP D to C, although there is now international consensus to aim for GMP C for ART laboratories [19]. Once the clinic has specified its air quality requirements, compliance with the designated classification has to be demonstrated in the formal process of qualification. Qualification is mostly done on a yearly basis by a testing organization that performs normative tests compliant with the ISO 14644-1 and ISO 14644-3 standards [20]. Qualification is followed by monitoring in order to control performance, both in rest state and in operation [21].

Air quality monitoring [22] consists of the enumeration of particles and microorganisms, both in rest and in activity. Before starting, a monitoring program needs to establish the sampling frequency and locations, the number of samples per location, the sample volume, and the test methods. This way of working, which is not yet familiar to ART and other tissue/cell establishments, derives from pharmaceutical guidelines. The EU directive refers to annex 1 of the EU GMP [23] that specifies the techniques for particle and microbial testing, similar to those described in the US cGMP [22] and the US Pharmacopeia for the production of medicines for human use [24]. These pharmaceutical guidelines can guide the ART establishments in setting up a monitoring program.

Furthermore, the EU GMP makes a distinction between environmental monitoring at rest state and monitoring of the aseptic process in operation. Environmental monitoring at rest state verifies whether the environment is ready for the forthcoming activity, whereas aseptic process monitoring aims to ensure that the people, processes, and environment remain under control during operation.

Particle counters can be part of a BMS or an EMS. It is possible to monitor VOCs in laboratory air. Photo ion VOC detectors, measuring in the ppm range and with a 4- to 20-mV output, are commercially available and can easily be connected to any real-time EMS. Monitoring VOCs may lead to the detection of non-compliance of cleaning and disinfection procedures by cleaning staff outside of working hours and can avoid the introduction of dangerous and toxic products released by non-approved cleaning agents.

The maintenance schedule for serving and filter replacement should be defined (by particle count for HEPA filters and analysis of filter saturation for active carbon and chemical VOC filters) and records should be kept of filter replacement dates and batch numbers. The preventive maintenance schedule should be defined in a service-level agreement between the laboratory and the company performing the maintenance.

Temperature and relative humidity

The absolute value of ambient temperature in the cleanroom is not really important for MAR (occupational health and safety rules should be respected) but should not exceed 25°C in order to control microbial contamination. If the environment of a cleanroom is cold and dry, microbiological contaminants will not grow. If the ambient relative humidity (RH) and temperature of the cleanroom environment exceeds 50% and 25°C, the risk of bacteria growth increases. On the other hand, humidity that is below 35% promotes static electricity, personal discomfort, and irritation of mucous membranes and eyes. An ambient RH between 40% and 50% minimizes the impact of bacteria and respiratory infections and provides a comfortable working environment. Also, incubators do not function well if ambient temperature is above 30°C. However, for optimal lab performance, it is important to keep ambient temperature constant to avoid fluctuations in the surface temperature of equipment (heated stages and incubators), and therefore the ambient temperature should be monitored and alarmed.

During the design phase of a new cleanroom, attention should be given to the positioning of workstations and incubators so that they are not located directly in front of or below HEPA filtered air conditioning outlets.

Ambient temperature and RH are usually monitored by a BMS.

Light

The effect of direct sunlight and hard white fluorescent light on mammalian zygotes and embryos is well documented [25, 26] and most laboratories limit the amount of light exposure to gametes and embryos. In total, 95% of this light energy originates from microscope halogen lamps during manipulation and handling [27], and, in particular, the blue region (400–500 nm) of light is harmful [28]. Therefore, the use of green filters on microscopes is recommended.

Gas supplies

There is now convincing evidence that low oxygen concentrations for embryo culture are associated with increased live birth rates [29]. IVF incubators depend on a supply of gas in order to regulate their internal atmospheres. Depending on the incubator's design, this is either 100% CO_2 and 100% N_2 for incubators with integrated gas mixing units, or custom-made mixtures of 5%–6% CO_2, 5% O_2, and 89%–90% N_2 for incubators without integrated gas mixing capacity (MINC™ benchtop incubator, Cook Medical; BT37 benchtop incubator, Origio/Planer). All gases should be of the highest quality and VOC filters should be installed on gas lines. Incubators with gas mixing units do have sensors and can give an alarm when the gas supply is failing, but this is not the case with incubators that run on premixed gasses. The latter can be monitored by placing a small Petri dish-sized infrared CO_2 sensor [30] inside an incubator chamber.

Laboratory equipment and real-time monitoring

Real-time monitoring (RTM) has long been seen as simply impractical because of the lack of accurate CO_2 sensors, the difficulty in connecting too many points, and the cost of cabling and adding sensors and data transmitters. Today, with the universal availability of low-cost wireless technology, the internet, smartphones, and tablets, this is no longer the case, and there are now affordable solutions that provide vital, real-time information to

monitoring systems and the people who need it, such as the laboratory manager. RTM systems can reduce "loss" by equipment failure and thus provide the manager with increased safety and reliability. Also, regulators see the benefits of monitoring; this requirement is now integrated into professional guidelines [31], regulatory requirements [10], and accreditation standards [11].

It is possible to connect analogue sensors for temperature and gas levels (CO_2, O_2, and VOCs). If feasible, monitoring sensors that are independent from the equipment should be used. This makes it possible to detect equipment sensor drift, allows verification of manufacturers' performance claims, and may detect environmental factors such as electrical failure.

Air pressure, RH, airflow sensors, and particle counters can be connected to an RMS, but these parameters are usually integrated into a BMS. Laboratory ambient air monitoring should be part of the EMS since deviations in ambient temperate have consequences on the temperature regulation of microscope heated stages.

Digital signals that can be monitored in real time include door status and equipment alarm signals. It is even possible to read digital Recommended Standard (RS) 232 or RS 485 interphases.

Modern web-based systems provide accurate and effective control of equipment. The data are remotely accessible over a secure internet connection and intelligent alarms warn the laboratory manager in case of an unexpected event or equipment malfunctioning or failure. To increase reliability, technical alarms (sensor break, monitoring equipment failure, or network failure) should be possible, and this aspect should be considered when a monitoring system is chosen. Of course, with modern technology, it is possible to send alarms by telephone, email, or SMS, but the alarm messaging program should be bi-directional so that alarm acknowledgement is possible (and logged). In case of no reaction within a predefined timeframe, an automatic cascading system should be activated.

Culture system

Temperature issues

Although the optimal temperature for oocyte handling and embryo culture is not really known, limited decreases in temperature can alter the cytoskeleton [32] and spindle [33] of oocytes, and there is limited recovery after cooling and rewarming [34], indicating that human meiotic spindles are exquisitely sensitive to alterations in temperature and that the maintenance of temperature close to 37°C during *in vitro* manipulations is important for normal fertilization and subsequent embryo development. These temperature effects are irreversible so it is important to avoid suboptimal temperatures. Temperature issues can occur during follicle puncture, during manipulations on heated stages on stereo microscopes and injection microscopes, in incubators, and during embryo transfer. Temperature should be measured in culture dishes under oil and in tubes with calibrated probes. The choice of measuring probe is important. Thin, fast-responding, non-shielded type T thermocouples can be used to detect small temperature gradients and are excellent at detecting hotspots on heating stages, whereas more precise, small probes fixed in culture dishes are more suitable for precise temperature measurements in incubators. Although the most stable and accurate sensors are resistance temperature detectors (Pt100 and Pt1000), they are not easily available in small sizes to affix inside a culture dish. For this purpose, thermistor probes are probably a better choice. Thermistors with 0.1°C accuracy are now widely available and at a very reasonable price. They have a fast response time and because of their high sensitivity, they are ideal in detecting temperature changes in culture dishes. Of course, accurate temperature measurement is only possible through the use of suitably calibrated sensors and instruments, and the accuracy of these measurements will be meaningless unless the equipment and sensors are correctly used. Good knowledge of measurement science is a basic requirement, one that is lacking in many laboratories.

Culture media and pH

The choice of culture medium is beyond the scope of this chapter. There is no ideal pH for culture media, as this varies from medium to medium and manufacturer to manufacturer, but it usually fluctuates within a range of 7.1 to 7.4. The pH of bicarbonate-buffered medium is regulated by the concentration of CO_2 dissolved in the culture medium, and this is regulated by the partial pressure of CO_2 in the incubator air. It is therefore important to carefully monitor incubator performance by RTM. In large-volume standard incubators, it is easy to integrate infrared CO_2 sensors. In small-volume desktop incubators, this is more challenging, but in some brands it is possible. While with modern and well-controlled incubators it is possible to maintain stable pH values, pH will increase while culture media are outside the incubator. To slow down this pH increase, oil is often layered over culture media. Besides this protective effect on pH, an oil overlay also reduces evaporation and heat loss and provides protection from particulate air contaminants [35].

The protective effect on pH is, however, quite limited in time, as shown in Figure 2.1. When culture medium is directly exposed to ambient air, the pH rise starts immediately. When a culture dish is removed from the incubator and the lid is left on the dish,

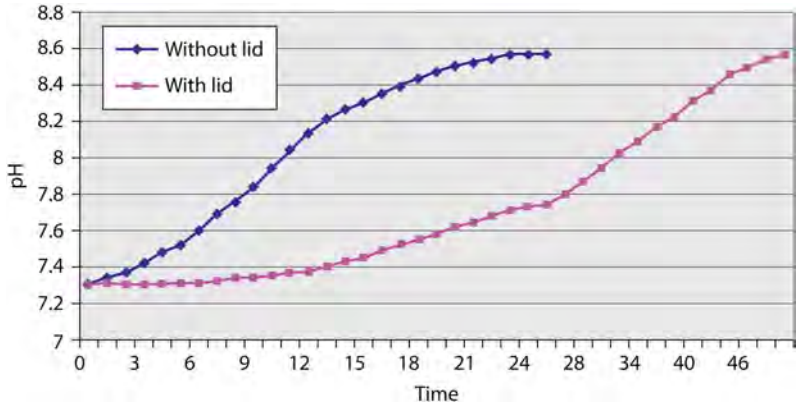

FIGURE 2.1 pH of culture medium under oil in ambient air over time (minutes).

then the pH starts to rise after 10 minutes. It is therefore good practice to leave lids on culture dishes during zygote and embryo scoring. These pH problems can be avoided by working in isolators with CO_2 (and temperature) regulation.

For quality control purposes, the pH of each batch of culture medium should be measured after proper pre-equilibration and should be within the range specified on the certificate of analysis. A conventional pH meter with a glass electrode is technically challenging (samples for measurement have to be removed from the incubator, measurements should be done at 37°C, and measurements have to be done in ambient air) and time-consuming. Better solutions are the continuous pH recorders in incubators or the use of a point-of-care blood gas analyser (only possible for culture media, not suitable for HEPES or MOPS buffered media).

Osmolality

The osmolality of commercial media ranges from 255 to 298 mOsm/kg [36]. Most IVF labs culture embryos in droplets of media under oil overlay. Microdrop preparation can influence culture media osmolality, which can impair embryo development [37], so this technique should be standardized and staff should be trained in culture dish preparation. With recent developments such as non-humidified benchtop incubators and single-step media with prolonged culture without medium change, monitoring osmolality has become an important part of quality control (after opening and during storage) and process control (measurement of spend culture medium and after five to six days of culture). For this purpose, each laboratory should have a freezing point depression osmometer.

Contact materials

Disposables such as oocyte retrieval needles, culture dishes, ICSI needles, and transfer catheters are used extensively throughout the whole IVF procedure, and the choice of disposable should be defined and its performance documented in a validation procedure. Disposables for embryo culture are available from many different manufacturers. Unfortunately, some have been shown to have toxic effects on gametes and embryos by sperm motility assay [38] or mouse embryo assay (MEA) [39].

The American Society for Reproductive Medicine (ASRM) practice guidelines [40] require that material that comes in contact with sperm, eggs, or embryos should be non-toxic and should be tested by the vendor with an appropriate bioassay or animal model. In Europe, all materials and reagents that come into contact with human material for transplantation must be approved as a medical device (MDR) [41]. This includes, but is not limited to, aspiration needles, transfer catheters, plastic ware, glassware, culture media, and protein sources. The European Society of Human Reproduction and Embryology (ESHRE) guidelines [31] require that culture media should be mouse embryo tested, and the European Directives require that these disposables should be tested with an adequate bioassay by the supplier, but there is no consensus or standard on how this MEA test should be performed. Variables that have an effect on MEA sensitivity [42] are the starting point (oocytes, zygotes, or two-cell embryos), number of embryos per volume of culture medium, culture medium and use of albumin, exposure protocol of the disposable (medium volume and duration of exposure), and the use of an oil overlay, so manufacturers can easily modify their assay conditions and either aim to maximize sensitivity (with a high rejection rate) or reduce sensitivity (with a low rejection rate). Product inserts or certificates of analysis are not informative and the end-user cannot judge the real value of the company's statement "MEA tested." Laboratories should therefore request this information and select suppliers based on their transparency in providing information on test conditions, exposure protocols, and acceptance criteria.

Laboratory personnel

The number and qualifications of laboratory personnel are critical factors for maintaining stability in the laboratory. The recommended staffing levels are one full-time equivalent "bench" or "hands-on" embryologist per 120 stimulation cycles per year [43]. As in any discipline in which technical proficiency can directly influence a measurable outcome, monitoring performance is essential to confirm that a procedure is carried out correctly and optimally. The aim of this monitoring is to discover departures from protocol and to identify opportunities for correction and improvement. Examples of performance parameters are the number of two pronuclei and the number of degenerated oocytes per total number of mature eggs injected (ICSI), number of embryos recovered intact and viable per number cryopreserved and per number thawed/warmed (cryopreservation or vitrification), number of clinical pregnancies per number of embryo transfers (embryo transfer), number of embryos continuing development per number of embryos biopsied, number of embryos with molecular signals per number of embryos biopsied (embryo biopsy), number of oocytes survived and intact per number of oocytes vitrified (oocyte vitrification), and number of gestational sacs per total number of hatched embryos [44].

Witnessing

One of the definitions of quality is to satisfy stated or implied needs or, in other words, to meet patients' expectations. Traceability of cells during IVF is a fundamental aspect of treatment, and involves witnessing protocols. Failure mode effect analysis of a human double-witness system has clearly demonstrated the loopholes and risks of manual witnessing [45]. Automated electronic systems based on barcodes or radiofrequency identification tags can replace manual witnessing [46] and reduce the risk of gamete exchange. It is our experience that such an electronic witnessing system reduces staff distraction and stress, increasing staff efficiency.

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3

KPIs FOR THE IVF LABORATORY

Alison Campbell

Introduction

Key performance indicators (KPIs) are essential tools to monitor and control quality and improvements in the in vitro fertilization (IVF) laboratory.

Numerous variables play into the success and demonstrable performance of an IVF laboratory. These variables include patient management, gamete quality, laboratory and culture environment, equipment, processes, consumables, and the skill and degree of experience of the laboratory team. The IVF laboratory plays a key role in the success of IVF treatments and therefore is often the focus of great scrutiny. In addition, a high number of outcome measures can be used to measure the success of a laboratory, making the design and selection of KPIs complex.

The simplest and arguably the most practical and effective approach to KPI use is to identify high-level, or headline, KPIs, with defined minimum numbers of processes or treatments to enable reliable analysis. Alongside these, the facility to drill down deeper and further into additional indicators, if required, in order to pinpoint and address a potential weakness in the system is beneficial.

These headline KPIs can be useful to identify areas or processes which may need further scrutiny, or to facilitate the efficacy of a change in process or practice. Longitudinal monitoring is also important for trend analysis and to provide an early warning of potential issues.

Contextualizing KPIs

Because of the many factors which can impact the success of fertility treatment and outcomes within the IVF laboratory, including scientific, clinical, environmental, and demographic factors, it can be helpful to try to put KPIs into context, particularly when comparing different laboratories or periods of time. At CARE Fertility, we have developed a simple tool which has proven helpful in contextualizing success rates based on patients being treated within a timeframe. Figure 3.1 shows an example of the output of this tool. It uses four parameters: age of oocyte provider, AMH, oocyte number, and single embryo transfer to classify patients' prognoses from very poor through to very good. The proportion of patients in these simple categories enables us to anticipate and to compare results across multiple clinics in a different way, and to understand and quickly determine if results may not meet KPI targets in a particular time period. More often than not, a period where KPIs may not be met aligns with a period with a high proportion of patients being treated with very poor or poor prognosis, according to the criteria used in this tool. And this has already been predicted.

Using KPIs

It is important to consider the factors which make KPIs valuable in the IVF laboratory. They need to be measurable, reliable, and trusted. Users should have confidence and belief in the quality of the data utilized for calculating these indicators of performance, which will enable their reproducibility and reliability. Use of expert consensus-championed PIs is wise, and enables access to carefully considered levels to benchmark against. As defined in the Vienna consensus for laboratory performance indicators (PIs), the high level, and most important indicators, are referred to as "key"—KPI; and these relate to the core activity in the IVF laboratory. Other indicators, referred to as PI, are helpful for scrutinizing specific areas of practice or process and, whilst they may not be assessed as frequently as KPI, accurate data should be collected to enable more detailed analytics, as needed. Reference indicators (RIs) can also be of use for providing a proxy indication of something, or for benchmarking between practitioners or laboratories, as these indicators relate to aspects that are outside of the laboratory and, as such, are less controlled or influenced.

With so many variables which cannot be controlled by the laboratory (e.g., patient clinical factors) or that the laboratory may have little influence over (e.g., medical practices), as described earlier, whilst somewhat challenging, it is important to ensure that laboratory KPIs are closely, and not tenuously, linked to laboratory activity. Large data sets can help minimize the impact of outliers, although many IVF laboratories may require long durations to collate sufficient numbers, by which time things may have changed.

Reference populations and KPI

Due to the impact that patient factors can have on laboratory outcomes relating to IVF and intracytoplasmic sperm injection (ICSI), along with clinical outcomes, when assessing KPIs, a reference population that excludes outliers and variables which can skew results is recommended. The following criteria for inclusion in the reference population were proposed by the ESHRE and Alpha expert consensus group:

- Oocytes from patients <40 years
- Autologous, fresh oocytes undergoing IVF or ICSI
- Fresh or frozen ejaculated sperm
- No preimplantation genetic testing (PGT)

In the interest of size of data set and considering current practice and outcomes, exclusions could be minimized by revising this list to include vitrified/warmed oocytes within the reference population; considering the age of the patient at the time of oocyte cryopreservation. This is because outcomes between thawed oocytes are widely reported to be similar to fresh oocytes.

Using a reference population for KPI monitoring gives a less heterogeneous set of data and therefore enables more reliable benchmarking of results across different laboratories, within a network, region, country, and beyond; providing that the reference population is agreed by all participants and the data is

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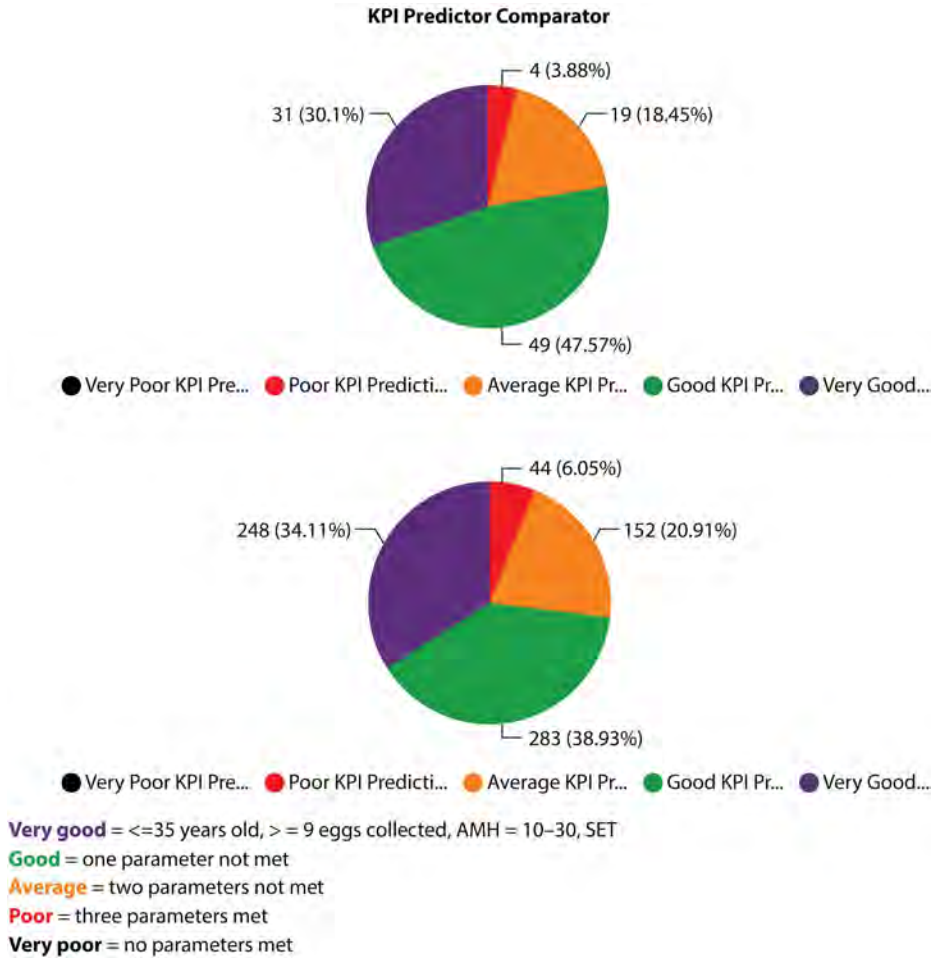

FIGURE 3.1 KPI prediction by simple patient factors.

recorded accurately. Once established, the reference population should be maintained and not revised regularly as this allows longitudinal monitoring and trend analysis to be undertaken.

Having a narrower reference population e.g., oocytes from patients <30 years, can be useful, but unless the clinic is conducting high numbers of treatments, the benefits can outweigh the drawbacks, such as the length of time it takes to undertake sufficient treatments for sample numbers to be of value.

Some clinics prefer to use several reference populations, one being "gold standard" patients. This population should have very good prognosis and may include patients undertaking their first IVF/ICSI treatment, include oocytes from young patients where the number of eggs may be within an "optimal" range, normal semen parameters and single embryo transfer, etc., for example.

Working within a network of clinics

The use of expert consensus guidelines can be an invaluable tool for laboratories to benchmark their own performance against reported industry norms or indicators. However, some laboratories may perform different proportions of complex cases and offer different treatments to other laboratories, and may not gain fully from generic KPIs.

Clinic groups, or networks, are becoming more common and one of the advantages for the laboratories within them is the facility for direct comparisons of KPI according to subpopulations of patients or treatment types, which may not be as straightforward to undertake with laboratories working with less consistency in practice.

Funnel plots are particularly useful in providing visual comparisons of multiple laboratories' performances relating to specific KPIs whilst considering the number of treatments performed, and identifying where they sit within control limits or target values.

Figure 3.2 shows an example of a funnel plot for the CARE Fertility group when they were 10 clinics. During the period depicted, all were performing above the lower control limits. Each diamond represents a clinic plotted within a funnel according to the number of IVF treatments undertaken per year, and their individual fertilization rate for IVF, in this case.

Variation within one KPI: e.g. IVF fertilization rate

Some KPIs have several variations, and users may need to select which works best for them in their laboratory. Convention and

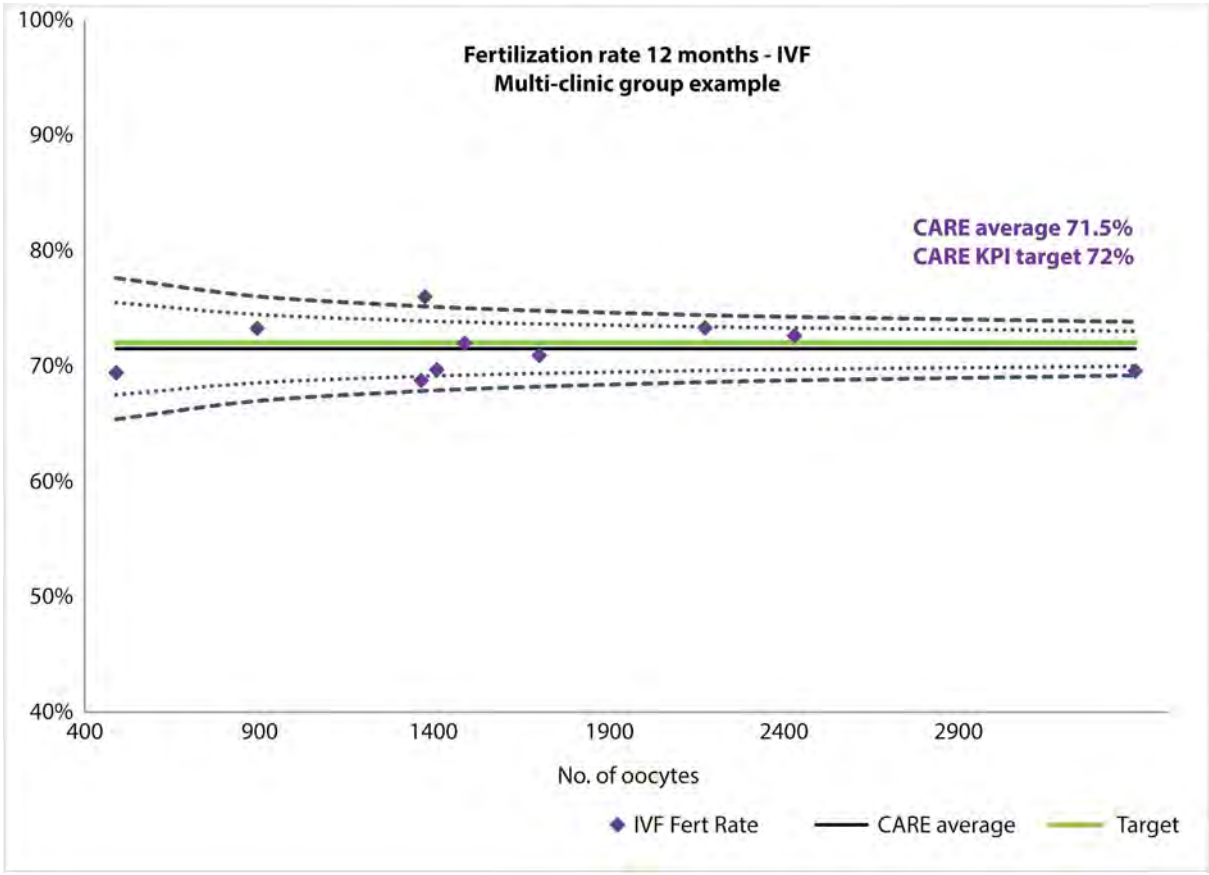

FIGURE 3.2 Funnel plot example for IVF fertilization rate.

expert consensus suggests that the IVF fertilization rate should be calculated as follows:

IVF normal fertilization rate (%) = number of oocytes with 2 pronuclei and 2 polar bodies divided by the number of cumulus oocyte complexes (COC) inseminated (multiplied by 100)

The consensus KPI values associated with this are a competency level of ≥60% and a benchmark (aspirational target) of ≥75% (Vienna consensus 2017).

This is a typical example of a KPI with several, justifiable alternatives. For example, the denominator in this KPI calculation may be impacted by clinical operating practice, which is often outside of the laboratory team's control, deeming it less useful as a KPI if clinical practice is not standardized. For example, follicular flushing, whilst generally not recommended or considered best practice, is still performed in some clinics, or by some practitioners. Flushing of smaller follicles, in particular, can result in the aspiration of less mature cumulus–oocyte complexes and therefore lower "IVF normal fertilization rate." KPI selection, therefore, requires careful consideration and users should be mindful of the limitations of them.

The use of instinct for indicating

Unless updated very regularly, with large data, many KPIs will, by design, be somewhat outdated. Therefore, there remains a place in the IVF lab for continuous scrutiny and challenge; and laboratory staff should remain mindful of the importance of their observational skills, experience, and instinct to detect early signs of potential issues in the laboratory.

In many IVF laboratories 20–30 years ago, there was a daily, audible indicator of quality! The computer-controlled freezers, with the noisy pressure pumps could be heard, often in the afternoons, demonstrating high-quality supernummary embryos being cryopreserved. If this irritating but comforting noise wasn't heard, it was a useful indicator that embryo quality (at least that day) may be poor. In today's busy labs, with flexible working and increasingly varied role profiles, embryologists may have less continuity or spend less time within IVF laboratories to be able to make such sensitive and live observations to flag potential issues ahead of KPI generation.

Interdisciplinary team communication is vital to ensure scrutiny and early warnings. For example, if the ultrasonographer comments that, with the newly introduced gonadotropin, they have noted a different pattern of follicular growth, this should be recorded and an assessment of oocyte maturity and fertilization in the IVF laboratory may be brought forward in this case.

Another real-life example of the importance of observing and reporting the unusual in the IVF lab is the appearance of late vacuolation in the peri-compacting embryo. This may not be detected in core KPI, like blastulation rates, but may be an indication of osmolality deviations in the culture environment, and needs further scrutiny.

Broad shoulders in the IVF laboratory

Some IVF laboratories have a "broad-shoulders" approach to KPIs. Whilst this may seem unconventional and uncomfortable for some, the use of transparent and regular review of individual practitioners' results, by laboratory process, can be highly effective in identifying and ensuring best practice and continuous improvement.

For example, at CARE Fertility, the results of each laboratory team member's own results, for several outcome variables, are automatically and regularly generated and reviewed. For example:

- Fertilization rate by sperm preparation, oocyte recovery, cumulus removal, IVF, ICSI
- Implantation rate by embryo transfer
- Cryo-survival rate by oocyte/blastocyst vitrification and by warming
- Implantation rate by embryo warming

A similar approach is made for medical practitioners.

This system, if utilized, requires clear communication that this is undertaken together, in the interest of our patients and primarily to highlight best practices. It is also very important that everyone involved understands the high number of confounding factors within the IVF process and that these broad-shoulders results are purely considered as potentially indicative and not absolute. If one practitioner is excelling in one area, for example, with 90% fertilization observed following their ICSI, then scrutiny of their practice may be warranted, along with coupling them up with the lower performing practitioners for observational and training sessions.

In general, it appears that the more attention that is given to the small details, and the more visible the IVF laboratory's performance, the better the outcomes.

Published recommended KPIs: Are they still relevant?

A comprehensive and commonly used consensus report of an expert meeting on the development of assisted reproduction laboratory PIs was published in 2017 [1]. Twelve KPIs, five PIs, and two RIs were recommended for "fresh" IVF and ICSI treatments. Competency levels along with aspirational benchmarks were proposed for these 19 indicators. The KPIs are summarized and defined in Figure 3.3.

For cryopreservation, the proceedings of an expert consensus meeting were published around a decade ago. It proposed 14 KPIs with benchmarks for cryopreservation.

With evolving clinical practice, and the associated increase in the proportion of single blastocyst transfer, pre-implantation

KPIs	✓ Competency	★ Benchmark	KPIs	✓ Competency	★ Benchmark
1. IVF Fertilization rate	≥60%	≥75%	7. Day 3 development rate	≥45%	≥70%
2. ICSI Fertilization rate	≥60	≥75	8. Day 5 development rate	≥40%	≥60%
3. Failed Fertilization rate	<5%	<5%	9. Successful biopsy/tubing rate	≥90%	≥95%
4. ICSI degenerati on rate	≤10%	≤5%	10. Blastocyst cryo-survival rate	≥90%	≥99%
5. Day 2 cleavage rate	≥95%	≥99%	11. Cleavage stage implantation rate	≥25%	≥35%
6. Day 2 development rate	≥50%	≥80%	12. Blastocyst implantation rate	≥35%	≥60%

Definitions	
1. 2PN/COC inseminated	7. 8 cell stage embryos/2PN
2. 2PN/oocytes inseminated (ICSI)	8. Blastocysts/2PN
3. Cycles without 2PN/stimulated cycles	9. Amplified DNA/biopsied embryos
4. No. oocytes lysed/inseminated (ICSI)	10. Blastocysts intact/Blastocysts warmed
5. Cleaved embryos/2PN	11. Sacs (ultrasound detected)/cleavage embryos transferred
6. 4 cell stage embryos/2PN	12. Sacs (ultrasound detected)/blastocysts transferred

FIGURE 3.3 Vienna consensus reference indicators.

genetic testing for aneuploidy (PGT-A), and "freeze-all"—whereby embryos are not transferred fresh within the stimulated cycle but vitrified and warmed later for a "frozen embryo transfer" (FET)—several of the established KPIs may not be so relevant now. A number of recent publications consider this, and also ask whether KPIs are transferrable if patient treatment plans change e.g. from fresh to freeze-all [2, 3]. Due to these significant changes in blastocyst cryopreservation practice and results, the Vienna consensus group proposed a KPI for blastocyst cryo-survival.

Another KPI which was not included within the Vienna consensus but has recently been demonstrated to be useful, is day 5 usable blastocyst rate. KPIs such as this have the advantage over clinical outcome-related KPI in that they can be used to detect the efficacy of a controlled change, or raise alert to a negative trend. With the numerator in the KPI calculation being embryo number (as opposed to number of cycles/embryo transfers), this has the advantage of having the potential to detect KPI shifts in clinics with lower cycle volumes [4]. The Vienna consensus was critically appraised soon after its publication [5].

A recent publication explored the potential need for fine-tuning of the Vienna consensus according to female age. Interestingly, it concluded that most laboratory outcome measures were reliable irrespective of female age. However, KPIs relating to extended embryo culture should be fine-tuned to consider female age, due to good quality blastocyst rate being independently associated with it [6].

The future of KPIs

Across IVF laboratories worldwide, there are varied approaches to the use of KPIs, both in the value these are given within the IVF laboratory and in the complexity and detail within the approach, which ranges from basic, *ad hoc* use to highly sophisticated, automated systems.

With a movement toward digitalization and automation within IVF clinics and laboratories generally, KPI monitoring of the future is likely to become less laborious, rapid and sensitive, with the potential to incorporate large and live data sets.

Several commercially available tools already exist to enable laboratory data to be processed semi-automatically, with the facility for laboratory staff to customize reports, graphs, dashboards, and functions. These may use CSV files or other formats. These tools allow users to switch between parameters and subpopulations, and can provide side-by-side comparisons.

As data sets become increasingly detailed and digitalized, and data storage and transfer more flexible, artificial intelligence is likely to be increasingly utilized, along with deep learning, to detect and anticipate IVF laboratory KPIs [7].

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Further reading

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4

QC IN THE CLOUDS

Digitizing Quality Control

Giles Anthony Palmer

> The most profound technologies are those that disappear. They weave themselves in the fabric of everyday life until they are indistinguishable from it.
>
> **Mark Weiser, Computer Scientist [1]**

The rise of quality control to meet the clouds

Quality management is the cornerstone of modern clinical embryology. No longer a novel idea, a long-established doctrine that states that a successful *in vitro* fertilization (IVF) laboratory requires a high level of quality control, ensuring constant improvements by a continuing cycle of implementation, evaluation, reflection, and corrective action [2].

In fact, it is hard to remember when quality control in the IVF laboratory was not so fiercely defended. Nowadays it is either highly recommended or mandatory [3–6], but in the early 1980s, after the birth of the first child following IVF, standards relied on self-motivation; gradually national bodies and societies developed quality guidelines and published best practice guidelines featuring the necessity of having a total quality management system [7, 8].

One of the first to address quality control *per se* was Lynette Scott in 1993, where many aspects of embryo culture were under scrutiny using a mouse embryo bioassay to look at the effects of such variables as type of water, media composition, contact materials, and incubators conditions [9]. Another more clinically oriented study by Matson in 1998 described mechanisms of internal quality control, external quality assurance, and audits as being useful tools for monitoring laboratory performance, cementing the idea in the psyche of the clinical embryologist that "moving the work from being a subjective art form to an objective science" was beneficial to IVF success [10].

Despite this mainstream adoption, the tools of quality control have not progressed greatly in several decades. Most clinics use antiquated ways of recording laboratory data with only a cursory contemplation of the results [11].

Why in this electronic age is data often buried in folders and binders with analysis rare and only examined at times of clinic inspections?

One reason is perhaps that it has not become standardized. Today, the practice of quality control in laboratories across the world can *at the very least* be described as heterogeneous. Knowledge of what each laboratory undertakes to pursue its version of quality excellence is unknown and is based loosely on the legacy of former laboratory managers and the latest opinions on optimum laboratory conditions and manufacturers' recommendations.

The ever-evolving IVF laboratory has a myriad of parameters to check, and with each new addition to the laboratory, every latest piece of equipment brings its own quality control challenges.

What are the factors that we should know intrinsically about our own labs in the process of quality control?

The evaporation rate of our dewars? Or the gas consumption rate of our incubators? The optimum temperature for the heated stage of the intracytoplasmic sperm injection (ICSI) rig or even the best temperature on the warmed surface where the oocyte collection is performed?

We are told that everything in the laboratory is important [12] but to what appropriate level should it be monitored? What are the optimum environmental conditions for the lab? Which are the vitally important checkpoints, and which are superfluous?

To answer these questions, we must go to the root of the problem; quality control is time-consuming and the manner to how it is generally performed can interfere with workflow.

Collection and serious analysis of laboratory data is still hindered by using pen and paper. Any attempt to seriously interpret this data would require at least transcribing to an electronic worksheet but entails a cumbersome step which may introduce errors [13]. In contrast, the use of a mobile application (app) using cloud computing would reduce paperwork and provide a modern, convenient, and insightful way to look at data.

Cloud computing has changed the face of how businesses handle information technology since the mid-2000s [14]. It delivers to any clinic or company computing as a service and, with no in-house servers, provides a network of multiple computers and servers connected to each other over the internet. These services are monitored closely so problems are fixed whenever they occur, and users do not have to worry about maintenance and system upgrades. Cloud computing allows data to be accessed anywhere and creates a remote way of monitoring the periodic quality control data.

Quality control using the cloud has been present for many years in other industries and is an important part of good practices used in fields such as aerospace and defence, pharmaceuticals, manufacturing, electronics, and the automotive sector (Advantive Inc., USA). Stakeholders use this service as a proactive tool to improve product performance where non-compliance, similar to the IVF industry, may lead to fines, operational shutdowns, and stern legal intervention.

Quality control reporting has at its very core the monitoring of periodic drift. Drift may seriously influence the outcome and can signal a decline in equipment performance and well-being if left unchecked. Wadewale and Desai [15] describe six basic types of drifts and if not electronically recorded they can be difficult to recognize, subtle changes may be overlooked, and abrupt changes may be either missed or fleetingly disregarded. Using electronic means allows a way to precisely classify the data, reflect, and adapt to such changes quicker, whether these are sudden, incremental, or gradual deviations, recurring, sudden, or simply "noise" (Figure 4.1).

The "new normal" of work-from-home, work-from-anywhere, flexible hours, and our new mindset [16] make cloud computing

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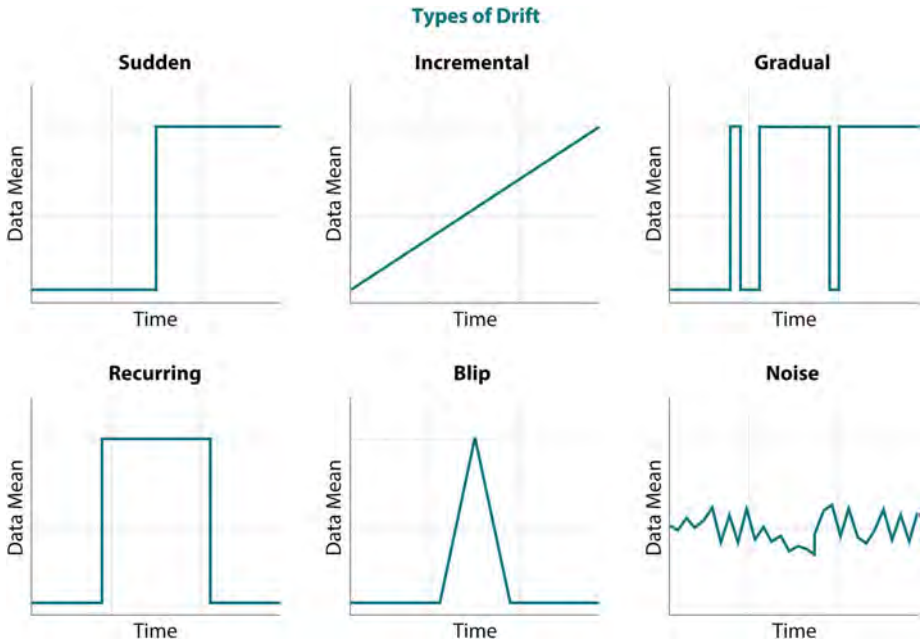

FIGURE 4.1 Types of drift. (Adapted from [15] with permission.)

a prerequisite for following laboratory quality control remotely using timely advances in technology.

One such cloud-based app specially design for the IVF laboratory is Reflections (IVFQC, Althea Science, USA). One of a suite of apps that aims to improve recording and action on quality control data. Accessible from wherever there is an internet connection, it provides the clinic with an inspection-ready electronic ledger of lab logs, recorded parameters, and statistical analysis. Whether data is in paper or electronic form, the records must be analyzed, and this cloud-based application provides a platform to add instrument parameters and perform statistical and fluctuation reports in a clear and concise way (Figure 4.2).

Specifically designed for the IVF laboratory, and accessible by a personal computer (PC), smartphone, or tablet, the format of entering data can be fully customizable to mimic any previous tabulations that were transcribed using paper and clipboard.

In the absence of standardized guidelines and in the void of global information surrounding the quality control practices in the assisted reproductive technology (ART) laboratory this author reported the finding from a study looking at the habits of users of this app [17] in a global setting. This novel "call-to-action" [11] involved a study of 36 laboratories in 12 different countries conducted to assess differences and similarities between laboratories using this adaptable cloud-based quality control app.

Data from equipment and quality control recordings were grouped into domains, according to their function in the laboratory, such as "incubators," "air quality," "heated stages," and "cryo-storage vessels," and the corresponding data was analyzed both individually and as a whole.

It was no surprise in the results that the embryologist attention was mainly focused on the incubators, still the "work horse" of the lab where 50% of all data entries were attributed to this domain, followed jointly by warming stages and cryo-room readings at 11%; 9% of all data points were used for checklists where compliance to certain protocols were recorded electronically on the app.

The study showed the differing global habits and different ranges that clinics accepted for minimum and maximum thresholds such as incubator O_2 and CO_2 concentrations and temperature values in instruments such as warming stages, heated surfaces, refrigerators, and freezers.

Regarding recording daily measurements, the study showed that the participating laboratories all measure incubator parameters, while 91% recorded warming stages and 41% the medical gas manifold. Interestingly, only 36% of clinics manually monitored dewars, despite the recommendations of visual observations being a minimum requirement in risk management of cryo-storage of reproductive tissue [18].

The intensity of data collection also varied between the clinics. To quantify this disparity a surrogate indicator of quality control diligence, the MAD score (mean average data score), was formulated. It highlights the most conscientious clinics by using the number of data entries per day divided by the number of instruments monitored (Figure 4.3). Great variation was observed between the clinics.

The higher the score, the more manual quality control readings were being conducted regardless of the size and volume of work performed by the clinic. If daily checks are to be standardized it would be fair to say that the MAD score would be similar.

It remains to be seen if the laboratories with a higher MAD score enjoy a higher success rate, but, in a follow up study, authors noted that clinics display clear differences in reporting habits, and clinics that have a higher MAD score are in regions which have a rigorous regulatory body [19]

Although equipment in many laboratories is monitored in real time and continuously logged, the frequency of manual inspections should not be underestimated, and at least a daily visual check of equipment needs to be performed [20]. Often delegated to more junior members of the laboratory team, the daily log can help tremendously the young scientists' orientation into clinical embryology and induction into the requirements and trouble shooting skills which are essential traits of the profession [21].

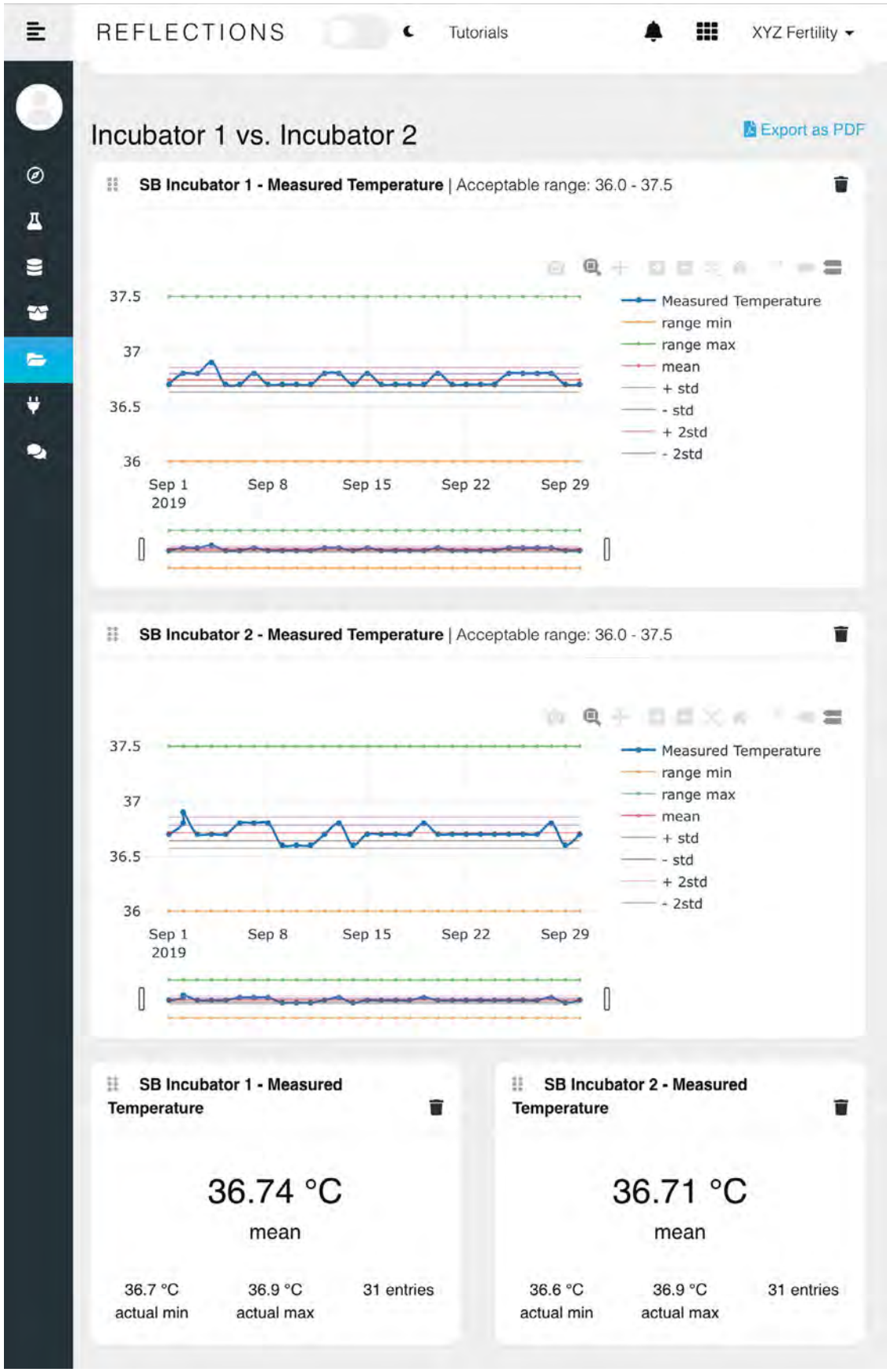

FIGURE 4.2 A typical screen display showing temperature input over time and statistical data in a laboratory setting.

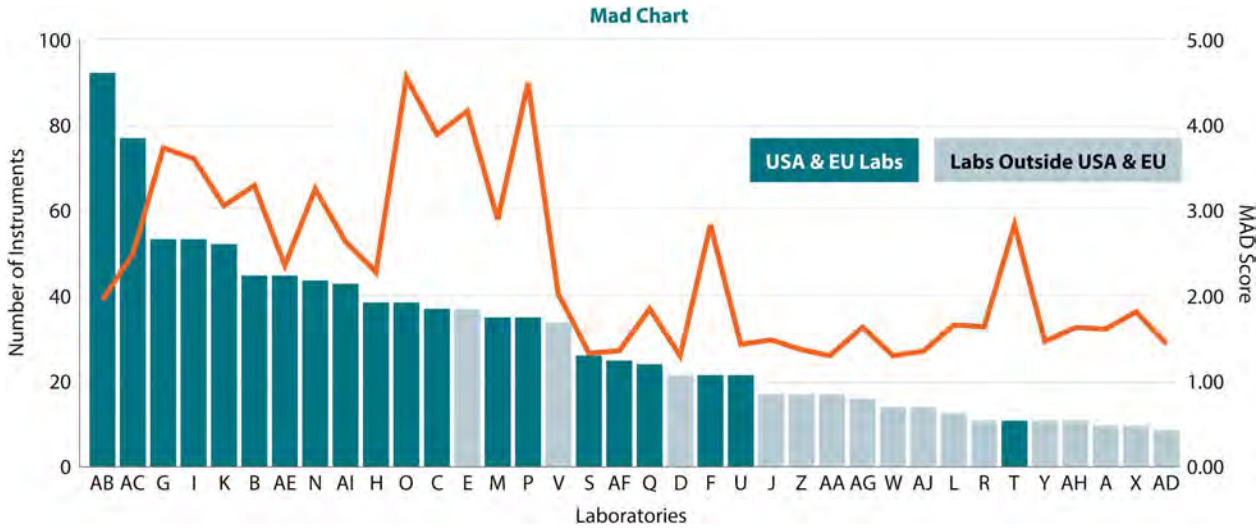

FIGURE 4.3 The highest MAD scores originated from countries where traditionally the IVF health sector had been more rigorously governed; of the top 50% of clinics in this study in relation to the MAD score, 89% were located in Europe or North America. (Adapted from [19].)

Finally, the daily duty of performing an electronic "lab log" can safeguard against data entry being inadvertently missed or ignored. Alarms and reminders may alert the embryologist of any deviation from the desired result.

Simply put, cloud computing gives the broadest access to the data; allowing more senior embryologists to manage single or multiple sites synchronously; following task completion by their staff and reviewing data entry from afar.

When clouds appear like towers

Few would disagree that quality control has made the practices in the IVF laboratory safer, continually on a path of persistent improvement through corrective and preventative actions, but it remains to demonstrate improving clinical outcome. Although cloud computing gives immediate access to statistical and graphical interpretation of laboratory data it remains shielded in "silos," departmentalized, and fragmented from other data streams in the IVF clinic.

A question remains: Can any of these measured parameters be linked with clinical outcome? Notwithstanding there being more than 200 cofounders that can impact IVF outcome [22], every step of the patient's journey is now traceable through electronic medical records; from first rendezvous, diagnosis, and treatment planning to tracking and tracing gametes and embryos during their *in vitro* residency and subsequent fate. Surely linking this data to quality control data is the first step to resolving this problem.

Despite the fear of leaving a clinic vulnerable and exposed to litigation in the event of an adverse condition, each clinic with a robust quality control system should not be duly concerned. At last, we should be able to use all the data from a clinic like "business intelligence" where insights into performance, goals, and operations can be analyzed, forming data-driven decisions for improving success.

While many innovations in our field have been adopted quickly into the IVF laboratory, there appears to be some resistance to change concerning data processing and digitization of quality control data. Responsibilities of the clinical embryologist have changed in recent years, giving rise to increasing screen time as growing demands on regulations and documentation changes the daily routine to include extensive administrable duties [23]. Perhaps the duties entailed in quality control are unpopular tasks seen as inconveniences and time-consuming.

To avoid the dissatisfaction that has been cited in similar circumstances, with clinicians asserting that electronic clerical duties are overwhelming, compete with patient "facetime" [24, 25], and lead to burnout [26], we must work smarter to avoid these failing. Indeed, embracing this flurry of interest in automation, AI and big data may be a necessary watershed in the role of the embryologist where technical skills might become less important while a growing leaning toward information technology will prove advantageous [27].

The digital lab

We have always belonged to an industry moving at a fast pace incorporating advances from various areas of science in its 40-plus years of development [28] with a penchant toward refinement with the promise of automization [29, 30] and recently the use of big data and AI [31] to make our decisions more precise, more accurate, and repeatable. Until this goal is reached, clinical embryology remains very different to other medical labs; it is largely manual in nature, requiring good hand–eye coordination with precise, delicate skills taught through a process of practise and competence assessments [32].

However, the workplace itself is changing, with veteran members leaving the workplace giving rise to new generations of embryologist better equipped to deal with the legacy of this expansion of information technology in our industry [33].

"Liquid expectations," the expectations that the user experience (viz. young embryologists and patients alike) that is enjoyed in other technologically advanced activities like online booking, streaming services, e-commerce, and logistics [34], may very well seep into this industry, heralding a quicker implementation of new digital innovations.

Indeed, more and more assignments are heading for the cloud—pushing everything to be accessible anywhere and anytime.

The increasing use of the smartphone has created better opportunities of engagement for patients and ART professionals alike [35], seeing that a transparent, compliant electronic audit trail is preserved. Telemedicine is now widespread within the health services [36, 37] and is benefiting from this integration accordingly. Patients can feel more engaged using an app such as the "SART mobile," launched by the Society for Assisted Reproductive Technology [38], which connects patients with resources and information about infertility in the United States, while Salve (Salve Technologies Limited, UK) uses a cloud-based "patient engagement platform" to streamline client workload to improve the patient experience.

Even home sperm assessments through mobile devices are being explored, measuring accurately and precisely motile sperm concentration all from a convenient smartphone [39].

For the embryologist, too, there are apps such as ART Compass LLC and the Proficient Lab LLC that use cloud computing for many aspects of laboratory quality management that were previously confined to the PC. Mobile access to training records, benchmarking, personal KPIs, and more are pushed to the phone or tablet, eliminating paperwork, optimizes training and competency records while keeping the clinical embryologist engaged in their person development and obligations.

As an increasing number of companies are offering novel products to the ART industry, compatibility and connectivity between various appliances and instruments has becomes a concern. This synergy has been accelerated through the use of an application programming interphase (API) allowing any two systems to link cloud services together. Like an "electronic handshake" these secure keys form an important development to facilitate a more extensive service where various desirable features from different companies can be easily accessible remotely on a single device.

One area where APIs are crucial is in the monitoring of crucial IVF laboratory equipment 24/7. Mandatory in many countries [4, 40], we require a modern way of following mission critical streaming data. While many systems rely on a relay system of multiple points of telecommunications or radio frequency transmitters to alert clinic staff, there are now available easy-to-install smart devices. These sensors can send real-time monitoring data direct to the cloud without intermediate servers via an API, completely bypassing any traditional networks and providing a system without routers, servers, or access points (PharmaWatch™, American Pharma Technologies LLC, USA).

Unavoidable risks in cryo-storage, too, may be reduced through several recent advances concerning the safety of gametes and embryos stored onsite. There has been a call for reckoning [41] following catastrophic events in 2018 that resulted in the loss of thousands of cryo-stored oocytes and embryos [42, 43]. Tank surveillance through the cloud, especially using thermal imaging (Cryosentinel LLC), offers a quick visual interpretation of dewar integrity and may well appease current fears about reaction times in the event of a catastrophic vacuum failure [44].

Also, new technologies may aid current short comings in the chain of custody. Quality control for decades has been assisted by the traceability and accountability given by electronically witnessing laboratory events. This "benchtop tagging" using either barcode or RFID tagged samples (IMT Matcher™, RI Witness™) assures compliance with protocols and reduces mismatch errors through proximity alerts in the embryology lab; and it has been absent from the cryo-room.

However, location tracking using thermo-tolerate RFID tags either submerged in liquid nitrogen itself [45] or embedded in a capsule [46] can now locate samples from within the dewar, and with the use of an automated storage system (TMRW Life Sciences Inc) can alleviate the need for customary inspections and audits, greatly improving frozen sample management through novel software and the use of robotics.

Tracking samples in transit would complete the "cold-chain-of-custody" using a specialized dry shipper dewar where transportation of frozen gametes and embryos can be closely scrutinized using cloud computing. Already, conditional monitoring of many metrics other than just core temperature such as orientation, proximity, and shock, can be relayed to and from the cloud (SmartPak™, Cryoport Systems, LLC), mitigating risk and providing assurance for clinic and patient alike to the correct transport conditions and precise location of the precious cargo.

Since introduction in the mid-1990s, mobile phones have become firmly embedded in our society. The advent of 3G in mid-2001 offered mobile data handling capabilities which rapidly diffused [47], and the recent emergence of 5G technology will bring major improvements to cloud-computing services with low to zero latency and connections to devices, not just phones.

The evolution of the mobile technology has progressed from a simple communication device to a ubiquitous tool for data analysis [48], opening up possibilities of quick and easy access to laboratory quality data at our fingertips, but at some cost. . . .

Dark clouds

Increased access to clinic data does have its drawbacks. Safety of data storage is clearly paramount, and many countries have strict guidance for the transmission of data both within the walls of medical establishments and in the clouds.

Such is the case in the United States, where the Health Insurance Portability and Accountability Act (HIPAA) is a series of federal regulatory standards that outline the lawful use and disclosure of health information. It consists of three major components—privacy, security, and breach notification rules. All data usage must be HIPAA compliant [49].

Similar strict laws exist in Canada that govern personal and identifying data with the Personal Information Protection and Electronic Documents Act (PIPEDA) [50]; in Australia with the Privacy Act (Privacy Act), both recently amended to internet-based data handling [51]; and the General Data Protection Regulation (GDPR), a regime of personal data protection requirements adopted by the European Parliament [52]. Security and privacy with cloud computing remains a constant battle, with many services using two-factor authentication and end-to-end encryption (familiar to users of mobile banking services), resulting in a more secure way to store and precure data, but the industry must always be watchful.

Cybercrime flourished with the increase of home/remote use of systems in the light of the 2020 COVID-19 pandemic [53], and robust measures must be in place to prevent malicious access of criminal activities, ensuring clinics stay vigilant to cybercrime, and in particular ransomware attacks, which has led to debilitating consequences in several clinics worldwide [54].

Serious consequences ensue for any breach of confidentiality, and whereby most security breaches happen as a result of staff oversight, not familiar with data security, it is recommended regular data awareness courses would help the "end users" understand their obligations learning data handling etiquette [55] and remaining being vigilant to digital threats.

We may be becoming more connected as a society, but because of security issues, data fragmentation is a major problem that

prevents countries and organizations from sharing information. This may be solved by blockchain technology: using the cloud, or rather multiple networks within clouds, information packages can be uniquely tagged "block by block" onto the existing data. This creates a chain of information, an immutable ledger [56], that can be tracked and verified floating in the ether. Most recognized for its use in cryptocurrencies, this recent development has opened opportunities for the healthcare sector, offering an effective way of exchanging data and research material across health systems and even borders [57].

Finally, the system cannot "go down." When relying on a third party to manage your data handling needs business continuity must be maintained at all times. This is being addressed by large cloud-computing providers who consistently back up data and employ redundancy within their electronic architecture to ensure that an individual failure has an immediate fall-back system. Additionally, "cross region deployment" of cloud servers scattered in different geographic areas and back-up "warm standby" servers aim to produce a "fault-tolerant service" [58].

QC in the fog

Sensors are getting smaller. Micro-electro-mechanical systems (MEMS) have enabled simple and inexpensive data collection in smart devices with low power usage, facilitating the emergence of networks of interconnecting devices in what has been described as the "internet of things" (IoT) [59].

This "ecosystem" of electronic devices where network connectivity and computing capability extends to objects, sensors, and everyday items not normally considered computers, allows these devices to generate, exchange, and consume data with minimal human intervention.

The internet of people becomes the internet of things and would not be possible without harnessing cloud computing fuelled by the development of the mobile network standard 5G [60, 61] offering super-fast connectivity.

According to Cisco Systems, Inc. there were 7.6 billion active IoT devices in 2020, a figure which will grow to 24.1 billion by 2030 [62]. At present, this technology is most visible in "smart homes" where home appliances are monitored and controlled via the world wide web [63, 64], but is rapidly being deployed in many other domains such as intelligent grids, waste management, farming, and energy management. There is increasing interest from healthcare markets [65] with a tremendous potential of IoT to improve patient safety, staff satisfaction, and operational efficiency.

The greatest body of work has been done with diabetes sufferers, with several applications using IoT smart insulin pens as a continuous glucose monitoring device preventing hyperglycaemia and hypoglycaemia [66].

Other "smart devices" are being trialled for the treatment of such conditions as asthma [67] and Parkinson's disease [68]. Intelligent medicine packaging using embedded sensors and minute RFID tags can shadow patient compliance to drug administration at home monitoring when the package seal has been opened [69].

Familiar to smart watch owners and "life loggers" there are many wearables bordering on being called a medical device. Interacting with skin directly or through clothing, these devices can aid health and well-being; many patents now exist to measure specific human physiological parameters such as heart rate, respiration rate, and blood pressure [70].

Similarly, haptic or touch technology applies the forces of pressure or vibration to interface with the operator. This "force feedback" can be most recognized in gaming consoles and has been present on wristwatches and health monitors in the form of tapping since 2015 [71].

Indeed, Amazon has patented a haptic wristband to steer storeroom employees to the correct inventory and alert them if their package is incorrect [72].

Could this *new* sensory perception guide embryologists in their operations in the IVF laboratory and prevent mistakes?

It would not be a large stretch of the imagination if these new devices could log our activities around the IVF laboratory, tracking our movements and monitoring our equipment, offering complete transparency?

One study describes quality control in an IVF setting using a network of well-positioned IoT sensors measuring real-time key environmental parameters such as temperature, humidity, and volatile organic compounds (VOCs) content [73].

IoT, together with AI, is already used in quality control systems to maintain industrial machines and can analyse drift and predictive maintenance. The system can be further trained to predict the remaining useful life of the machine before it requires maintenance or replacement [74].

Equally, we may be able to harness this tech to detect changes in our daily routine and act as an early warning system, monitoring performance, self-diagnosing a fault, and sending for an engineer!

The ideal place to analyse and act on most IoT data is near to those very devices, and to this aim, "fog computing" has been developed. Fog in nature is low-forming cloud, and this phrase, coined by Cisco Systems, Inc. in 2015 [75], denotes a decentralized computer system consisting of "nodes" capable of performing both networking and computational operations at the same time that is closer to both the ground level and the user.

Fog computing promises low latency, as these nodes are closer to the user and can provide instant responses, require little bandwidth owing to the pieces of information are aggregated at different points along the network with no loss of connection.

High security is assured by the huge network of nodes in a complex distributed system with the possibility of blockchain security [76].

In its classical usage, quality control in the IVF laboratory is simple documentation to show that equipment and instruments are functioning within a predetermined range, but using IoT and wearables we could move toward a passive method of data collection leading to complete transparency of all procedures in the lab.

What started with the adoption of technological solutions for electronic witnessing in the early to mid-2000s [77] could now lead to a recording system finally not interfering with the workflow to create big data on everything connected to the lab!

As we draw closer to "ubiquitous computing," first described by Mark Weiser [78], where we will have access to computing anywhere and in any situation, we rely on the technology of cloud computing to deliver us fast, reliable, and secure computing from any location.

In our own setting, a mix of mobile telephone applications, continuous monitoring systems safeguarding critical equipment, numerous IoT devices, and wearables could track and monitor events and conditions around the IVF laboratory. Precision timing of laboratory events, compliance to protocols, staff competency, and equipment monitoring could all be effortlessly recorded and monitored. High tech, low impact on workflow, these innovations could change the way we view quality control data collection in a "smart lab" in the near future (Figure 4.4).

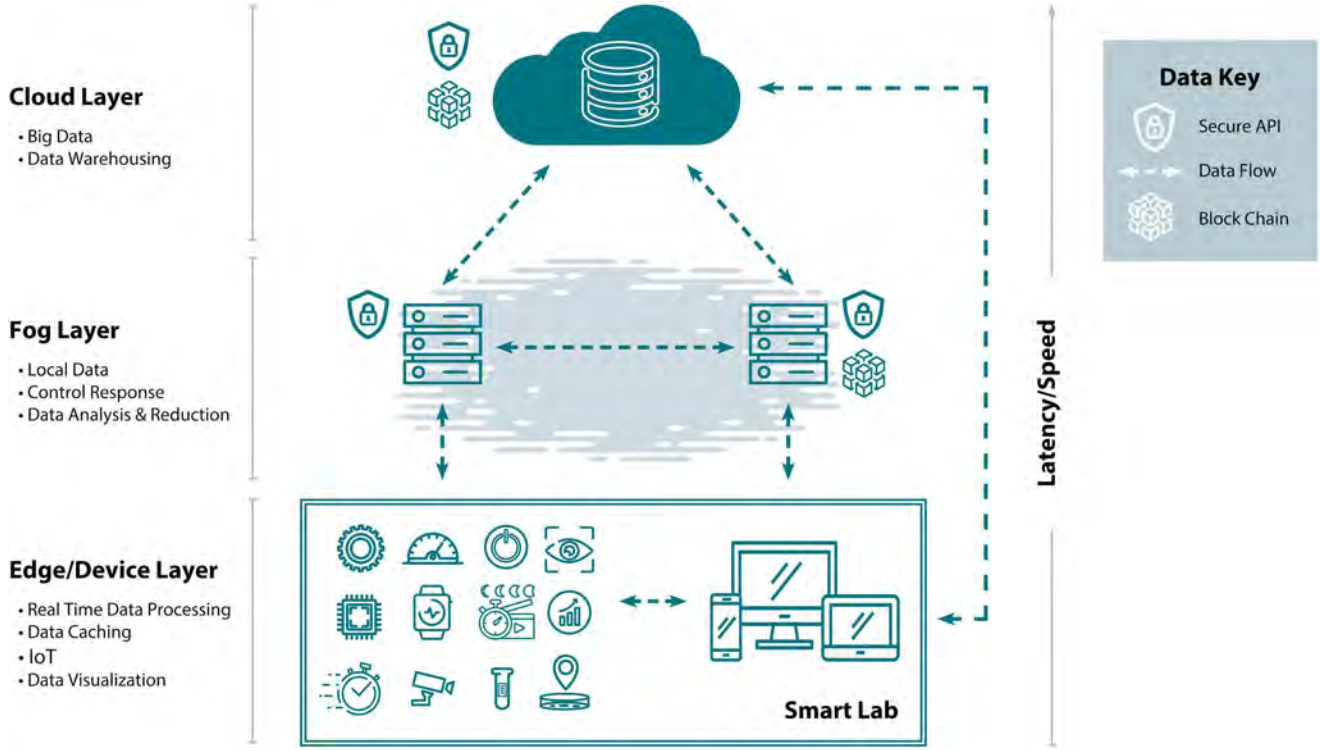

FIGURE 4.4 Smart lab.

As processes in the ART laboratory have constantly been improved and refined with the implementation of quality management, it is time to realize that data can now be processed with minimal effort and inconvenience with the maximum computational power using existing cloud services.

Quality control in the IVF laboratory can surely only benefit from a more digital integration and a more active uptake around the world by embryologists; it only remains to be seen if this forecast of new technologies can give a brighter outlook into the way we perform quality control—giving this cloud a silver lining.

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5

THE ASSISTED REPRODUCTION TECHNOLOGY LABORATORY

Current Standards

Cecilia Sjöblom

Introduction

Quality assurance (QA), quality control (QC), and accreditation are concepts that seem to touch on a wide range of functions in our society. QC systems and standardization are especially needed in units for assisted reproduction technology (ART) to ensure the reproducibility of all methods and that all members of staff are competent to perform their duties. The necessity of a QC system becomes even clearer when considering the possible risks of ART.

Over the years that ARTs have been practised, extensive knowledge has been gained on how to run an ART laboratory and what methods to use to achieve ultimate success. Facing the future, we encounter other variables such as the safety and efficiency of the laboratory, and quality and standardization become key features. Professional, national, and international guidelines on how ART should be performed have been established over the years, and many countries have legislation concerning how ART should be practised [1]. Among others, England, Australia, and the United States have instituted a system whereby the ART clinics have to be licensed to practise these techniques and the clinic and the laboratory are audited by a third-party authority in order to ensure correct practice [2–5]. However, with the increased knowledge of the importance of implementing quality systems, most clinics choose to conform to any of a range of available standards.

This chapter first provides an overview of the most common laboratory standards together with some regional/national guidelines and regulation. Then, it provides a simple "how-to" guide for laboratories seeking to conform to internationally recognized standardization. Then, most importantly, it goes beyond the standards to establish some key determinants of success, which are interdependent for maintaining high-quality standards, safety, and improved results in the *in vitro* fertilization (IVF) laboratory.

Standards

International standards and regulatory frameworks

International Organization for Standardization (ISO) 9001, with its current version 9001:2015 Quality Management Systems—Requirements [6], is the most widely used standard in ART clinics and involves the quality system of the whole organization. This standard covers the need for quality management and the provision of resources (both personnel and equipment), and a substantial section involves customer satisfaction and how to improve services. A more detailed overview of ISO 9001:2015 is presented in Chapter 32 [7].

ISO 17025:2012, specifying general requirements for the competence of testing and calibration laboratories [8], is the main international standard for laboratory accreditation. It is based on the European norm (EN) 45001 [9] and was originally modelled on the corresponding ISO/International Electrotechnical Commission (IEC) guide [10]. The scope of this standard is specialized and is aimed towards assurance of methods and includes both the quality system and the technical part of the activities such as validations of methods, QA, QC, and calibration of equipment. In 1997, Fertility Centre Scandinavia became the first IVF laboratory to be accredited according to this international standard [11].

With an increase in laboratory accreditation, it was evident that ISO 17025, aiming to standardize testing and calibration laboratories, could not fully accommodate and cover the complexities of a medical testing laboratory. ISO 15189, on medical laboratories, particular requirements for quality, and competence, was issued to aid the accreditation of methods used in medical testing. It was first issued in 2003, with the current fourth edition issued in December 2022 [12]. It is used for the accreditation of medical laboratories and brings together the quality system requirements of ISO 9001 and the competency requirements of ISO 17025 and addresses the specific needs of medical laboratories.

Most medical laboratories in Europe and Australia are accredited according to ISO 15189. There are differences between the two laboratory standards, with ISO 15189 focusing on patient outcome without downgrading the need for accuracy, and it emphasizes not only the quality of the measurement but also the total service provided by a medical lab. The language and terms are familiar to the medical profession, and it highlights important features of pre- and post-investigational issues while also noting ethics and the information needs of the medical laboratory. ISO 15189:2022 is risk-based and addresses the need for equivalency of quality management systems and competency requirements between laboratories. The need for this becomes more obvious at a time when potential and actual patients are increasingly mobile—the systems to collect medical data on these patients must be standardized independently from their location.

IVF laboratories located in the European Union (EU) are required to adhere to the Directive on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells, usually called the European Union Tissue and Cells Directive (EUTCD) and its guide/supporting documents [13–17]. The European Society of Human Reproduction and Embryology (ESHRE) has issued a position paper on the EUTCD [18], and it is important to underline that regardless of ESHRE's recommendations, each EU country interprets the Directive differently. However, one part of the EUTCD is very clear: the demand for a quality system. The Directive states that "Tissue establishments shall take all necessary measures to ensure that the quality system includes at least the following documentation: standard operating procedures (SOPs), guidelines training and reference manuals." Certainly, by achieving accreditation to ISO 15189, this demand will be fulfilled along with several other demands of the Directive. The EUTCD is currently being updated, and

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in July 2022, the European Commission published a "proposal for a regulation of the European parliament and of the council on standards of quality and safety for substances of human origin intended for human application and repealing Directives 2002/98/EC and 2004/23/EC" [19]. It will take into account technological, epidemiological, and clinical developments in ART [20]. While welcoming the initiative and being largely supportive of the proposals, ESHRE is preparing a position statement on the proposals [21].

Joint Commission International (JCI) is a non-profit organization with the main focus on improving patient safety accredit, and they certify hospitals and healthcare organizations worldwide. JCI has a range of standards including Accreditation Standards for Clinical Laboratories [22]. The World Health Organization (WHO) in collaboration with JCI has developed a core program for patient safety solutions. It brings attention to patient safety and practices, which can help reduce the risks involved with medical procedures. The most recent advice builds on "nine patient safety solutions" including patient identification and recommends actions in four basic categories: (i) risk management and quality management systems; (ii) policies, protocols, and systems; (iii) staff training and competence; and (iv) patient involvement [23].

The Clinical and Laboratory Standards Institute (CLSI) is another global not-for-profit standards development organization, and while mostly applicable to the United States, the CLSI standards are of great help for improving laboratory quality and safety [24].

In the United States, the College of American Pathologists (CAP) in collaboration with the American Society for Reproductive Medicine (ASRM) has developed a standard that meets requirements of reproductive laboratories. The Reproductive Accreditation Program promotes the quality and safety of laboratories and is recognized by the Society for Assisted Reproductive Technologies (SART) to meet membership requirements for IVF facilities [25].

Other standards that might be less suitable for the IVF laboratory are the Good Manufacturing Practice/Good Laboratory Practice (GMP/GLP) guides. These standards apply to research laboratories and the pharmaceutical production industry. They include demands on the laboratory facilities that will be difficult to meet with the limited resources that many IVF clinics have [26, 27].

In addition to these quality system-driven standards, there are many IVF-specific standards and guidelines including WHO laboratory manuals for the examination and processing of human sperm [28] and the Alpha/ESHRE consensus papers on embryo assessment [29] and ART laboratory performance indicators [30]. The Alpha consensus group has published a consensus for cryopreservation establishing key performance indicators (KPIs) and benchmarks for both slow freezing and vitrification [31].

National and regional standards

While the ISO standards cover the fundamental needs for quality systems in the IVF laboratory, many regions and countries have specific guidelines, laws, and regulations. It is important to note that while some of these regulatory frameworks are standards and others are license requirements or law, when it comes to inspections and audits, the laboratory is expected to conform.

Europe

With the EUTCD in place, all IVF laboratories handling gametes and embryos are required to have a quality system and to fulfil the demands of the Directive and the national interpretation of it. This has led to most of the IVF laboratories in the EU holding or working towards formal accreditation to ISO 15189 or ISO 17025. As described earlier in this chapter, ESHRE has published revised guidelines for good practice in IVF laboratories, providing an easy-to-navigate guide to support laboratory specialists and also to fulfil some of the demands of the Directive [32]. In the UK, where all IVF clinics are required to be licensed by the Human Fertilisation and Embryology Authority (HFEA) [33], there are further guidelines regulating the IVF laboratory as detailed in the HFEA Code of Practice (HFEA CoP) [34]. Specifically, the CoP contains demands for risk management, sample identification, and embryology staffing as described later in this chapter. Following the departure of the UK from the EU, new HFEA EU exit legislation was introduced [35]. Following this, the HFEA has made changes to the standard licensing conditions reflecting some of the core requirements of the EUTCD with regards to laboratory air quality, traceability, medical devices, and importing of tissue [36].

Australia and New Zealand

In Australia, the Reproductive Technology Accreditation Committee (RTAC) undertakes the licensing of IVF clinics. While the RTAC CoP [5] is far less comprehensive than its UK counterpart, it contains critical criteria with a focus on risk management, staffing, and sample identification as well as further guidelines covering the requirement of a quality management system. In addition to the code, Fertility Society Australia issues technical bulletins, which act as educational communication to all units and certifying bodies, offering advice and guidance. It is not enforceable [37]. In New Zealand, RTAC licensing is optional, but most clinics hold an RTAC license. While the majority of IVF clinics hold an ISO 9001 certification, most are also accredited by National Association of Testing Authorities (NATA) to ISO 15189 for some of the crucial methods such as semen analysis. However, very few laboratories hold ISO 15189 accreditation for the overall IVF laboratory processes.

Asia

At the time of publication, there were very few IVF laboratories in Asia accredited according to ISO 15189 or similar standards and the laboratory accreditation was not widespread. However, there is an increased interest and need for standardization. Many private IVF centres throughout the region have ISO 9001 certification.

Memorial Hospital in Istanbul, Turkey, was the first IVF laboratory in the region to achieve ISO 15189 accreditation (acknowledging that Turkey is a transcontinental country at the junction of Europe and Asia).

With the introduction of strict regulations of ART in China, it has become increasingly challenging to obtain approval to operate an ART centre. The Ministry of Health issued the first series of regulations on ART in 2001 which remain in current [38]. These regulations have detailed requirements with respect to facilities, staff, equipment, clinic management, QA/QC, indication and contraindication of IVF, intracytoplasmic sperm injection (ICSI), egg donation, and preimplantation genetic testing (PGT), among others, along with the ethical aspects of various issues. In addition, since 2006, the department requires control inspections of ART providers to be done every two years and that there is provision for accredited training of ART specialists. The Chinese Society of Reproductive Medicine of the Chinese Medical Association is actively engaged in detailed

ART treatment guideline establishment and implementation. In 2015, the Health Commission issued the Guidelines for the Configuration Planning of ART (2015 edition) to guide the scientific planning of all provinces (autonomous regions and municipalities) for the development of ART from 2015 to 2020 with a new edition published in 2021 [39]. It is proposed in the Guidelines that a Plan for the Application of ART (2021–2025) be formulated by all regions, with the medical institutions carrying out ART in their respective provinces (autonomous regions and municipalities). It is clarified in the Guidelines that, when formulating this Application Plan, all regions shall pay more attention to guaranteeing social public welfare, optimizing the efficiency of resource supply, and improving the service quality. The compilation principles include quality and safety priority, classified guidance and management, reasonable planning and layout, and stable and orderly development [39].

ART in India is governed by the recently gazetted Assisted Reproductive Technology (Regulation) ACT (December 2021) and a law on surrogacy (January 2022) [40]. The Indian Council of Medical Research (ICMR) has issued National Guidelines for Accreditation, Supervision, and Regulation of ART Clinics (not legislated) [41]. The Guidelines cover issues such as staff qualifications and laboratory procedures, but neither the ACT nor the ICMR Guidelines have a formal demand for quality systems. In November 2021, the Indian Society for Assisted Reproduction (ISAR) published Consensus Guidelines on Safety and Ethical Practices in In vitro Fertilization Clinics with the aim of helping embryology laboratories across India to standardize their practices and improve outcomes [42]. The guidelines cover a wide range of topic but importantly highlight the need for QC, staffing training and qualifications, safety/risk management, and ID and traceability. ISO certification is not widespread for individual IVF clinics, but larger hospitals that have IVF departments are commonly ISO certified.

In Japan, IVF services have historically been charged out of pocket with a Grant system covering some of the costs; however, this was changed at the end of March 2021. From April 2022, IVF and related services are covered by the national insurance system. Leading up to the introduction of IVF in the national insurance system, the Japan Society of Obstetrics and Gynecology (JSOG) developed a clinical guideline which not only forms the basis for what to include and what not to include in the insurance system but also provides some guidance on the evidence level for treatment practices and technologies. Even though JOSG has issued other guidelines covering IVF, there is no single guideline that comprehensively covers laboratory practices and the need for quality systems. As a result, some clinics have created their own umbrella organizations for implementing common quality practices within IVF called the Japanese Institution for Standardizing Assisted Reproductive Technology (JIS-ART) [43].

In Singapore, the Ministry of Health has introduced stringent licensing requirements for assisted reproduction services in private hospitals and clinics. It covers demands for QC, facilities, embryology training, and sample identification [44]. All IVF providers in Singapore are accredited according to the international version of the RTAC CoP [45].

Middle East

ART in many Muslim countries is covered by a number of fatwas (religious opinion concerning an Islamic law issued by an Islamic scholar) [46]. The first fatwa relating to ART was issued in 1980 by His Excellency Gad El Hak Ali Gad El Hak, the Grand Sheikh of Egypt's Al-Azhar Mosque. The core requirement is that the couple is married, and the use of donor sperm or oocytes is prohibited [47].

Apart from the fatwas, there are very few regulations and standards for IVF laboratories in the Middle East and few laboratories are formally accredited to international standards, but many larger hospitals hold JCI and ISO accreditations.

Saudi Arabia has a comprehensive fatwa containing demands for documented SOPs, safeguarding of sample ID, and prevention of mix-ups, among others. The Ministry of Health has started setting standards, and some centres have had audits by the authorities [46].

Centrally located, luxurious, and tourist friendly, the United Arab Emirates (UAE) attracts IVF patients from all over the Middle East and Europe. The UAE has stringent laws regulating IVF and requires that all centres are licensed by the Department of Health (DOH) with all laboratories being regularly audited. The Federal Law No. (07) Concerning Medically Assisted reproduction was updated in 2019 [48], with the DOH Standard for Assisted Reproductive Technology Services and Treatment issued in 2022 to direct the implementation of the new law. The Standard demands that laboratories must obtain accreditation either from the CAP Reproduction [25] or ISO [12]within 6–12 months of establishment. Existing laboratories are required to meet these accreditation demands by February 2023 [49]. The regulations include demands on the embryology staff having master's degree and PhD and stipulates requirements for ongoing training, laboratory facilities, documented protocols and procedures, and QC.

A recent review by Dr Chokri Kooli provides a great overview of ART laws and regulations in Muslim countries in the Middle East and beyond [50].

Latin America

Registro Latinoamericano de Reproduccion Asistida (Red LARA) covers most of the Latin American clinics. Although membership in the organization is voluntary, 70%–80% of clinics participate in the data collection, accreditation, and continuous professional development training programs. The accreditation includes external audits and follows the Standard Rules for the Accreditation of the ART centre and its laboratories of embryology and andrology [51] involving, among others, QC, KPIs, staff requirements, equipment, and materials.

Each country has individual health authority regulations that must be adhered to when establishing a new IVF centre/laboratory, with many taking advice from colleagues in the United States. ASRM is offering accreditation of laboratories outside the United States; however, more commonly, embryologists establish laboratory facilities and processes according to their knowledge considering their financial possibilities and what they learn from clinics from around the globe.

Each country also has local embryology societies. In Argentina, Asociacion para el Estudio de la Biologia de la Reproduccion (ASEBIR) has a special interest group on laboratory quality for the members of the society. ASEBIR has developed a calculator named Cassandra that calculates the staff number and time required for performing all embryology laboratory activities in order to assure safe staffing levels [52].

North America

In 2004, the Canadian federal government passed the Assisted Human Reproduction (AHR) Act with Health Canada holding

the responsibility for federal functions related to assisted human reproduction [53, 54]. In 2016, Health Canada announced that they intended to make sections of the Act enforceable, notably section 10 of the AHR Act, on the Safety of Sperm and Ova Regulations which came into force in 2020 [54]. The Guidance Document Safety of Sperm and Ova Regulations covers a range of areas such as gamete and embryo handling, quality management, staff requirements, and facilities [55]. Additionally, The Canadian Fertility and Andrology Society (CFAS) has published a number of clinical practice guidelines and is working towards comprehensive professional standards concerning the laboratory activities involved in IVF prepared by its ART Lab Special Interest Group, along with training and competency requirements that include the continuing professional development of all ART laboratory scientists [56].

In the United States, the practice committees of the ASRM and the Society for Reproductive Biologists and Technologists (SRBT) have recently issued updated comprehensive guidelines for human embryology, andrology, and endocrinology laboratories [57, 58]. This publication provides guidance on embryology laboratory staff minimum requirements for education, training, continuing education, and experience. It further gives a very clear overview of US laboratory certification and accreditation requirements, regulatory obligations with regards to the US Federal Food and Drug Administration (FDA), and implementation of Quality Management Systems [58].

How to achieve laboratory accreditation

It is important to underline that in no way are all the quality standards independent of each other. The new ISO 15189:2022 is modelled on ISO 17025:2017, with the major difference being the medical laboratory terminology used in ISO 15189. In turn, both are aligned to ISO 9001:2015, and as such, the new ISO 15189 standard is risk based with the patient in focus prompting laboratories to take the risk to patients into account in both processes and quality management. ISO 15189:2022 also allows for more flexibility than previous standards with formulations like "where applicable" following "shall" stipulations. This allows the laboratory to take their own circumstances into account when applying the standard. The quality system requirements of both standards (17025 and 15189) are based on ISO 9001. As a result of this, laboratories within ISO 9001-certified clinics seeking accreditation will have major parts of the system requirements of the two laboratory standards already in place. It could be recommended that the first step towards accreditation is to get the clinic certified to ISO 9001; further details on this subject are found in Chapter 32. The requirements discussed throughout the continuation of this part of the chapter will be for laboratory accreditation to ISO 15189 or ISO 17025 on top of (over and above) what is already required for certification to ISO 9001. For example, scope, organization, and document control are found in all the standards, and many of the demands are the same, but the requirements further discussed in this chapter will be what ISO 15189 has (hereinafter referred to as the standard) in addition to what has already been implemented through ISO 9001 certification. Correlation tables for ISO 9001, 17025, and 15189 can be found in the standards themselves.

Getting Started

The first step towards an accreditation is to make sure that everyone in the organization wants to achieve the same goal. The full understanding of how everyone benefits from an accreditation will make the process easier. A good way to ensure this is to have staff meetings throughout the process and involve all staff from the very beginning. The most frequent mistake organizations make when trying to implement a QC system is not to involve everyone. Divide the project into smaller sections and give out personal responsibilities enabling all staff to be included in the preparation work. This will also make the implementation easier.

A good way to make sure that all demands in the standard are covered is to make up a table of contents using the ISO 15189:2022 standard table of contents as a template. An assessment can then be made of what needs to be added to the quality manual and other documentation. It is important to note that while the standards have demands for management structure, internal audit, or document control, the laboratory standards have some more specified demands not found in ISO 9001, and these need to be added to the specific procedures.

Methods and SOPs

Examination processes (ISO 15189:2022; 7.3)

The methods and processes we use in the embryology laboratory and their efficacy have a direct impact on the pregnancy results of the clinic. It is therefore hugely important that we standardize these methods and make sure that they are reproducible. In simple words, an ICSI should be done in the same way using the same disposals and equipment by all embryologists in the lab, ensuring that an ICSI done by embryologist A on a Monday is performed in exactly the same way and with the same level of skill as an ICSI done by embryologist B on a Friday. Ensuring the performance of correct methods is achieved through several steps. First, we need to make sure that the processes and methods we use are correct and up to date with the latest developments in ART. Hence, a clear starting point should be a literature search, together with the knowledge gained from workshops, external training, and visits to other clinics. The standard stipulates that we "shall select and use methods which have been validated for their intended use to assure the clinical accuracy." Once the details of the methods have been agreed between the embryology team members, they need to be documented. A document describing a method or process used in a laboratory is commonly called an SOP. A good SOP should follow a set format, and the old ISO 15189:2012; 5.5.3 contains a very good guide for SOP layouts [59]. The SOP title should be followed by a short clinical description of the method. The analytic principles need to include a theoretical description of the method and review of the current literature. The SOP should outline the competence demands on embryologists performing the process. Collection and handling of gametes and embryos should include the sampling procedures and the physical environmental issues such as temperature. Remember that all variables in the SOP, such as those referring to the measurement of temperature, have to give a precise range, followed by a description of how the temperature is measured, the accuracy of the thermometer, and how often and how it is calibrated. There should be clear descriptions of how the sample is labelled and, considering the risks associated with the work in an IVF laboratory [60], the marking should be logical and clear in order to eliminate completely the risk of mixing of samples (for further details, refer to the "Sample identification, witnessing, and prevention of misidentification" section later in this chapter). The description of the procedural steps should be written in an

uncomplicated way so that they can be easily followed by any new member of staff under supervision.

All equipment used for the method should be listed with references to handling instructions and calibration protocols. Any safety routines and occupational hazards involved should be discussed and clearly known by the embryologists. References to any textbooks or publications concerning the method should be included last.

The standard demands that the procedures used should meet the requirements of the users of the laboratory service, preferably applying methods that have been published in established/authoritative textbooks, peer-reviewed texts, or journals. If in-house methods are used, these need to be appropriately validated for the intended use and fully documented by the laboratory. The standard requires that all documentation relating to the processes and all supporting documentation be readily available to personnel (7.3.1 c), preferably digitally as the use of paper should be minimized in the clean facilities (embryology laboratory).

When the SOP is written, it needs to be communicated to all members of the embryology team, and it is important to allow them to comment, give feedback, and suggest changes before the document is formally issued and implemented. The way to check that all embryologists follow the new SOP is to undertake audits, and it is suggested to audit all processes three months after the issue of the SOP. If the audit findings include discrepancies between the written SOP and the embryologists' hands-on working procedure, then either the SOP needs to be changed to reflect the actual hands-on procedure or the member of staff needs to be retrained and reminded of the importance of following the agreed SOP. No embryologists can insist on doing things "their own way" in a standardized high-quality IVF laboratory.

Once the SOPs are fully implemented and the audits show that we have achieved the required reproducibility, then we need to ask: is it working? Is the method we agreed upon successful? The standard calls this "verification" and "validation" (7.3.2), and it is the process that confirms that the techniques and methods used in the IVF laboratory are suitable to produce good embryos, viable pregnancies, and live births. All methods must be validated regularly, and the SOP should include information on how often and how validations are done. The EUCTD includes demands for validation, and in the UK, the HFEA CoP [34] requires that all processes in the IVF laboratory be validated. Some methods and techniques used in the laboratory can be difficult to validate, and it is acceptable to use retrospective analysis of fertilization, damage, and pregnancy rates to validate ICSI and IVF. Appropriate validation of new techniques can become very difficult when considering the sample size needed to prove a null hypothesis or small increase in pregnancy rates. An accurate validation of a new culture medium will need hundreds of patients in each study group. Adding to the complexity of validation practice is the fine line between validation and research, and questions are raised regarding the need for ethical approval to undertake validations [61]. However, it is highly recommended to regularly validate other practices in the lab, such as changes of osmolarity during preparation of dishes, temperature fluctuation during denudation, and temperature distribution in incubators. Validation of temperature in a culture medium in different types of dishes on all heated stages in the laboratory should confirm the appropriate range of surface temperature of the heated stage.

Handling of gametes and embryos

Pre- and post-examination process (ISO 15189:2022; 7.2, 7.4)

The standard has specific demands on how the sample—that is, gametes and embryos—should be collected and stored, noting that the pre-examination process can influence the outcome of the intended process. The samples must be correctly and safely identified, and any laws regulating the identification of patient samples have to be considered (for further details, refer to the "Identification, witnessing, and prevention of misidentification" section later in this chapter). The sample should be accompanied by a written, standardized request of what procedure the sample should be used for. It is a common occurrence that the requests for treatment are unclear and that couples who could have had conventional IVF end up having ICSI due to poor communication. Senior embryologists with considerable experience in assessing sperm samples are more suitable to making the final decision on IVF or ICSI in conjunction with the couple on the day of treatment when the sample has been washed than the referring doctor who takes the decision on IVF or ICSI based solely on a semen analysis report. Other procedures where clear requests are crucial are frozen embryo transfer (ET) cycles to ensure that the embryo is thawed at the correct time assuring endometrium/embryo synchronization. For collection of sperm, the date and time of collection should be noted by the patient and the date and time of receipt should be recorded by the laboratory. Noting sperm sample collection time is important as an ejaculated sample needs to be processed within 40 minutes of production. Delay in processing with prolonged sperm—seminal fluid (reactive oxygen species [ROS] exposure)—can cause increased sperm DNA damage/mutational loads resulting in poor fertilization outcome and embryo development. Section of the standard 7.2.4.2 provides a guide on what information the laboratory must include. Assuring patient consent is another crucial part of the pre-examination process (12) and the post-examination process (7.2.4) specifically with regards to cryo-preserved material.

Usually, the procedures for collecting samples at pre- and post-examination are documented in the applicable laboratory SOPs for sperm processing and oocyte collection. However, it is important to include the specific demands of the standards for these procedures and the documentation of them.

Laboratory sheets and reports

Reporting and releasing results (ISO 15189:2022; 7.4)

The details from assessments of gametes and embryos we document in the laboratory on lab sheets are referred to in the standard as reports. The reporting of results should always be accurate, clear, unambiguous, and objective. This requires that the lab sheets be standardized and follow a set format. They should be filled out in a neat manner—no scribbling allowed. All entries and comments on a lab sheet should be accompanied by a date and signature. For sperm assessment, sources of errors and uncertainty of measurements should be stated and properly calculated for each method. Formal reports, such as seminal fluid analysis reports, should also be checked and signed off by the senior andrologist/embryologist before being issued.

Many laboratories have computerized databases and enter the information from the lab sheets into the database. It is important to understand that the handwritten lab sheet is considered source data and therefore needs to be archived correctly, not destroyed

after computer entry. If the laboratory wants to go paper free, it has to indeed be paper free and allow for direct data entry onto the computer without an in-between paper sheet. When considering the need for signatures and witnessing, a complete paperless IVF laboratory could be difficult to create. The standard provides a good overview of what it required with regards to reports in (12).

The embryology laboratory

Facilities and environmental conditions (ISO 15189:2022; 6.3)

A laboratory needs to ensure that the environmental conditions of the laboratory are suitable for the safe handling of gametes and embryos and do not invalidate the results or adversely affect the quality of any procedure. In simple words, this means that the IVF laboratory must be designed in such a way that the outcome of any procedure is optimal and not affected by environmental parameters. The standard further requires us to consider the safety of patients, visitors, laboratory users, and personnel.

Live birth results following IVF treatment vary from country to country and from clinic to clinic and often within a clinic from month to month. It is a general consensus that patient demographics, such as age and cause of infertility, are the main factors affecting the outcome. Considering a varying population of patients, it is of great importance that parameters in the laboratory are stable. Defining the environment and setting limits for acceptable working conditions will help with reducing variables and result in the patient being the only factor that varies. Exactly what this encompasses will always be down to interpretation and international, national, or regional regulations; however, the standard has some clear demands, and some environmental factors cannot be ignored.

General laboratory layout

The theatre for oocyte retrieval and ET should be in close vicinity to the laboratory. The laboratory layout should further ensure safe handling of gametes and embryos; small, crowded laboratories impose a significant risk for accidents, resulting in loss of gametes and embryos.

The laboratory should never double as an embryologist office. There needs to be a minimal allowance of paper in the laboratory as this can increase the amount of particles in the air. Therefore, only patient records necessary for ongoing treatment should be kept in the laboratory. Also, the laboratory is not the place for cardboard boxes as these involve a high risk of fungus infections. Furthermore, the laboratory is not a storage room for disposables; only a weekly stock of disposables should be kept inside the lab, and further storage can be managed elsewhere. The equipment held in the laboratory should be limited to only that which is absolutely necessary; again, the laboratory is not a storage room for old lab equipment.

Access rules

The standard stipulates that access to the laboratory facilities is controlled (6.3.2). The laboratory should have limited access ensured by use of locks, swipe cards, or other access controls. It should also hold documentation verifying who has access to the laboratory. There should be documented and implemented rules for what is required for access to the laboratory including demands for change of clothes and shoes, the use of hair cover and masks, and the washing of hands. Although some embryologists insist that changing clothes and covering hair are of no importance, it is important to understand that embryology and handling of gametes and embryos are sterile processes with a need to protect the samples from microbes and contaminants. The correct degree of cleanliness is impossible to reach if the embryologists are using their own clothes or only minimal cover such as laboratory coats. Best practice is to change clothes and preferably use scrubs, which are made of low-lint, no shedding material; cotton is high lint and not advisable. Many embryologists complain that these types of scrubs are uncomfortable and that they will not use them as cotton is comfortable, but it is important to understand that we did not become embryologists to be comfortable—we need to do what is best for gametes and embryos. Further, all hair should be covered, and again some might see the cap as a fashion item that looks much better if hair is allowed outside it, but they need to be reminded to tuck in all hair before entering the laboratory. Changing into cleanroom shoes goes without saying. Best practice is to have all-white shoes with white soles in the laboratory. This makes it easy to spot any spillage on them. Also, the rack for these shoes should be designed so that the shoes are hung up with soles facing out, allowing for daily inspection of the cleanliness of the shoes. If coloured shoes are used outside the laboratory, it will be easy to spot anyone who has forgotten to change the shoes. Hands should be washed using a proper disinfectant soap before entering the laboratory. Furthermore, jewellery, nail polish, long fingernails, and perfumes should not be worn in the laboratory.

Health and safety

The laboratory is required to ensure the safety of its entire staff. This includes providing an environment that minimizes the risk of transfer of any contagious contaminants through the use of class II biosafety cabinets when handling unscreened patient materials. Further, installing low-oxygen alarms and ventilation in cryo-storage facilities (note the embryology laboratory should never double up as a cryo-storage facility).

Temperature

The optimal IVF laboratory temperature is a matter of great debate; however, it has to be defined to a limited range. Some embryologists argue that an elevated laboratory temperature benefits the embryos through reduced risk of cooling during transport from the incubator to the heated stage. However, high laboratory temperatures will provide a perfect environment for microbes and contaminants. All laboratory equipment is designed to operate at room temperature, usually defined as $23 \pm 2°C$, and unless the laboratory can show process verification at a different temperature, this range will be the one demanded by the standard. A laboratory without temperature control cannot be accredited.

Light

The embryo is extremely sensitive to light exposure; however, there is a wide range of opinions on whether light in the laboratory or from microscopes will harm embryos or not. It has been very elegantly demonstrated in a large study on hamster and mouse embryos that cool fluorescent light increases the ROS production and apoptosis in blastocysts and reduces the development of live-term fetuses [62]. The embryos were handled under minimal light conditions, and the test groups were exposed to 5–30 minutes of cool white, warm white, or midday sunlight. A total of 44% of blastocysts exposed to cool white light and transferred to recipients developed to term of pregnancy (day 19), compared with 73%

in the control; 58% of blastocysts exposed to warm white light developed to term (day 19). When embryos were exposed to only one minute of sunlight, only 25% of embryos developed to term, with 35% being resorbed. In light of these findings, best practice should be to have a dim light in the laboratory and to close out any daylight.

Air quality

Another area of great debate is the demands of clean air in the laboratory, and this has also been affected by regional interpretation of the EUCTD. The standard requires that attention is paid to sterility and presence of dust, and it is highly recommended that laboratories periodically monitor the particle count and presence of volatile organic compounds (VOCs) in the air, together with microbial monitoring using contact plates for surfaces, such as replicate organism detection and counting containing Sabouraud dextrose agar (SDA; for detection of fungus) and trypticase soy agar (TSA; for detection of bacteria) and similar (TSA and SDA) settlement plates for air sampling. The plates should be exposed in key positions in the laboratory, theatre, and treatment rooms for four hours. Acceptable limits are zero colonies inside the flow hoods or handling chambers and <10 colonies outside the hoods in the laboratory.

General cleanliness

An IVF laboratory should always be clean, and the laboratory standards demand that documented frequent cleaning procedures are implemented and that cleaning is confirmed by active signatures. The use of harsh detergents is not recommended, and cleaning should be undertaken using 70% alcohol followed by sterile water or other products tested for embryology use such as Oosafe® (SparMED, Stenløse, Denmark) [63]. Steam cleaners are suitable for the cleaning of floors.

Culture medium, devices, and disposables

(ISO 15189:2022; 6.6, 6.8, 6.4)

All devices used in ART, such as culture media and consumables, will affect the outcome of the treatment. First, the laboratory needs to decide on their own requirements for culture medium, oocyte collection needles, culture dishes, and so on. This includes limits in toxicity and results from mouse embryo assays for culture media, oocyte pickup needles, or plastic ware. There is solid evidence that many of the devices and disposables we use in the embryology laboratory are indeed reprotoxic, and it is our duty to make sure that we do not use items that will expose the embryos to stress [64]. It is important to consider any national, regional, or local regulation that applies. EUTCD stipulates that all devices that come into contact with cells, gametes, or embryos need to be tested according to the EU devices directives [65, 66] and be Conformité Européenne (CE) marked. The laboratory also must define requirements for the safe transport of devices from supplier to the laboratory and how they will be inspected when they arrive to ensure they meet the limits specified. For example, there has to be a system to ensure that the box containing the culture medium is still cold when it arrives. This can easily be done by inserting a temperature probe into the box upon arrival, or requesting that the medium provider pack a temperature data logger with the medium, which you can attach to your computer when the medium arrives and ascertain that the temperature inside has been constant and correct throughout the transport. Moreover, consumables then have to be verified before taken into use. Some laboratories choose to culture excess embryos or undertake sperm survival assays in new batches of culture medium; however, this type of verification is not demanded by the standard, and it could be argued if it is really necessary. If all the devices conform to the EU devices regulation, they should already have been stringently tested. ISO 15189 only demands that the laboratory actively checks the test reports issued by the manufacturer and confirms that the reports comply with their own limits for use.

When the devices are accepted for use, it is crucial that they are stored correctly to ensure their continued suitability for use. The laboratory must safeguard correct storage by defining the exact storage environment. Limits for temperature in refrigerators and freezers are crucial, and culture medium should be stored in a pharmaceutical refrigerator that guarantees a constant temperature throughout, whereas a normal kitchen refrigerator is not acceptable [67]. The environment in general storage rooms is also important as plastic ware stored at high temperatures will not be suitable for use.

All purchased supplies, reagents, and consumables should be included in the laboratory inventory. Information in the inventory shall include lot number (batch number), date of reception, and date taken into use. The inventory for equipment should include unique identification, date of arrival, date placed in service, last calibration or service, and periodicity of service and calibration. The laboratory is required to keep a list of approved suppliers and to critically evaluate all suppliers on an annual basis.

The batch or lot number of any device that comes into contact with a given patient's gametes or embryos needs to be recorded on that individual patient's records.

It is not appropriate to have a list of batches currently used in the lab and to draw conclusions from this using the date and guesswork of what device was used for what patient.

It is of great advantage to have a computerized case file system whereby each cycle has a batch record page attached. This page includes a full list of culture media and laboratory ware and the batches in use, and with a simple mouse click, it marks what materials were used in every step of the cycle, from culture media down to pipette tips.

Equipment

(ISO 15189:2022; 6.4)

A laboratory should have all the equipment needed to ensure provision of the best service. The standards require a documented program for preventive maintenance, and it is the responsibility of the laboratory manager to regularly monitor and ensure appropriate service, calibration, and function of all equipment. All equipment used in an accredited laboratory has to be clearly labelled with a unique identifier, date of last calibration or service, and date or expiration criteria as to when recalibration/service is due. Together with this, all equipment used should be included in an equipment record containing information listed in ISO 15189:2022; 6.4.7. There should be clearly documented processes for the validation of equipment function before it is taken into use (6.4.3). The standard of equipment used in IVF laboratories is generally very high, but even the best equipment can fail and not function optimally if it is not appropriately maintained. All embryologists should have solid knowledge of how to operate all equipment, and there should be written implemented procedures in place for action taken if there happens to be an equipment failure. Crucial equipment such as incubators should always be

connected to auto-dialers enabling staff to promptly respond to any faults out of hours.

Equipment should be verified by test runs; for example, before a new centrifuge is taken into use in the laboratory, a series of mock sperm preparations have to be undertaken and documented.

Monitoring and traceability

(ISO 15189:2022; 6.3, 6.4, 6.6, 7.3)

Chapter 2 presents a detailed report of the monitoring of equipment and laboratory parameters and the traceability of reference equipment [68].

Monitoring of KPIs

Most clinics that have a quality system in place monitor KPIs. Similar to the monitoring of laboratory environmental parameters, each clinic has to agree on documented limits of performance. Usually, when monitoring parameters such as live birth, clinical pregnancy, and fertilization, there is no upper limit; however, a lower limit is necessary, along with documented plans for immediate action whenever a KPI falls under the agreed limit.

The KPIs that are essential for monitoring in connection with the laboratory include, but are not limited to, fertilization rates for IVF and ICSI, damage rates for ICSI, survival of embryos after thawing, and pregnancy results from ET. Benchmarking and KPI monitoring are hotly debated topics, and it must be underlined that trying to benchmark against a different laboratory's KPIs is a futile exercise, as laboratory performance is affected by factors such as patient selection, among other things. The best benchmarking for KPIs is done against an in-house-determined "gold standard." This is a subsection of good-prognosis patients, and the indicators for this group should be very much constant. For example, a drop in the overall KPI for fertilization with no drop in the corresponding "gold standard" indicates that the issue is related to the material coming into the laboratory. However, a drop in the KPI for the "gold standard" definitely suggests that there might be a problem with performance.

KPIs should be monitored for the whole laboratory and for each embryologist and doctor. It is important to underline the importance of confidentiality when monitoring individual performance, considering the need for the training of any embryologist falling under the given limit, but not ignoring the stress and decrease in self-confidence this can lead to. All members of staff need to understand that the monitoring is not a way of punishing people but rather to ensure that all embryologists perform to the same high standard, minimizing variables. Another important outcome of individual performance monitoring is to identify persons with exceptionally high results so that others can learn more and thereby increase the overall success.

The Alpha/ESHRE consensus group has published a detailed guide of ART laboratory performance indicators with clear examples and explanations [30]. Similarly, the ESHRE Clinic PI working group has published performance indicators for clinical practice in ART [69].

Quality assurance

Ensuring the validity of results (ISO 15189:2022; 7.3.7)

QA makes sure that you are doing the right thing in the right way, and QC makes sure that what you have done is what you expected. In short, QA is process-oriented and QC is product-oriented. When discussing QA/QC, it is easy to get confused; however, the terminology is not important—what is important is that the laboratory has control mechanisms in place to ensure that they perform according to the SOPs and to the highest standard. (12) and (12) demand that the laboratory has both internal QC (IQC) and external QA (EQA) in place for monitoring of the validity of the methods used. This includes the demand of internal and external controls and inter/intra-laboratory comparisons and validations. The laboratory is required to determine the uncertainty of results. This can be difficult with a subjective parameter such as embryo scoring; however, it can easily be done for the assessment of sperm. Through assessment of a series of sperm samples by all laboratory staff involved in the preparation of sperm, a coefficient of variance can be calculated, usually resulting in a 10%–15% variance.

The standard also demands that all embryologists/andrologists assess sperm samples and photos or movies of embryos on a regular basis, usually at least every three months. It is the responsibility of the laboratory manager to document the results from these comparisons, calculate variations, and address any deviance. To collect samples and photos and arrange these types of intra-laboratory comparisons takes time, and, over and above this, the standards also demand that the laboratory participates in inter-laboratory comparisons. A laboratory can share photos of embryos and samples of sperm with other centres and set up an inter-laboratory comparison scheme, although the standard clearly states that self-developed programs like this should not be used when organized external schemes are available. In the UK, most laboratories participate in the UK National External Quality Assessment Service (UK NEQAS) andrology and embryology morphology scheme, which uses online resources, DVDs, and/or formalin-fixed samples for assessment [70]. UK NEQAS collaborates with Swiss software developing company Gamete Expert and use their platform for the EQA program [71].

A web-based inter-laboratory comparison scheme is run by Dr. James Stanger and includes schemes for the assessment of all stages of human preimplantation embryos, sperm morphology and concentration, and ultrasound measurement of follicles (www.fertaid.com). The scheme provides monthly assessments of embryos and sperm and allows the laboratory manager to use the information for intra-laboratory comparison. As each of the different schemes has some 200–300 participants around the world, the intra-laboratory comparison scheme provides a solid reference for the laboratory management to implement corrective actions when deviations are found [72]. All these forementioned EQAs are in substantial agreement with the ISO/IEC 17043:2010 Conformity assessment—General requirements for proficiency testing, which is a requirement by the standard [73].

Patient contact

Advisory services (15189:2022; 5.3.3)

In most IVF clinics, the embryologists have no or very little contact with the patient and also very little input into the exact treatment options. In an accredited laboratory, the standard demands that the laboratory actively provides advice on choice of treatment and clarification of any laboratory outcomes. As discussed previously, some decisions such as fertilizing oocytes using IVF or ICSI should be taken by a senior embryologist rather than a

doctor. The ultimate approach is to have the couple/patient sit down with the embryologist after oocyte and sperm collection for a "post-oocyte pick up (OPU) chat." This gives the opportunity for the embryologist to discuss with the couple/patient issues such as the quality and numbers of sperm and oocytes and advise them on the best procedure ahead. This short chat should also include reminding the couple/patient of risk and success; that is, there is always a risk for failed fertilization, failed cleavage, or failed blastocyst development. If the couple/patient has been reminded of these risks, it makes it somewhat less stressful to make a call to them in the unlikely event of a failed fertilization.

Evaluations and audits

(ISO 15189:2022; 8.8)

Audits can be internal or external, vertical or horizontal, or process-oriented or system-oriented. Therefore, it is easy to get confused and caught up in terminology and to miss out on the great opportunity that audits provide for improving the system and our service to patients. To find nonconformities at an audit is not bad—it is proof that the system is working and we are capable of recognizing our weaknesses and faults and ready to learn and improve on them. For general internal audit principles, see Chapter 32 [7].

Internal audits

The laboratory standards are more precise in what exactly should come out of an audit and what is needed for a correct audit process. When preparing, writing, and implementing internal audit procedures, ISO 15189 is precise on what exactly is needed. The current standard requires laboratories to take a risk-based approach to audit intervals with processes which are high risk to patients be audited frequently. Poor outcomes of previous audits also require a specific process to be audited more frequently to assure that the corrective action has been efficient.

External audits

If the laboratory aims to seek formal accreditation to ISO 15189, the National Authority for Conformity Assessment performs the external audits. A formal accreditation is always advantageous, but in many countries, this option is not available, and as it is a rather pricey process, some laboratories choose to state that they adhere to the standard without formal accreditation.

When a laboratory is ready to be formally accredited, they need to apply for accreditation and the national authority will assess whether they have the appropriate expertise to perform the audit. If not, they can seek help from other members of the International Laboratory Accreditation Cooperation (ILAC) [74] or European Accreditation [75] who have the appropriate experienced auditors. Together with the application, the laboratory has to supply evidence of a fully compliant quality system and it is essential that all methods for which accreditation is sought have gone through a series of internal audits. Result documentation from these audits is supplemental to the application. The accreditation body then arranges a pre-audit to assess the readiness of the laboratory, and, pending the outcome of this pre-audit, an accreditation audit will be arranged. When the accreditation audit has been done, the lead auditor or any technical experts can only recommend that the laboratory be awarded accreditation. This recommendation is then passed on to the board of the accreditation body, which will decide if the laboratory is to be awarded accreditation.

Beyond the standards

While the embryology laboratory could be seen as any other clinical medical laboratory, there are some major differences to do with the delicacy of the samples it handles. Whereas a mistake in the day-to-day pathology laboratory can mostly be rectified by resampling, a mistake in the embryology laboratory can lead to major irreparable trauma for the patients [60]. Therefore, it is of great importance that we acknowledge these differences and implement processes that help safeguard us from incidents. Although some national and regional guidelines acknowledge these differences, IVF laboratories worldwide need to understand and address this. There are three major areas concerning not only the safeguarding of patients' gametes and embryos but also aiming to protect the embryologists working in the laboratory: (i) training of embryologists to make sure that the staff handling these delicate samples and undertaking the complex IVF processes are properly trained, (ii) appropriate sample identification processes, and (iii) implementation of risk management processes.

Training and accreditation of embryologists

Personnel (ISO 15189:2022; 6.2)

Clinical embryology is a highly skilled profession, and the main contributors to IVF success are the skills and knowledge of the embryologists. When considering the impact that the training of embryologists has on results, it is evident that there is a need for formalized training programs in every clinical IVF laboratory.

When looking at the international ISO standards, the requirement for personnel is not clearly defined. The standard states that the laboratories need to specify the competence requirements for all processes and activities including requirements for education, qualification, training, re-training, technical knowledge skills, and experience (6.2.2a). Further, areas of responsibility should be clearly outlined together with duties in the documented job descriptions. There should be clearly documented procedures in place for the introduction and training of new staff and the reintroduction of staff after long periods of absence or leave, together with documentation on how proof of competence is issued. The management of the laboratory should formulate goals for each member of staff with respect to continuing education and professional development (6.2.4). These goals should be assessed and discussed at annual appraisals, which should be documented but kept confidential.

In recent years, there has been an increased focus on the training and accreditation/certification of clinical embryologists. A formal training programme has been in place for embryologists in the UK since 1995. The original program was provided through Association of Clinical Embryologists (ACE, the ACE Certificate) including a minimum of two years training with both practical and theoretical components. In 2019, ACE voted to merge with the Association of Biomedical Andrologists and the British Andrology Society with the Association of Reproductive and Clinical Scientists (ARCS) formed in 2020. ARCS is a unified professional society covering all aspects of reproductive science and research and is now the driving force behind embryology professional development [76]. Trainee embryologists enrol through a training program managed under the National Health Service (NHS) Scientist Training Program (STP) [77]. This is a three-year graduate entry program that is covered by a fixed-term employment and, upon finalization, awards the holder a master's degree in reproductive science from an accredited university. Post STP

training, clinical embryologists follow a career pathway towards registration through either the Academy for Healthcare Science (AHCS) or the Association of Clinical Scientists (ACS) [76]. After registration, embryologists can pursuit membership of the Royal Collage of Pathologists.

In 2008, ESHRE introduced a certification for embryologists with the aim of certifying the competence of clinical embryologists working in IVF and of developing a formal recognition for embryologists [78]. It provides two different pathways to certification: a clinical embryologist track open for embryologists with at least a BSc degree in natural/life sciences, at least three years hands-on experience with human gametes and embryos in an ART laboratory, and a minimum of 50 hands-on core embryology procedures and a senior clinical embryologist track for candidates with either an MSc or PhD, at least six years hands-on experience in an ART laboratory, and a minimum of 50 hands-on core embryology procedures. All ESHRE members who meet the requirements can apply. The assessment includes a logbook outlining the procedures included in the training and the minimum cases done and passing a multiple-choice examination. The certification process is validated and recognized in accordance with Union Europe´enne des Me´decins Spe´cialistes (UEMS) and their Council for European Specialists Medical Assessment (CESMA). ESHRE also offer a continuous embryology education credit system, with the credits being needed for three-yearly renewal of the certificate. In the 10 years from its start in 2008, the program has certified 773 clinical and 493 senior clinical embryologists. In 2012, the certification was opened up for non-European candidates, and in 2018, a pilot long-distance on-site exam for a small group of 22 candidates was organized in India, simultaneously with the main exam that was held in Geneva [79]. The 2020 exam was cancelled due to the COVID 19 pandemic, and in 2021, the exams were held online for the first time.

In Canada, CFAS has issued guidelines for an applied training program and evaluation and development of competencies for ART laboratory professionals. While CFAS is not a certifying or regulatory body, the CFAS program aims to develop standards that all ART laboratory professionals should conform to and to verify individuals are up to those standards [56].

In the United States, the Practice Committees of the ASRM) and the SRBT have recently published comprehensive guidelines for human embryology, andrology, and endocrinology laboratories [58]. These guidelines clarify embryology laboratory staff minimum requirements for education, training, continuing education, and experience together with recommended minimum staff numbers to ensure safe operations.

In Australia, Scientists in Reproductive Technologies (SIRT) are in the process of formalizing embryology training, aiming for a future certification and continuous professional development system.

With the ever widening availability of the ESHRE certification and their exams on clinical embryology being the most widely accepted tests of knowledge from laboratory science in ART, there is still a need for clinics to find ways of formalizing training for their embryologists. Every clinic should have documented training procedures clearly stating the minimum of supervised procedures a trainee has to undertake before being signed off for independent work. For the ESHRE certification, this includes 50 procedures of each of OPU, semen analysis and preparation, insemination, ICSI, zygote and embryo evaluation, ET, cryopreservation of oocytes/embryos, and thawing of oocytes/embryos. Obviously, the outcome of those procedures needs to be evaluated too, and the trainees have to meet the set KPIs of the clinic to be approved. To ensure the theoretical component—that the trainee knows why and not only how—it is suggested that essays set on subjects such as preimplantation genetic diagnosis and embryo development are included along with a small examination. It is also crucial to fulfil the need for continued professional development, allowing embryologists to attend conferences and workshops and to participate in research.

Sample identification, witnessing, and prevention of misidentification

One of the most crucial tasks in the IVF lab is to ensure the correct identity of gametes and embryos. Over the years, there have been numerous reports of misidentification resulting at best in a cancelled cycle if the mistake is identified before embryo transfer and at worst in tragedy if realized after the embryo transfer or indeed birth. These errors are generally the result of trained personnel not following the known procedure for reasons such as distraction, tiredness, or being rushed [80, 81]. Alternatively, it is caused by poorly written or non-existent policies and protocols (active failure vs. latent condition). The solution to misidentification is the development of robust identification procedures that are risk assessed (for further details, see the "Risk identification, management, and prevention" section).

The EU tissue directive includes demands for appropriate sample identification with the core being a unique identifier for each sample. However, the most stringent guidelines involving safe sample identification procedures are provided by the HFEA CoP [34]. In the UK, it is a licensing requirement to have robust ID systems (Mandatory Requirement T71, HFEA CoP), and all IVF laboratories must put in place processes to ensure that no mismatches of gametes or embryos or identification errors occur. With this comes a demand for double witnessing of the identification at all critical prints of the IVF laboratory process. The witnessing has to be signed at the time of the checked step, and records must be kept in each patient's case file. Together with this license requirement, the guidelines stipulate that all samples of gametes and embryos be labelled with at least the patient's full name and two of the following identifier: date of birth, hospital number, NHS number/Community Health Index (CHI) number or unique donor identifier. It is important to note that a patient's name or date of birth is not a unique identifier. The witnessing is mandatory and required every time gametes or embryos change vessel (dish or tube), and the person checking should have a full understanding of the process they are witnessing, allowing only trained clinic staff named on the HFEA license to undertake the check. At semen sample handover, oocyte retrieval, and embryo transfer, the patient is required as an active participant in the identification.

In Australia, the RTAC CoP Critical Criterion 7 on Identification and traceability sets out the requirements with a minimum of three forms of identification used to ensure the traceability of all persons and specimens [5]. Double witnessing is strongly recommended but not mandatory; rather, the CoP requires the laboratories to annually audit and risk assess the process. The technical bulletin on Patient and Sample Identification (Technical Bulletin 4) is very detailed and provides robust guidelines for identification; however, it is not enforceable [37]. Similarly, the ESHRE laboratory guidelines include a section on identification of patients and traceability of their reproductive cells [32].

While most laboratories use manual double witnessing, identification checks can also be electronic, with several witnessing

systems being available for embryology purposes. The technology applied includes radiofrequency technology and barcodes. The advantages of automated systems are that their accuracy is not affected by lack of concentration or poor protocols [81], and they have a significantly lower error rate than human error (0.001% compared with 1%–3%) [82]. So by introducing electronic witnessing, we can possibly reduce errors in misidentification and potentially add an extra level of patient safety [82, 83]. A recent study showed that apart from improving safety, applying an electronic witnessing approach could also improve timing and efficacy of processes [84]. From a patient perspective, improved ID processes and risk minimization through electronic witnessing is welcomed. In a survey of 408 patients undergoing IVF, more than 90% were concerned about errors and 92% of them confirmed that the introduction of electronic witnessing would reduce their concern about a biological mix-up in the laboratory [85].

However, it is important to underline that all the current electronic witnessing systems are based on some type of sticker being attached to the tubes and dishes and mistakes can certainly occur in printing and labelling. Moreover, while the systems are not foolproof, they are expensive and some are bulky, taking up a substantial space. The development of electronic witnessing systems for IVF is only at its infancy, and the technology will more than likely be refined in the future.

In addition to the HFEA CoP, RTAC bulletin, and ESHRE guidelines, which are IVF specific, there are several standards and recommendations on the subject of patient and sample identification. The CLSI guideline on Accuracy in Patient and Sample Identification [83] describes the essential components of processes and systems that need to be implemented for accurate patient and sample identification. It covers the whole process from the pre-examination phase to the reporting of results, underlining the importance of staff training, risk assessment, and the use of unique identifiers, and it relates to both manual and electronic systems. The previously mentioned "nine-patient safety solutions" from WHO/JCI have patient identification at its core [23]. The ISO standards also have demands of correct sample labelling; however, they offer little information on safe solutions.

There are certainly huge advantages to the use of manual double witnessing, but there is always a slight risk that a procedure like this can cause mistakes, as we cannot double the embryologist workforce. One major source of incidents in the IVF laboratory is insufficient staffing, and to be interrupted while working with embryos can have disastrous consequences. In a busy IVF lab setting, scientists need to switch repeatedly between the patients' material they are working on and the patients' material they are being asked to check [86, 87]. In practice, the principal operator interrupts their workflow to locate a "witness" and the "witness" is interrupted from their own task to carry out the double check. Daniel Brison [88] estimated that, in a well-staffed IVF lab, each embryologist was witnessing 15–20 other procedures in a morning on top of their own workload. Many laboratories today have very few embryologists, and with a witnessing routine in place, this will not only increase the workload but also add a heightened risk of distraction when an embryologist has to interrupt others' work to get them to witness a certain step in the procedure. Moreover, human beings and systems under stress will underperform in rushed situations and stress is known to affect human performance in many sectors, including the IVF laboratory. Most clinics have periods when patient throughput is increased without compensation in relation to staffing levels. Systematic overtime, overloaded work schedules, high cognitive loads, and chronic staff shortages contribute to error-inducing environments [89–91]. In addition, other forms of stress such as inadequate training and lack of guidance have been identified as sources of identification errors [92].

When introducing a robust, safe ID system in the laboratory, the best way of starting is to avoid reinventing the wheel. Even if your laboratory is located outside the UK, the HFEA CoP Guidance note 18 provides a great guide on how to ensure that the correct gametes are mixed and the right embryos are transferred [34]. To make it simple, the IVF laboratory must have written protocols for witnessing and each step involved has to be risk assessed (documented). As a part of the standardization introduced into a laboratory, there will be written SOPs and flow charts, and it is easy to identify each step where a gamete or embryo changes tube or dish. Simply add a witnessing signature to the laboratory sheet to each of those steps (the procedure itself should already have a signature on the sheet). An exception to the witnessing requirements is the so-called forced functions, such as when a clinic receives only one sperm sample on a given day, and so, there will be a forced function when the sample is transferred from one tube to another. If the clinic makes use of this, it has to be risk assessed.

With the first step in the process being reception of gametes, semen samples, or oocytes and the last step being embryo transfer, the HFEA CoP underlines the need for the patient to be involved in this crucial identification step. Here, it is important to implement a process that involves positive patient identification, which is the foundation for error prevention [93]. In simple words, the embryologist will ask the patient to audibly read out his/her name and any other identifier you have chosen such as date of birth and at the same time have a witness—the doctor or nurse—to confirm this positive identification step being done.

The witnessing action itself also needs to be done correctly. It should include three major components: (i) the ID-labelled vessel that holds the gametes or embryos; (ii) the new vessel that the sample is being moved in to, labelled with the same ID; and (iii) the patient documentation (i.e., the laboratory sheet containing the full identification of the patient). In addition to these three components, the embryologist performing the "move" (principle operator) reads the name and unique identifier aloud from the sample vessel, the new vessel, and the laboratory sheet, followed by the witness reading aloud the same.

Other hugely important factors are the strength and quality of the identifier itself. The need for a strong unique identifier together with the name is paramount. With the date of birth being too weak and not considered unique, the clinic or laboratory needs to create a couple-specific identifying number such as a unit number or couple number. A patient-specific number such as a medical records number is not advisable as the embryo mostly belongs to the couple and not one patient only. This identification, name, and couple number then need to be affixed to the vessel in a clear, safe manner. The most widely used labelling is handwriting with a nontoxic pen. Usually, the ID is written on the side of tubes and bottom of dishes (mirrored from the outside) to allow easy noticing. Printed stickers are also being used; however, it should be made clear that stickers contain glue and, when placed in a humidified incubator, this results in an increase of VOCs, which in turn can be toxic to the embryo. Another way of labelling is etching the ID into the plastic using a small syringe, but scratching of plastic will also increase VOCs and can be toxic to the embryo. Moreover, the etched details appear very faint and cannot be considered safe from a clear witnessing point of view.

FIGURE 5.1 Sperm preparation areas RED and BLUE each containing all equipment needed for complete sperm preparation. (a) Documentation for the patient, assigning work area RED to this patient. (b) Labelled sample pot and preparation tubes are double witnessed when brought into the area. (c) Only the sample currently being prepared in area RED is centrifuged in the area's designated centrifuge. When the preparation is complete, the area is sterilized before being assigned to the next patient.

Finally, the ID should always be affixed to the part of the vessel actually carrying the sample. Labelling the lid of a dish or a tube is not acceptable.

If a process involves gametes or embryos changing vessel several times during a short time period, such as sperm preparation or embryo freezing/thawing, then it can be acceptable to witness the whole area. For example, a laboratory preparing sperm can have multiple biosafety cabinets with one designated centrifuge and other equipment assigned to a specific defined work area. Note that each work area must have a designated centrifuge and two samples cannot be centrifuged together if this approach is adapted. When a sample is being brought into this area, all tubes involved can be witnessed at the same time with the prospect that only one sample will be handled through the whole process from start to finish (Figure 5.1). Obviously, this process needs to be risk assessed if adapted.

Correct labelling together with witnessing procedures will help minimize the risk of misidentification, but it is also absolutely imperative that only one couple's samples are handled at any one time. Preparation of a number of sperm samples, or cryopreservation or thawing of multiple patients' embryos at the same time, poses a huge risk for mix-ups and should never be done.

Risk identification, management, and prevention

According to the WHO, one in six couples experience difficulties in conceiving and would need some form of assisted reproduction method [94]. Worldwide, more than eight million children had been born as a result of an ART treatment in 2019, and it is estimated that over two-and-a-half-million treatment cycles are undertaken annually, resulting in 500,000 deliveries every year, possibly taking that number to more than 10 million at the time of writing [95]. With the increase in IVF cycle number worldwide, it has become evident that just like in other areas of medicine and healthcare, errors are inherent. But it is important to remember that these errors most often result from a complex interplay of multiple factors; only rarely are they due to the carelessness or misconduct of single individuals. Historically, rather than addressing the source of errors, prevention strategies have relied almost exclusively on enhancing the carefulness of the caregiver [96]. A culture of blame and finding a scapegoat has commonly been the response to adverse events, and this is an approach that can never improve the system and prevent the incident from reoccurring. The portioning of blame to an individual usually comes with a promise that "it will never happen again" [97]. The crucial changes in the approach to risk management in IVF clinics are presented in Table 5.1. In order to prevent errors and identify risks, IVF laboratories must introduce robust risk management including an analysis of systems and structures in advance of those risks actually materializing, thus embedding risk management into the daily routine for embryologists. The international standard ISO 31000:2018 Risk Management Guidelines [98] is the most widely acknowledged tool for addressing, managing, and preventing risk. Implementing this standard will not only vastly decrease the risk of adverse events and near misses but also provide tools for how to learn from incidents when they happen and prevent them from happening again. ISO 31000 will provide a clear guide on how to set up a risk management policy and clearly outlines what needs to be included.

Errors and incidents result from failures, and these can be categorized as active failures or latent conditions [99, 100]. Active failures result from violation of the agreed protocols, lapses, or mistakes. Latent conditions or errors include error-provoking conditions such as workload, fatigue, knowledge, supervision, and equipment and weaknesses in defence including unworkable procedures or switching off a malfunctioning alarm. Latent conditions are embedded in all systems as it is not possible to foresee all error-producing situations. However, as they pre-exist, active failures may be able to be identified prior to adverse events occurring. Therefore, these conditions tend to be the targets of risk management systems.

The first step towards risk management in the laboratory is to have a clear overview of the protocols and procedures undertaken by the embryologists. This should be provided already as part of the quality system and demand for SOPs. With the use of process maps and flow charts for the procedures, it will be easy to identify areas and procedures that could be high risk, but total risk management has to include all processes and procedures. Mortimer and Mortimer [101] provide a simple summary of risk management by asking and answering three basic questions: what can

TABLE 5.1 Shift in Approaches to Risk Management in *In Vitro* Fertilization Clinics

Outdated Approach	Modern Approach
Main goal	
To protect the IVF clinic's reputation	To improve patient safety and minimize risk of harm to and misidentification of embryos and gametes through better understanding of systemic factors that affect the risk for incidents
Reporting	
Acknowledge only reports submitted in writing	Variety of methods to report: paper form, electronic form, telephone call, anonymous reporting, and person-to-person reporting
Investigation	
Investigate only the serious occurrences	Encourage reporting of "near misses" and investigate and discuss the potential causes
Interview staff one on one when there is an adverse incident	Have root cause analysis meetings with the entire team
Corrective/preventive action	
Blame and train (or dismissal)	Perform a criticality analysis chart and determine the root cause of the "near miss" or the adverse occurrence
Work with department involved to develop corrective action	Work with the team to develop a safety improvement plan
Information from investigation kept confidential	Develop corrective action and share with the whole IVF team
Communication	
Talk to the patients only if necessary and be vague about incident/findings	Advise clinic director to speak directly with the patients and talk with them about any unexpected outcome and error; keep them appraised of steps taken to make the environment safe for the next patient
Long-term follow-up	
Assume that action is taken to correct the problem that occurred, and notice only when it happens again that no action is taken	Monitor and audit to determine that changes have been initiated and that the changes have made a difference

Source: Adapted from [96].
Abbreviations: IVF, *in vitro* fertilization.

go wrong? What will we do? If something happens, how will we resolve it? There are three core tools for helping us address risk and to answer those questions: failure mode and effects analysis (FMEA), root cause analysis, and audit.

A comprehensive way of proactively addressing risk is to make use of FMEA. Like many approaches that improve quality and safety, FMEA has its origins in the army, space, and aviation industry, but it is now used as a tool for error prevention in a wide range of industries, including healthcare. The aim of FMEA is to try to think of every possible way a process can go wrong, how serious it would be, and how the process can be improved to avoid failure. It is important that all embryologists on the team are involved in assessing each process using FMEA. A simple format for FMEA is illustrated in Table 5.2. The first step is to identify the process to be assessed, using the examples of insemination, mixing of oocytes, and sperm for IVF. Then, identify what could go wrong (potential failure mode); for example, an embryologist forgets to inseminate, mixes the wrong oocytes and sperm, loses oocytes, and bumps a dish. Then, ask "what could be the result of this failure?" It could be failed fertilization, creation of an embryo or indeed child with the "wrong parents," and decreasing the chances of pregnancy. Then, assess the seriousness of the suggested failures using a 1–10 scale with 1 being no effect and 10 being critical. For failed fertilization, one could argue a seriousness of 8, but the creation of a mixed-up embryo has a severity of 10. Once severity has been established, address the different causes of the failure; in this case, being rushed, low staffing levels, poor processes, lack of checklists, and no witnessing system. Then, rate how often this would happen, from 1 being no known occurrences (has never happened in any IVF clinic) and 10 being very high risk (with this happening regularly). Forgetting to inseminate happens in all clinics, but one could argue that it is very rare, so an occurrence of 2 or 3 would be appropriate. Then, discuss and list the current controls (e.g., use of daily worksheets or reminders) followed by assessing what chance there is that we would detect the failure. With forgetting to inseminate, this will be evident the morning after when the oocytes are found without sperm and are unfertilized, and we can assign this a 1 representing

TABLE 5.2 Sample Failure Mode and Effects Analysis Worksheet

Item/ Function	Potential Failure Mode	Potential Effects of Failure	**S** Severity Rating (1–10)	Potential Cause(s)	**O** Occurrence Rating (1–10)	Current Controls	**D** Detection Rating (1–10)	RPN (Risk Priority Number)	Recommendations and Action	Action Taken	**New S** (1–10)	**New O** (1–10)	**New D** (1–10)	**New RPN**

detection every time it happens. However, with the case of a mix-up, this could go completely undetected and should be assigned a 9–10; the fault will be passed to the customer undetected, or, in IVF terms, the resulting embryo will be transferred leaving the patient or child to detect the failure. Then, calculate the risk priority number (RPN) by multiplying the severity, occurrence, and detection; for forgetting to inseminate, this is $8 \times 3 \times 1 = 24$. Now the initial analysis is done and the embryology team has the task of lowering the RPN. There needs to be an active discussion on how the procedure can be changed, allowing everyone on the team to come up with suggestions. Remember that we can sometimes grow accustomed to our own best practices but should consider the suggestions from trainees, who after all provide us with a fresh pair of eyes. Preventing failure in insemination could include the introduction of daily worksheets and checklists together with witnessing and improved ID checks. When these suggested changes have been discussed, documented, and implemented, a new value for severity, occurrence, and detection is assigned and the new RPN should hopefully be significantly lower than the original.

The FMEA exercise is not only a mathematical exercise resulting in reduced risk through the actions taken but also a great way of making all embryologists aware of what risks are involved in each step of the IVF process, and this awareness itself can help reduce risks. There are some excellent studies published on applying FMEA to embryology laboratory procedures including core embryology processes [102, 103], PGT [104], and witnessing [105].

Even the best risk management systems have incidents and near misses. So, what can be done when an incident occurs? The answer is root cause analysis, which is the reactive component in a risk management system. A root cause analysis is simply an analysis of the very reason for the incident occurring. A simple example is when recently trialling a new incubator, the lid accidentally fell over the hand of the embryologist while placing dishes inside, resulting in spillage of the medium and loss of 1 out of 23 oocytes. The root cause analysis included discussing the incident at the lab meeting: Had it happened before? Were there any near misses previously where the lid had been falling without incident? But also we discussed how we place dishes in the incubators: Are we sometimes carrying more than one dish? We further contacted the supplier to see whether it was a fault of the incubator itself. It was concluded that the lid of our trial incubator did not recline and was a risk if left open without holding on to it. We implemented a procedure where only one dish could be carried and placed in the incubator at any one time, always allowing one hand to be free to hold up the lid. At no time is it appropriate to revert to the old, outdated way of thinking where we apportion blame; this can never result in improvement. More complex root cause analyses could focus on the failure to inseminate as used for the FMEA, instead of looking at it proactively, doing a root cause analysis after the fact. Mortimer and Mortimer [101] provide an interesting example of root cause analysis of poor fertilization results, with the outcome being a complete reformulation of the fertilization medium.

Many root cause analyses I have been involved in concluded that the level of staffing was inappropriate. It is important to underline that staffing issues such as overworking and poor training are the main contributors to incidents. There is also the issue with staff who are not accepting professional responsibility and do not take enough care to undertake their duties or follow protocols; they should not continue to work in the laboratory [101].

Finally, a very effective tool in addressing and analysing risk is audits. All incidents followed by a root cause analysis will include suggestions for change and continuous improvement. To ensure these have been implemented and are indeed effective, one needs to undertake internal audits (see the "Evaluations and audits" section).

Another side to safe practice is to have robust contingency plans. There should always be a documented, agreed plan B. This will include having a backup for all equipment, such as a minimum of two microscopes, heated stages, and centrifuges. For more expensive equipment such as ICSI rigs, oocyte aspiration pumps, and controlled freezers, where sometimes the clinic cannot afford to have two sets, there needs to be a written agreement with another IVF clinic regarding utilization of their equipment.

Concluding remarks and future aspects

Throughout completing the long and work-intensive process of applying standardized systems in an embryology laboratory, one might ask what it has meant for the embryologists and the results of the clinic. There is no doubt that introducing and fully implementing a quality system standardizes methods and the ways in which embryologists perform their work. The troubleshooting, maintenance of equipment, and milieu are improved and standardized. This guarantees optimal handling of a couple's gametes and embryos and inevitably will lead to improved outcome.

The number of ART treatment cycles undertaken worldwide is increasing every year, and with the improvement of the techniques we use, more babies are born as a result of IVF. With the outcome improving, we are aiming towards a future where more focus will be on the safety of treatment and indeed the long-term health of children resulting from ART. With this comes a demand for standardization and improvement of quality. The introduction of quality management systems will ensure reproducibility and traceability, which will be crucial for the future follow-up of these children.

To face the future, we need to improve our understanding of the long-term effects of our laboratory procedures on embryo health, acknowledging that some of our methods might deliver in numbers but might be detrimental when considering the adult health of children conceived through IVF. A review of the follow-up of children born from IVF over 25 years in Sweden has revealed that in contrast to cleavage-stage transfer, children born after blastocyst transfer exhibited a higher risk of preterm birth and congenital malformations [106]. A study comparing euploidy rates in donor egg cycles between different fertility centres showed some centres achieving euploidy rates between 70% and 80% while other centres having rates as low as 40%–50% [107]. Considering the nature of donated oocytes as somewhat standardized, these results strongly indicate that there are laboratory practices that contribute to higher or lower euploidy rates. Taking these two studies into account, it is evident that suboptimal culturing and handling of embryos have long-reaching effects far beyond blastocyst development, successful pregnancy, and live birth. It indicates that what we do in the clinical embryology laboratory is closely connected to the adult health of children born from IVF. This further highlights the importance of standardization, along with implementing processes that go beyond the standards, working towards improved risk management, robust and thorough training of clinical embryologists, and processes to ensure correct identification and prevention of mix-ups.

Finally, it is important to acknowledge that quality management together with a never-ending commitment to improve our service, beyond standards, is the only way forward towards a future where we can guarantee safe, efficient IVF treatment for all patients and the birth of children who go on to live healthy lives.

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6

EVALUATION OF SPERM

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Introduction

Abnormalities in sperm production or function, alone or in combination with other factors, account for 40% of all cases of infertility. Although a battery of tests and treatments have been described and continue to be used in the evaluation of female infertility, the male has been essentially neglected. The majority of programs offering advanced assisted reproduction technologies (ART) employ only a cursory evaluation of the male—rarely extending beyond semen analysis. Several factors account for this disparity. First, most practitioners of ART are gynaecologists or gynaecologic subspecialists who have little formal training in the evaluation of infertile or sub-fertile men. Second, the urologists, who perhaps theoretically should have taken the lead in this area, have devoted little of their literature or research budgets to the evaluation of the infertile male. Third, and perhaps most important, is the inescapable fact that sperm function testing remains a very controversial area of research. Many tests have been described, yet few have been extensively evaluated in a proper scientific manner. Those that have continue to be criticized for poor sensitivity or specificity, a lack of standardization of methodology, suboptimal study design, problems with outcome assessment, and the lack of long-term follow up. Although many of these same criticisms could also be levelled against most diagnostic algorithms for female infertility, in that arena, the tests continue to prevail over their critics. Fourth, like female infertility, male infertility is certainly multifactorial. It is improbable that one sperm function test will prove to be a panacea, owing to the multiple steps involved in fertilization. In addition to arriving at the site of fertilization, sperm must undergo capacitation and the acrosome must allow for the penetration of the cumulus cells and the zona pellucida so the sperm head can fuse with the oolemma. In addition, the sperm must activate the oocyte, undergo nuclear decondensation, form the male pronucleus, and then fuse with the female pronucleus. Finally, with the advent of intracytoplasmic sperm injection (ICSI), sperm function testing has assumed a role of even less importance. In recent years, ICSI has been utilized in greater than half of the ART cycles conducted each year. Many programs are applying 100% ICSI cycles [1]. As fertilization, blastocyst, and pregnancy rates improve in the contemporary ART lab, more and more logical questions are being asked about the proper role for sperm function testing. This chapter reviews techniques available for sperm evaluation and examines the issues surrounding their utilization in the modern ART program.

Patient history

A thorough history of the infertile couple at the time of the initial consultation will frequently reveal conditions that could affect semen quality. Some of the important factors to consider are as follows:

1. Reproductive history, including previous pregnancies with this and other partners.
2. Sexual interaction of the couple, including frequency and timing of intercourse along with the duration of their infertility.
3. Past medical and surgical history: specific attention should be paid to sexually transmitted diseases, prostatitis, or epididymitis, along with scrotal trauma or surgery—including varicocele repair, vasectomy, inguinal herniorrhaphy, and vasovasostomy.
4. Exposure to medication, drugs, toxins, and adverse environmental conditions such as temperature extremes in occupational and leisure activities, either in the past or in the present.

Semen analysis

The hallmark of the evaluation of the male remains the diagnostic semen analysis. It is well-known that the intra-patient variability of semen specimens from fertile men can be significant over time [2]. This variability decreases the diagnostic information that can be obtained from a single analysis, often necessitating additional analyses. What is also apparent from literature that analyses samples from "infertile" patients is that the deficiencies revealed may not be sufficient to prevent pregnancy from occurring. Rather, they may simply lower the probability of pregnancy, resulting in so-called subfertility. Clearly, the overall prognosis for a successful pregnancy is dependent on the complex combination of variables in semen quality coupled with the multiple factors inherent in the female reproductive system that must each function flawlessly. The commonly accepted standard for defining the normal semen analysis is the criteria defined by the World Health Organization (WHO). These parameters for the fourth, fifth, and most recent sixth edition are listed in Table 6.1.

The normal or reference values for semen analyses have been altered with each new edition of WHO-defined criteria. The values from the current (sixth) edition have been derived from a retrospective look at the semen parameters of men with two to seven days of abstinence whose partner conceived within 12 months after the cessation of the use of contraception [3, 4]. There are significant changes in the parameters listed in the fifth and sixth edition compared with past editions. Some of these changes are due to observations made of the semen samples from the patients just mentioned. These real differences in declining sperm concentrations, motility, and normal morphology are thought to be due to environmental influences. However, the drastic changes in the morphology reference values are primarily due to the suggested use of the Kruger strict morphology method in the fifth and sixth edition. The value of this method will be discussed in the sperm morphology section of this chapter.

Collection of the specimen

When the semen analysis is scheduled, instructions must be given to the patient to ensure the collection of an optimum semen sample. Written instructions are useful, especially if the patient

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TABLE 6.1 World Health Organization Reference Values for Semen Analysis

Parameter	Reference Values		
	Fourth Edition	Fifth Edition	Sixth Edition
Volume	>2.0 mL	1.5 (1.4–1.7)	1.4 (1.3–1.5)
Sperm concentration	20×10^6	15 (12–16) $\times 10^6$	16 (15–18) $\times 10^6$
Total sperm count	40×10^6	39 (33–46) $\times 10^6$	39 (35–40) $\times 10^6$
Total motility	50%	40% (38–42)	42% (40–43)
Progressive motility	25	32% (31–34)	30% (29–31)
Vitality	50%	58% (55–63)	54% (50–56)
pH	7.2	7.2	7.2
Morphology	15%	4% (3.0–4.0)	4% (3.9–4.0)

Source: Data from [5–7].

Note: Liquefaction: Complete within 60 minutes at room temperature (fifth edition) and at 37°C (sixth edition). Appearance: Homogeneous, grey, and opalescent. Consistency: Leaves pipette as discrete droplets. Leukocytes: Fewer than 1 million/mL.

is collecting the specimen outside of the clinical setting. During the initial infertility evaluation, a semen specimen should be obtained following a two- to seven-day abstinence from sexual activity [2]. A shorter period of time may adversely affect the semen volume and sperm concentration, although it may enhance sperm motility. A longer period of abstinence may reduce sperm motility. Considering the natural variability in semen quality that all men exhibit, the initial semen collection may not accurately reflect a typical ejaculate for that patient. A second collection, with a two- to seven-day abstinence period, can eliminate the tension associated with the initial semen collection, and provide a second specimen from which a typical set of semen parameters can be determined. An additional cause of variable semen quality can be the site of collection. Understandably, many men are inhibited by collecting their semen sample at the clinic. Although collecting at home is less intimidating, it is not always practical due to distance or schedules. In the case where the semen sample is collected at the clinic, the second and subsequent collections are usually better than the first due to an increase in the patient's comfort level. The second collection may also be used to determine the optimal abstinence period for a patient. Masturbation is the preferred method of collection. The use of lubricants is discouraged since most are spermicidal. However, some mineral oils and a few water-based lubricants are acceptable. Since masturbation may present significant difficulty for some men, either in the clinic or at home, an alternative method of collection must be available. The use of certain silastic condoms (seminal collection devices) during intercourse may be an acceptable second choice. Interrupted intercourse should not be considered, as this method tends to lose the sperm-rich initial few drops of semen while transferring many bacteria to the specimen container [2, 4].

Care of the specimen

Appropriate care of the ejaculate between collection and examination is important. Specimens should be collected only in approved, sterile, non-toxic, plastic, disposable cups. Many other plastic containers are toxic to sperm, especially if the sperm is allowed to remain in the containers for the duration of time that it takes to deliver the specimen from off-site. Washed containers may contain soap or residue from previous contents, which can kill or contaminate the sperm. Delivery of the semen to the laboratory should occur within 60 minutes of collection, and the specimen should be kept at room temperature during transport. These recommendations are designed to maintain optimal sperm viability until the time of analysis.

Container labelling

The information recorded on the specimen container label should include the male's name along with a unique identifying number. Typically, a birth date, or a clinic-assigned patient number is used. Other helpful information recorded on the label should include the date and time of collection and the number of days since the last ejaculation. When the specimen is received from the patient, it is important to confirm that the information provided on the label is complete and accurate and documented accordingly.

Examination of the specimen

Liquefaction and viscosity

When the semen sample arrives in the laboratory, it is checked for liquefaction and viscosity. Although similar, these factors are distinct from each other [8, 9]. Liquefaction is a natural change in the consistency of semen from a semi-liquid to a liquid. Before this process is completed, sperm are contained in a gel-like matrix that prevents their homogeneous distribution. Aliquots taken from this uneven distribution of sperm for the purpose of determining concentration, motility, or morphology may not be truly representative of the entire specimen. As liquefaction occurs over 15–30 minutes, sperm are released and distributed throughout the semen. Incomplete liquefaction may adversely affect the accuracy of the semen analysis by preventing this even distribution of sperm within the sample. The coagulum that characterizes freshly ejaculated semen results from secretions from the seminal vesicles. The liquefaction of this coagulum is the result of enzymatic secretions from the prostate. Watery semen, in the absence of a coagulum, may indicate the absence of the ejaculatory duct or non-functional seminal vesicles. Inadequate liquefaction, in the presence of a coagulum, may indicate a deficiency of prostatic enzymes [10, 11].

Viscosity refers to the liquefied specimen's tendency to form drops from the tip of a pipette. If drops form and fall freely, the specimen has a normal viscosity. If drops will not form or the semen cannot be easily drawn up into a pipette, viscosity is high. This high viscosity remains, even after liquefaction has taken place. Highly viscous semen may also prevent the homogeneous distribution of sperm. Treatment with an enzyme, such as chymotrypsin [12], or aspiration of semen through an 18-gauge needle may reduce the viscosity and improve the distribution of sperm before an aliquot is removed for counting. Any addition of medium containing enzymes should be recorded, as this affects the actual sperm concentration. The new volume must be factored in when calculating the total sperm count.

Semen volume

Semen volume can be measured with a serological pipette that is graduated to 0.1 mL. The volume is recorded and multiplied by the sperm concentration to obtain the total count of sperm in the sample. A normal seminal volume before dilution is considered to be >1.3 mL [13].

Sperm concentration

A variety of counting chambers are available for determining sperm concentration. These include but are not limited to the haemocytometer, Makler counting chamber, and MicroCell. Regardless of the type of chamber used, an aliquot from a homogeneous, mixed semen sample is placed onto a 37°C chamber. The chamber is manufactured to a certain depth, which allows the sperm to distribute evenly in a very thin layer. Sperm within a grid are counted, and a calculation is made according to the formula for the type of chamber used. Accuracy is improved by including a greater number of rows, squares, or fields in the count. Sperm counts should be performed immediately after loading semen onto the chamber. As indicated earlier, a particular patient's sperm count may vary significantly from one ejaculate to another. This observation holds true for both fertile and infertile males, further complicating the definition of a normal range for sperm concentration. Demographic studies employing historic controls were used to define a sperm concentration of <16 million/mL as abnormal (sixth edition; [13]). Several investigators had observed that significantly fewer pregnancies occurred when men had sperm counts <16 million/mL; however, the prognosis for pregnancy did not increase proportionately with sperm concentrations above this threshold.

Sperm motility

Sperm motility may be affected by many factors:

- Patient's age and general health
- Length of time since the last ejaculation (abstinence)
- Patient's exposure to outside influences such as excessive heat or toxins
- Method of collection
- Length of time and adequacy of handling from collection to analysis

When the aliquot of semen is placed on the 37°C counting chamber, the count and motility should be determined immediately. If a chamber with a grid is used to count the sperm, the motility can be determined at the same time as the concentration by using a multiple-click cell counter to tally motile and non-motile sperm and then totalling these numbers to arrive at the true sperm concentration. The accuracy of the concentration and of the motility improves as more sperm are counted. If a wet-mount slide is used to determine motility, more than one area of the slide should be used, and each count should include at least 200 sperm. Prior to examining the specimen for motility, the slide or counting chamber should be examined for signs of sperm clumping. Agglutination refers to motile sperm sticking to other sperm. This can be head-to-head, head-to-tail, or tail-to-tail. This may indicate the presence of sperm antibodies in the semen. The severity of sperm agglutination is evaluated on a scale of 1 to 5. A score of 1 is isolated (<10 sperm/agglutinate); 2 is moderate (10–50 sperm/agglutinate); 3 is large (>50 sperm/agglutinate); and 4 is gross (all sperm agglutinated). The type and degree of agglutination should be recorded. This should not be confused with clumping of sperm to other cellular debris in the semen, or non-motile sperm stuck to each other (aggregation). In any case, sperm clumping may affect the accuracy of both the sperm count and the motility [1, 13].

Motility is one of the most important prerequisites for achieving fertilization and pregnancy. The head of the sperm must be delivered a great distance *in vivo* through the barriers of the reproductive tract to the site of the oocyte. Sperm must have sufficient motility to penetrate both the layers of coronal cells and the zona pellucida before fusing with the oocyte cell membrane (oolemma). An exact threshold level of motility that is required to accomplish fertilization and pregnancy, however, has never been described [12]. This may be due to variables in the equipment and techniques used in assessing motility.

Progression

While sperm motility represents the quantitative parameter of sperm movement expressed as a percentage, sperm progression represents the quality of sperm movement expressed on a subjective scale. A typical scale attempts to depict the type of movement exhibited by most of the sperm visualized on a chamber grid. Progression of sperm may also be calculated with sperm motility as a percentage of sperm exhibiting "progressive motility." With the advent of successful micro-assisted fertilization, progression has assumed more limited utility. Nevertheless, for those laboratories that quantify progression of motility separately, a score of 0 means no motility; 1 means motility with vibratory motion without forward progression; 2 means motility with slow, erratic forward progression; 3 means motility with relatively straightforward motion; and 4 is motility with rapid forward progression [13].

Sperm vitality

When a motility evaluation yields a low proportion of moving sperm (less than 50%), a vitality stain may be beneficial. This is a method used to distinguish non-motile sperm that are living from those that are dead. This technique will be discussed later in the sperm function section.

Additional cell types

While observing sperm in a counting chamber or on a slide, additional cell types may also be seen. These include endothelial cells from the urethra, epithelial cells from the skin, immature sperm cells, and white blood cells. The most common and significant of these cell types is referred to collectively as "round cells." These include immature sperm cells and white blood cells. In order to distinguish between them, an aliquot of semen can be placed in a

thin layer on a slide and air-dried. The cells are fixed to the slide and stained using a Wright–Giemsa or Bryan–Leishman stain. When viewed under 400× or 1000× power, cell types may be differentiated primarily by their nuclear morphology. Immature sperm have one to three round nuclei within a common cytoplasm. Polymorphonuclear leukocytes may also be multinucleate, but the staining method will typically reveal characteristic nuclear bridges between their irregularly shaped nuclei [1]. A peroxidase stain may be used to identify granulocytes and to differentiate them from the immature sperm. The presence of greater than one million white blood cells per one millilitre of semen may indicate an infection in the urethra or accessory glands, which provide the majority of the seminal plasma. Such infections could contribute to infertility [1, 14]. These samples can be cultured so that the offending organism can be identified, and appropriate treatment can be instituted. Besides bacteria, white blood cells on their own can contribute to infertility. They can especially be a detrimental factor in the *in vitro* fertilization (IVF) process. The white blood cells can be removed by centrifugation of the semen sample through a layer of silica beads; the toxins produced by the cells, called leukokines, may pass through the layer and concentrate in the medium below containing the sperm. If the sperm is to be used in the insemination of oocytes, the concentrated toxins will be in contact with the oocytes for several hours. These toxins may cause detrimental effects to the oocytes and to the embryos that develop from fertilization. The detrimental effects of white blood cells in a semen sample can be ameliorated by the application of ICSI, which eliminates the long-term exposure of oocytes to toxins.

Sperm morphology

Sperm morphology should be assessed using Kruger strict criteria according to WHO sixth edition standards (Figure 6.1) [15]. It is recommended that the slide be stained with Papanicolaou staining, although other methods can be utilized with proper evaluation and validation [13]. At least 200 sperm must be counted using bright field optics at ×1000 magnification with oil immersion [13]. WHO sixth edition criteria for assessing normal forms include the following:

- Head: Smooth; oval configuration; length, 5–6 μm, diameter 2.5–3.5 μm; acrosome, must constitute 40%–70% of the sperm head.
- Mid-piece: Slender, axially attached; <1 μm in width and approximately 1.5 μm in head length; no cytoplasmic droplets, >50% of the size of the sperm head.
- Tail: Single, unbroken, straight, without kinks or coils, approximately 45 μm in length (Figure 6.2) [13, 15–17].

As described by Kruger et al., sperm forms that are not clearly normal should be considered abnormal. The presence of 4% or greater normal sperm morphology should be interpreted as a normal result. Normal morphology of <4% is abnormal [16, 17]. Normal sperm morphology has been reported to be directly related to fertilization potential. This may be due to the inability of abnormal sperm to deliver genetic material to the cytoplasm of the egg. From video recordings, it appears that abnormal sperm are more likely to have diminished, aberrant, or absent motility. This reduced or unusual motility may result from hydrodynamic inefficiency due to the head shape, abnormalities in the tail structure that prevent normal motion, and/or deficiencies in energy production necessary for motility [18, 19]. In addition to compromised motility, abnormal sperm do not appear to bind to the zona of the egg as well as normal sperm. This has been demonstrated in studies employing the hemizona binding assay [20]. IVF has helped to further elucidate the role that normal sperm morphology plays in the fertilization process and in pregnancy.

Computer-assisted semen analysis

Computer-assisted semen analysis (CASA) was initially developed to improve the accuracy of manual subjective semen analysis. Its goal is to establish a standardized, objective, reproducible test for sperm concentration, motility, and morphology. This technique also characterizes sperm movement. The automated sperm movement measurements—known as kinematics—include straight-line velocity, curvilinear velocity, and mean angular displacement (Table 6.2). The use of CASA requires specialized equipment, including a phase contrast microscope, video camera, video recorder, video monitor, computer, and printer.

To perform CASA, sperm are placed on a chamber or in a capillary specific to the CASA device and then viewed under an internal magnification device. The video camera records the moving images of the sperm cells, and the computer digitizes these frames accordingly. The digitized images consist of pixels whose changing locations are recorded frame by frame. A total of 30–200 frames per minute are produced. The changing locations of each sperm are recorded, and their trajectories are computed (Figure 6.3) [21]. In this manner, hyperactive motion can also be detected and recorded. Hyperactive sperm exhibit a whip-like, thrashing movement, which is thought to be associated with sperm that are removed from seminal plasma and ready to fertilize the oocytes [21, 22]. Historically, the validity and reproducibility of results have kept CASA from becoming a standard procedure in the andrology laboratory. However, with advances in technology these devices have become more accurate, allowing for an opportunity for a more standardized and objective semen analysis within the field [23]. The accuracy of sperm concentration appears to be diminished in the presence of either severe oligospermia or excessive numbers of sperm. In cases of oligospermia, counts may be overestimated due to the machine counting debris as sperm. High concentrations of sperm may be underestimated in the presence of clumping. High sperm concentrations can also cause overestimations in counting due to the way the software handles collisions between motile sperm and non-motile sperm. In these cases, diluting the sample may improve the accuracy of the count [22, 23]. Sperm motion parameters identified by CASA have been assessed by several investigators for their ability to predict fertilization potential. Certain types of motion have been determined to be important in achieving specific actions related to fertilization, such as cervical mucus penetration and zona binding. However, the overall potential of CASA for predicting pregnancy remains to be elucidated. Persistent questions about the reproducibility and reliability of results and their interpretation continue to limit the routine use of CASA. The use of fluorescent DNA staining with CASA may help to improve its reliability. In addition, as the kinematics of sperm motion becomes better understood, CASA may play an integral role in determining the optimal method of assisted reproductive technique that should be utilized for specific types of male factor patients [24].

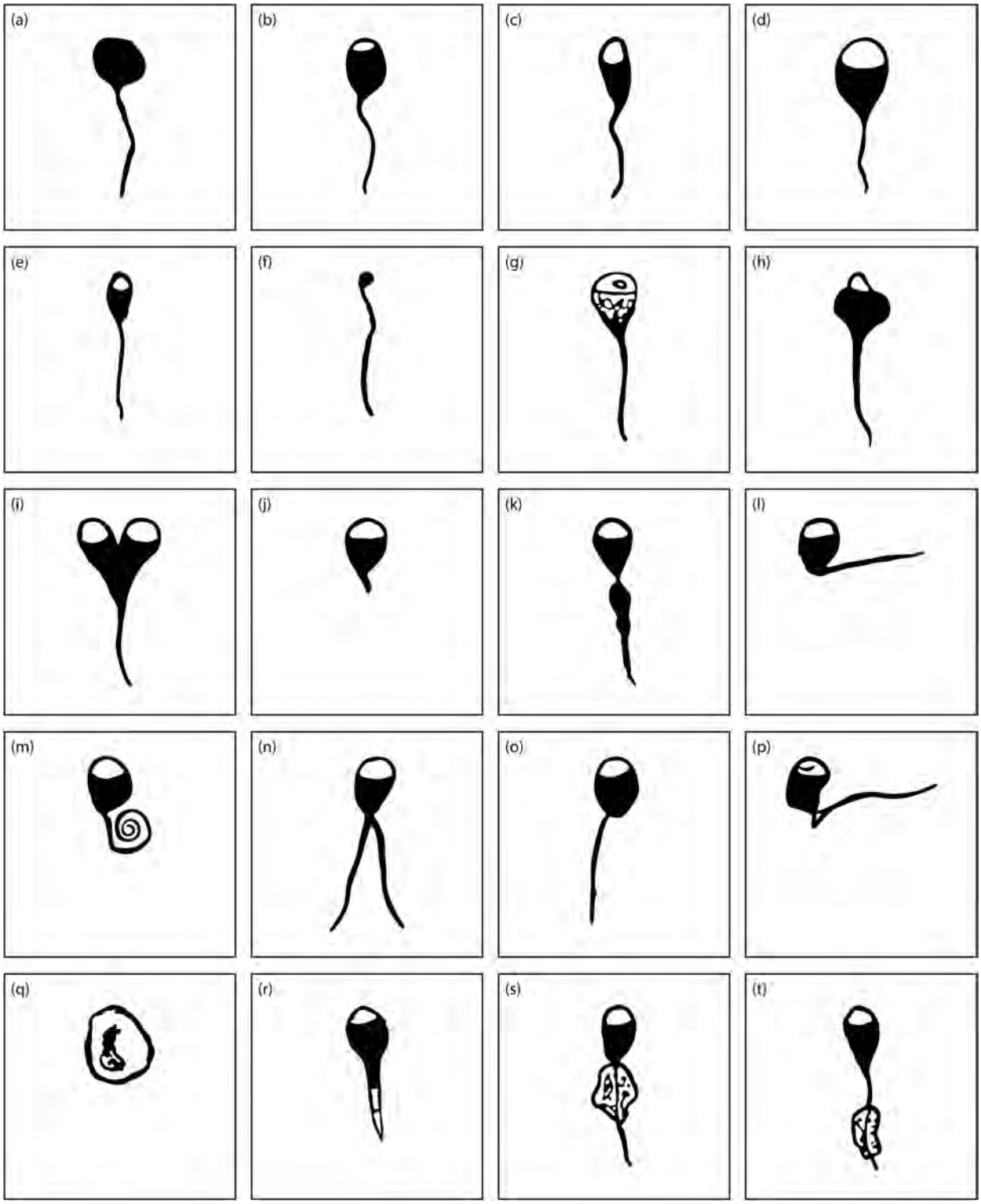

FIGURE 6.1 Different types of sperm malformations. (a) Round head/no acrosome; (b) small acrosome; (c) elongated head; (d) megalo head; (e) small head; (f) pinhead; (g) vacuolated head; (h) amorphous head; (i) bicephalic; (j) loose head; (k) amorphous head; (l) broken neck; (m) coiled tail; (n) double tail; (o) abaxial tail attachment; (p) multiple defects; (q) immature germ cell; (r) elongated spermatid; (s) proximal cytoplasmic droplet; and (t) distal cytoplasmic droplet. (From [17], with permission.)

TABLE 6.2 Kinematic Measurements in Computer-Assisted Semen Analysis

Symbol	Name	Definition
VSL	Straight-line velocity	Time average velocity of the sperm head along a straight line from its first position to its last position
VCL	Curvilinear velocity	Time average velocity of the sperm head along its actual trajectory
VAP	Average path velocity	Time average velocity of the sperm head along its average trajectory
LIN	Linearity	Linearity of the curvilinear trajectory (VSL/VCL)
WOB	Wobble	Degree of oscillation of the actual sperm head trajectory around its average path (VAP/VCL)
STR	Straightness	Straightness of the average path (VSL/VAP)
ALH	Amplitude of lateral head	Amplitude of variations of the actual sperm head trajectory about its average trajectory displacement (the average trajectory is computed using a rectangular running average)
RIS	Riser displacement	Point-to-point distance of the actual sperm head trajectory to its average path (the average path is computed using an adaptive smoothing algorithm)
BCF	Beat-cross frequency	Time average rate at which the actual sperm trajectory crosses the average path trajectory
HAR	Frequency of the fundamental	Fundamental frequency of the oscillation of the curvilinear trajectory around its average harmonic path (HAR is computed using the Fourier transformation)
MAG	Magnitude of the amplitude	Squared height of the HAR spectral peak (MAG is a measure of the peak to fundamental harmonic peak dispersion of the raw trajectory about its average path at the fundamental frequency)
VOL	Area of fundamental harmonic	Area under the fundamental harmonic peak in the magnitude spectrum (VOL is a harmonic measure of the power-bandwidth of the signal)
CON	Specimen concentration	Concentration of sperm cells in a sample in millions of sperm per mL of plasma or medium
MOT	Percentage motility	Percentage of sperm cells in a suspension that are motile (in manual analysis, motility is defined by a moving flagellum; in computer-assisted semen analysis, motility is defined by a minimum VSL for each sperm)

Source: Data from [22].

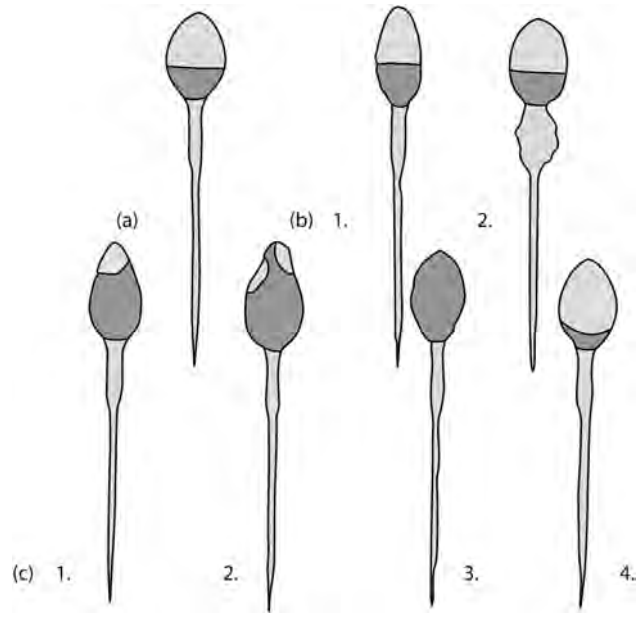

FIGURE 6.2 A diagrammatic representation of quick-stained spermatozoa. (a) Normal form; (b.1) slightly amorphous head; (b.2) neck defect; (c.1 and 2) abnormally small acrosome; (c.3) no acrosome; and (c.4) acrosome 70% of sperm head. (From [17], with permission.)

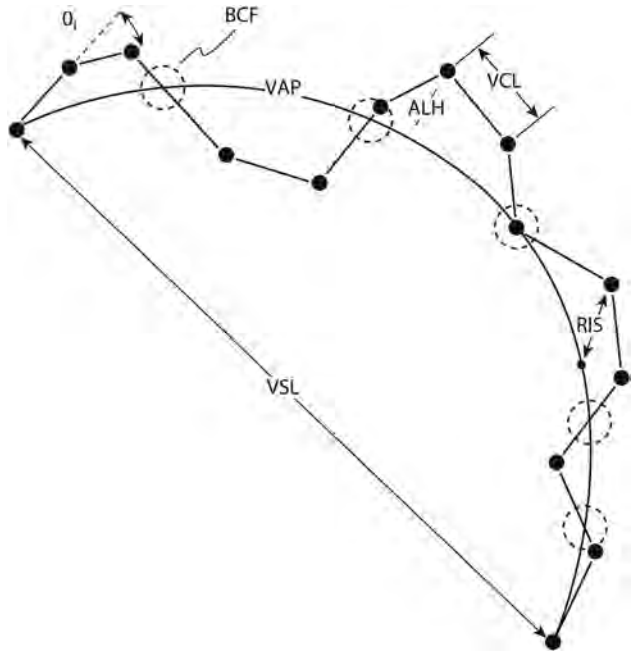

FIGURE 6.3 Examples of kinematic measurements involved in a single-sperm tracing.

Abbreviations: ALH, amplitude of lateral head; BCF, beat-cross frequency; RIS, riser displacement; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity. (Compiled from data in [22].)

Sperm antibodies

Because mature sperm are formed after puberty, they can be recognized as foreign protein by the male immune system. In the testes, the sperm are protected from circulating immunoglobulins by the tight junctions of the Sertoli cells. As long as the sperm are contained within the lumen of the male reproductive tract, they are sequestered and protected from the immune system, and no antibodies form to their surface antigens. If there is a breach in this so-called blood–testis barrier, an immune response may be initiated. The most common causes of a breach in the reproductive tract, which could initiate antibody formation, include vasectomy, varicocele repair, testicular biopsy, torsion, trauma, and infection [25, 26]. Once formed, antibodies are secreted into the fluids of the accessory glands, specifically the prostate and seminal vesicles. At the time of ejaculation, the fluids from these glands contribute most of the volume to the seminal plasma. These antibodies can then come into contact with the sperm and may cause them to clump. In women, the atraumatic introduction of sperm into the reproductive tract as a result of intercourse or artificial insemination does not appear to be a factor in the production of sperm antibodies. However, events that induce trauma or introduce sperm to the mucous membranes outside of the reproductive tract can induce antibody formation. Proposed examples of such events include trauma to the vaginal mucosa during intercourse or the deposition of sperm into the gastrointestinal tract by way of oral or anal intercourse [26]. There are several tests currently employed for detecting the presence of sperm antibodies. The two most common are the mixed agglutination reaction (MAR) and the immunobead binding test. The widespread application of ICSI has reduced the use and benefit of these tests and is commonly now used to circumvent any antibody formation.

The MAR

This test is performed by mixing semen, IgG- or IgA-coated latex beads or red blood cells, and IgG or IgA antiserum on a microscope slide. The slides are incubated and observed at 400× magnification. At least 200 sperm are counted. If antibodies are present, the sperm will form clumps with the coated latex beads or coated red blood cells. If antibodies are absent, the sperm will swim freely. The level of antibody concentration considered to be clinically relevant must be established by each centre conducting the test. The WHO considers a level of binding of ≥50% to be clinically significant. This test is used only for detection of direct antibodies in men, and is not specific for the location of bead attachment to the sperm.

The immunobead binding test

The direct immunobead test is performed by combining IgG- or IgA-coated latex beads and washed sperm on a slide. After washing, the sperm is placed on a slide with IgG- or IgA-coated latex beads and is read at 200× or 400× magnification. Similar to the MAR test, this test can be used for the detection of direct antibodies in men. If antibodies are absent, the beads will not attach to the sperm. If antibodies are present, the small beads will attach directly to the sperm. This test provides potentially greater information than the MAR, as results consider the number of sperm bound by beads, the type of antigen involved in binding, and the specific location where the bead is bound to the sperm.

This test may also be used to detect antibodies produced in a woman's serum, follicular fluid, or cervical mucus by incubating these bodily fluids with washed sperm that have previously tested negative for antibodies. To perform an indirect test, known direct antibody-negative sperm are washed and incubated for one hour at 37°C with the bodily fluid to be tested. The sperm are then washed free of the bodily fluid, resuspended, and mixed on a slide with IgG- or IgA-coated latex beads. The test is interpreted by noting the percentage and location of the bead attachment. Historically, the third edition WHO standard considered the level of binding of ≥20% as representing a positive test. The fourth, fifth, and sixth edition WHO standard considers a level of ≥50% to be a positive test. The level of binding of ≥50% is commonly considered to be clinically significant [12, 27]. The clinical value of anti-sperm antibody testing is predicated on the observation that the presence of a significant concentration of antibodies may impair fertilization. It has been reported that antibody-positive sperm may have difficulty penetrating cervical mucus. Although in these cases intrauterine insemination or IVF may improve the prognosis for fertilization, antibody levels >80%, coupled with subpar concentration, motility, or morphology, may necessitate the addition of ICSI in order to achieve the highest percentage of fertilization [24].

Sperm vitality

An intact plasma membrane is an integral component of, and possibly a biologic/diagnostic indicator for, sperm viability. The underlying principle is that viable sperm contain intact plasma membranes that prevent the passage of certain stains, whereas nonviable sperm have defects within their membranes that allow for staining of the sperm. Several so-called vital stains have been employed for this purpose. They include eosin Y, trypan blue, and/or nigrosin [28]. When viewed with either bright field or phase contrast microscopy, these stains allow for the differentiation of viable, non-motile sperm from dead sperm. This procedure may, therefore, play a significant role in determining the percentage of immotile sperm that are viable and available for ICSI. Unfortunately, dyes such as eosin Y are specific DNA probes that may have toxic effects if they enter a viable sperm or oocyte, which precludes the use of these sperm that have been exposed to the dyes for ICSI or insemination. Flow cytometry has also been utilized for the determination of sperm viability. Like vital staining, flow cytometry is based on the principle that an intact plasma membrane will prevent the passage of nucleic acid-specific stains. Some techniques, such as the one described by Noiles et al., employ dual staining, which can differentiate between an intact membrane and a damaged membrane [29].

Hypo-osmotic swelling test

Another means of assessing the sperm plasma membrane is the hypo-osmotic swelling test (HOST). This assay is predicated upon the observation that all living cells are permeable to water, although to different degrees. The human sperm membrane has one of the highest hydraulic conductivity coefficients (2.4 µL/min/atm at 22°C) of any mammalian cell [30].

As originally described, the HOST involves placing a sperm specimen into hypotonic conditions of approximately 150 mOsmol [31]. This environment, while not sufficiently hypotonic to cause cell lysis, will cause swelling of the sperm cells. As the tail swells, fibres cause the sperm tail to curl, and this change can be detected by phase contrast microscopy, differential interference contrast, or Hoffman optics. The normal range for a positive

test is typically considered to be a score ≥60%; that is, at least 60% of the cells demonstrate curling of the tails. A negative test is defined as <50% curling [32]. This test generated a significant amount of initial interest, and several investigators compared it to the sperm penetration assay (SPA) as an *in vitro* surrogate for fertilization, reporting good correlation [33, 34]. The use of the HOST is not increasing significantly, but can be beneficial in specific cases, such as those with nonmotile sperm, for example patients with ciliary dyskinesia [35].

Assays of the sperm acrosome

The acrosome is an intracellular organelle, similar to a lysosome, which forms a cap-like structure over the apical portion of the sperm nucleus [36]. The acrosome contains multiple hydrolytic enzymes, including hyaluronidase, neuraminidase, proacrosin, phospholipase, and acid phosphatase, which, when released, are thought to facilitate sperm passage through the cumulus mass, and possibly the zona pellucida as well (Figure 6.4). In fact, only acrosome-reacted sperm is capable of penetrating the zona pellucida, binding to the oolemma, and fusing with the oocyte [37]. Once sperm undergoes capacitation, it is capable of an acrosome reaction. This reaction is apparently triggered by the fusion of the sperm plasma membrane with the outer acrosomal membrane at multiple sites, leading to the diffusion of the acrosomal enzymes into the extracellular space. This leads to the dissolution of the plasma membrane and acrosome, leaving the inner acrosomal membrane exposed over the head of the sperm (Figure 6.5). Although electron microscopy has produced many elegant pictures of acrosome-intact and acrosome-reacted sperm, it is not always possible to know whether sperm that fail to exhibit an acrosome have truly acrosome reacted, or could possibly be dead. In addition, electron microscopy is not a technique that is available to all andrologists.

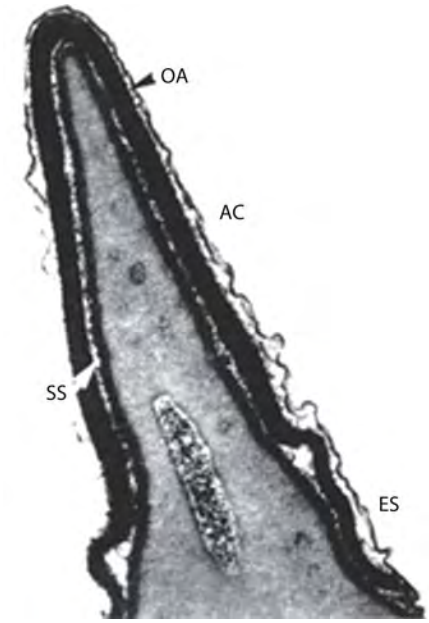

FIGURE 6.4 Sperm head with intact acrosome.

Abbreviations: AC, acrosomal cap; ES, equatorial segment; OA, outer acrosomal membrane; SS, sub-acrosomal space. (From [17], with permission.)

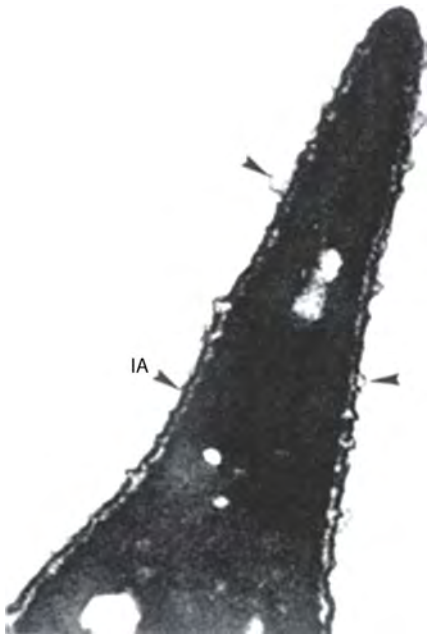

FIGURE 6.5 Acrosome-reacted sperm.

Abbreviation: IA, inner acrosomal membrane. (From [17], with permission.)

This has led to the necessity for the development of biochemical markers for the acrosome reaction. Throughout the 1970s, 1980s, and 1990s, multiple biochemical tests were described using a variety of lectins, antibodies, and stains. Although they apparently correlated well with electron microscopy, the tests were still time-consuming and difficult to perform [35, 38]. Contemporary assays for the determination of acrosomal status employ fluorescent plant lectins or monoclonal antibodies, which can be detected much more easily with fluorescence microscopy [39, 40]. This test is not routinely used, as the utilization of ICSI circumvents the need for acrosome binding to the zona pellucida.

Other biochemical tests

As noted earlier, one of the predominant enzymes that is present in the acrosome is proacrosin. The enzymatic action of acrosin is not necessarily correlated to the presence of an intact acrosome; therefore, assays for the presence of acrosin have been described [41]. Acrosin activity has been reported to be greater in fertile men than in infertile men [42]; however, there are no prospective evaluations correlating acrosin activity to fertilization rates in ART patients. Like all other tissues that require energy synthesis and transport, spermatozoa contain measurable levels of creatinine phosphokinase. Two isomers, CK-M and CK-B, have been described, and differences have been noted in the levels of these isomers in semen specimens from fertile and infertile men. Specifically, CK-M levels exceed CK-B levels in normospermic males, while CK-B levels are greater in spermatozoa from oligospermic males [43]. In this same study, researchers found that semen samples in which CK-M/CK-B ratios exceeded 10% exhibited higher fertilization rates in IVF than specimens with lower ratios. Few other studies have addressed this topic.

Sperm penetration assay

The SPA or hamster egg penetration assay was initially described by Yanagimachi et al. in 1976 [44]. It measures the ability of sperm to undergo capacitation and the acrosome reaction, penetrate the oolemma, and then decondense. In this test, oocytes from the golden hamster are first treated in order to remove the zona pellucida. As one of the functions of the zona is to confer species specificity, its presence would preclude performance of this test. However, zona removal obviously prohibits the SPA from being able to assess sperm for the presence of zona receptors.

Following zona removal, human sperm are incubated for 48 hours along with hamster oocytes, and the number of penetrations with nuclear decondensation is calculated. As originally described, it was hoped that the test would correlate with the ability of human sperm to fertilize human oocytes *in vitro*. Although the test was designed to assess the ability of sperm to fuse to the oolemma, it also indirectly assesses sperm capacitation, the acrosome reaction, and the ability of the sperm to be incorporated into the ooplasm. Unfortunately, however, intrinsic in the design of the test is its inability to assess the sperm's ability to bind to—and penetrate through—the zona pellucida. This factor continues to be one of the major criticisms that plague this test. Throughout the 1980s, multiple modifications of the SPA were published. These included modifications of the techniques for sperm preparation prior to the performance of the assay, such as inducing the acrosome reaction or incubation with TEST yolk buffer (Fuji Film Irvine Scientific, Irvine, CA), changes in the protocol methodology itself, and modifications of the scoring system [45, 46]. Published reports demonstrated widely varying conclusions, such as the finding that the SPA could identify anywhere from 0% to 78% of men whose sperm would fail to fertilize oocytes in ART procedures [47]. Most criticisms of the SPA literature centre on the poor standardization of the assay, the poor reproducibility of the test, and the lack of a standard normal range.

Although some reports suggest a correlation between the SPA and fertility, neither a large literature review [47] nor a prospective long-term (five-year) follow-up study demonstrated such a correlation [48]. In fact, a meta-analysis of 2906 subjects from 34 prospective, controlled studies suggested that the SPA is a poor predictor of fertilization [49]. In light of these considerations, support for this test has gradually waned.

Hemizona assay

Research has demonstrated a significant correlation between tests of sperm–zona pellucida binding and subsequent fertilization in ART. This led the European Society for Human Reproduction and Embryology (ESHRE) Andrology Special Interest Group to recommend inclusion of such tests in the advanced evaluation of the male [50]. Like the SPA, the hemizona assay (HZA) employs sperm and nonviable oocytes in an *in vitro* assessment of fertilization [51]. In this test, however, both gametes are human in origin. As described, the HZA assesses the ability of sperm to undergo capacitation, acrosome react, and bind tightly to the zona. Classically, oocytes that failed to fertilize during an ART procedure are bisected, and then sperm from a proven fertile donor (500,000/mL) is added to one hemizona, while sperm from the subject male is added to the other hemizona. Following a four-hour incubation, each hemizona is removed and pipetted in order to dislodge loosely attached sperm. A comparison or hemizona index (HZI) is then calculated by dividing the number of test sperm tightly bound to the hemizona by the number of control (fertile) sperm bound to the other hemizona:

$$\text{HZI} = \frac{\text{Number of test sperm bound}}{\text{Number of control sperm bound}} \times 100$$

This test assesses the ability of sperm to bind to the zona itself. Although the HZA is relatively expensive, labour intensive, and difficult to perform, there are some data that suggest that the HZA may help to identify individuals with a poor prognosis for success with ART (Figure 6.6) [52, 53]. A more recent prospective study employing receiver operating characteristic curve analysis has also suggested that HZA results may be used to predict subsequent fertilization in ART procedures with both high sensitivity and specificity [54]. Unlike several other tests of sperm function, a cut-off value (35%) has been identified as a predictor of IVF success. In addition, pregnancy rates in patients with values over 30 have been shown to be significantly higher than those in patients with values under 30 (40.6% vs. 11.1%, $p < 0.05$) [55]. The use of ICSI provides an alternative for successful fertilization in the event that a sperm to zona binding issue exists.

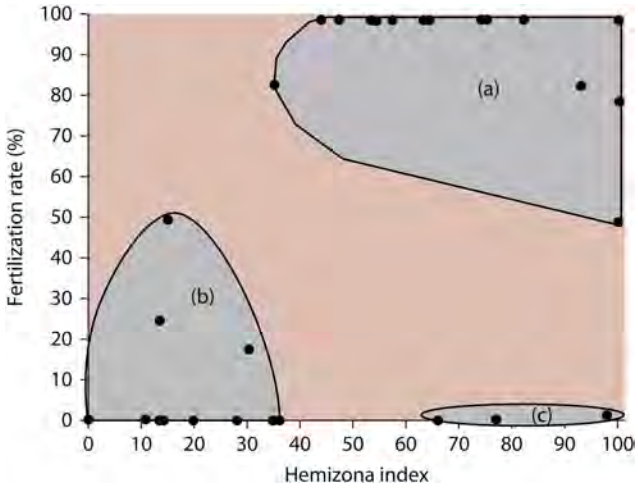

FIGURE 6.6 Cluster analysis of hemizona assay index and fertilization rate. (a) Good fertilization; (b) poor fertilization; and (c) false-positive hemizona assay index. (From [55], with permission.)

Mannose binding assay

Another historical test, the mannose binding assay, was used to assess the ability of sperm to bind to the zona. This *in vitro* procedure is based on a series of observations that suggest that sperm–oocyte interaction involves the recognition by a sperm surface receptor of a specific complementary receptor on the surface of the zona pellucida. This zona receptor appears to be a glycoprotein, the predominant sugar moiety of which is mannose [56]. In an elegant series of experiments, Mori et al. determined that sperm–zona binding could be curtailed by the addition of a series of sugars to the incubating media. Although many sugars impaired binding, the addition of mannose totally inhibited sperm–oocyte interaction [57]. *In vitro* assays in which labelled probes of mannose conjugated to albumin are co-incubated with semen specimens allow for the differential staining

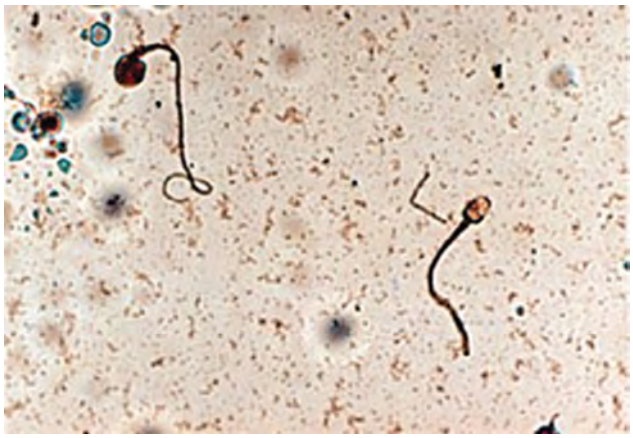

FIGURE 6.7 Mannose-positive (brown) and mannose-negative (clear) sperm. (Courtesy of Tammy Dey and Kaylen Silverberg.)

of sperm (Figure 6.7). Those that bind the probe are thought to possess the sperm surface receptor for the mannose-rich zona glycoprotein. Several investigators, including our group, have subsequently demonstrated that sperm from fertile populations exhibit greater mannose binding than do sperm from infertile males [58–60]. The application of ICSI has made this test obsolete.

Assays of sperm DNA integrity

The most current area of investigation into sperm function involves the assessment of sperm DNA integrity. Sperm chromatin has been demonstrated to be packaged very differently from chromatin in somatic cells. Specifically, the DNA is organized in such a manner that it remains very compact and stable [61]. As there are many ways in which this DNA organization or the sperm chromatin itself can be damaged, several assays of sperm chromatin assessment have been developed. There are two basic types of assays: direct assays, such as the "Comet" and "Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL)" assays; and indirect assays, such as the sperm chromatin structure assay or acridine orange assay [62]. The direct assays detect actual breakages in the DNA, while the indirect assays measure the relative proportions of single-stranded (abnormal) and double-stranded (normal) DNA within the sperm following acid treatment. Data from several studies suggest that infertile men have a significantly greater amount of DNA damage than fertile men [61, 63, 64]. There is also a suggestion that this finding is similarly present in the male partners of couples experiencing recurrent miscarriage. Despite these reports, at the present time, there is no conclusive correlation between the results of sperm DNA integrity testing and pregnancy rates achieved either naturally or with the ARTs. As such, the Practice Committee of the American Society for Reproductive Medicine recommended that the routine testing of sperm DNA integrity should not be included in the evaluation of infertile couples [65].

Conclusion

In summary, there have been many recent advances in the diagnostic evaluation of sperm and sperm function. Although many tests of sperm function have been described, there remains a lack of consensus as to the role of testing and the identification of the appropriate test(s) to perform. Owing to the complicated nature of sperm function, it is improbable that a single test will emerge with sufficient sensitivity, specificity, and positive and negative predictive values required of a first-line diagnostic tool for all affected men. A more likely scenario will be similar to that in female infertility, where a battery of tests—each evaluating a specific function—are employed as needed. Considering profound recent advances in gamete micromanipulation (e.g., ICSI), a more germane issue might be the overall relevance of sperm function testing in the contemporary andrology laboratory. Although this issue is quite controversial, it is likely that sperm function testing will continue to play a role in the evaluation of the infertile male. Just as ART is not the treatment of choice for all infertile women, it is not likely that micromanipulation will be the standard treatment for all infertile men. The gold standard of sperm function remains the ability to fertilize an oocyte *in vitro*. Therefore, in order to continue to address the preceding questions, it is incumbent upon investigators to design appropriate prospective trials to assess these tests thoroughly. Those tests that demonstrate a statistically significant correlation with fertilization *in vitro* must then undergo additional evaluation in order to assess clinical significance if we hope to develop an appropriate diagnostic algorithm.

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7

SPERM PREPARATION TECHNIQUES AND ADVANCED SPERM SELECTION FOR INTRACYTOPLASMIC SPERM INJECTION

Sinan Ozkavukcu, George Hughes, and Christopher LR Barratt

Overview

Sperm selection *in vivo*

Spermatozoa ejaculated during sexual intercourse undergo a challenging selection process *in vivo* [1]. As shown in humans and many other species, the number of spermatozoa reaching the uterine tubes is tens of thousands of times less than the number ejaculated [2]. The enhanced functional ability of the select cohort of spermatozoa that reach the uterine tubes is attributed to the sequence of events involved in capacitation and hyperactivation [3]. A combination of the challenging anatomical pathway created by the female reproductive tract and sperm competition contributes to eliminating the less functional spermatozoa [1]. Active and passive barriers present in the female reproductive tract, for example, the vaginal pH, cervical mucus, indistinct orifices of the uterine tubes, and the immune responses to the sperm, all aid in selecting a single spermatozoon for fertilization of the oocyte [4–6]. There is preliminary evidence that the accumulation of a higher number of spermatozoa in the ipsilateral uterine tube with the ovulating ovary was attributed not only to the peristaltic contractions in the uterine canal [7, 8] but also to the increased adnexal temperature and hormone concentrations during the late follicular phase [9]. For this reason, the separation of sperm with taxis methods (a form of sperm guidance towards or away from a specified stimulus, e.g., chemotaxis, thermotaxis) has become an increasingly popular way of improving sperm selection *in vitro* [10, 11].

Considering the routine practical applications of today's assisted conception laboratory, many complex mechanisms mentioned earlier can be bypassed to achieve fertilization and help couples dealing with infertility. Although conflicting data exist regarding the fact that the quality of spermatozoa that pass the cervix, uterus, and uterine tubes tend to be more functionally competent than the raw ejaculated sperm, the *in vitro* methods that are applied to isolate the best sperm cohort for *in vitro* fertilization (IVF) are called sperm selection/preparation methods.

Regardless of the insemination method applied in the laboratory, current sperm selection methods can isolate the most favourable cohort of spermatozoa within the heterogeneous population in semen and mathematically increase the chance of selecting the sperm with a high fertilization capacity [12].

Removal of the spermatozoa from the seminal plasma

The process of sperm washing aims to separate spermatozoa from the seminal plasma and thus remove decapacitating and damaging factors for the sperm. Apart from sperm selection, to perform sperm function tests correctly, the seminal plasma and spermatozoa should be separated as soon as liquefaction occurs. Long-term incubation of spermatozoa in seminal plasma increases their exposure to potentially detrimental factors, mainly caused by non-spermatogenic cells (e.g., immune cells), immature spermatogenic fractions, epithelial cells and cytoplasmic residues, via radical oxygen species (ROS) [13]. ROS products, which are beneficial to some extent for sperm capacitation, can cause damage when they accumulate at pathologic levels [14]. Indeed, a recent publication by Torra-Massana et al. demonstrated adverse clinical outcomes when sperm washing is delayed after collection. The study concluded that the optimum incubation time before sperm washing was 20 minutes, with significantly reduced fertilization rates in both donor and patient oocytes if this period was exceeded [15]. Other publications displayed similar findings in clinical outcomes. Evidence shows that once semen is washed and seminal fluid is separated from the sperm cells, further incubation of spermatozoa in a physiological solution does not cause additional damage [16–21].

Semen, unlike other body fluids, is not in a homogeneous suspension and is made up of contributions from several secretory organs. Ejaculation, a two-stage neuroendocrine reflex, occurs through the successive steps of emission and expulsion. While the seminal vesicle contributes 70% of the volume of semen, it is thought that the prostate and epididymis share the other 30% of the volume equally. Various cellular and biochemical studies have shown that the initial emission into the ejaculatory duct just before expulsion is a mixed secretion of the contents originating from the prostate and the epididymis tail, where the latter is the source of matured spermatozoa [22–26]. Therefore, the first ejaculated fraction is followed by high-volume secretion of the seminal vesicle with very few spermatozoa. During sexual intercourse, following ejaculation, the cervical mucus is washed with the sperm-rich first fraction, and the sperm is instantly carried through the uterus. This physiological process differs significantly from the semen collection procedure. In a clinical setting, the whole semen sample is collected into a container by masturbation, and exposure to seminal vesicle secretions might jeopardise the physiological capacity of the spermatozoa. Studies comparing the utility of split ejaculate fractions have gathered conflicting results, and the technique is not seen as practical in terms of semen collection in clinics [24, 26–28].

Sperm washing

It is recommended to centrifuge the semen to separate seminal plasma from cellular components after dilution with an appropriate medium. Sperm washing is not a sperm selection method as it does not separate live from dead and dying spermatozoa, cellular debris, bacteria, epithelial cells, or immune cells; instead, it collects them all as a packed pellet at the bottom of the centrifuge tube. It is, however, a simple method to remove the seminal plasma containing ROS, prostaglandins, toxicants, and any other contaminants (e.g., gradient medium, fixatives, antibodies,

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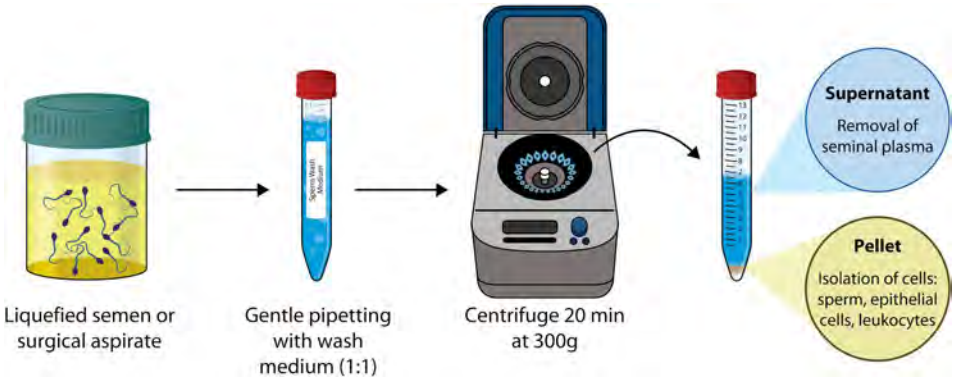

FIGURE 7.1 Basic methodology of sperm washing.

dyes) that are needed to be washed out. Sperm washing is easy to perform before several diagnostic examinations and before and after sperm cryopreservation [29]. A stand-alone sperm washing can be adapted for patients with an extremely low number of ejaculated sperm (e.g., severe oligozoospermia, cryptozoospermia), retrograde ejaculation, or cases with testicular/epididymal aspirations. The basic procedure of sperm washing is depicted in Figure 7.1.

Is centrifugation detrimental to spermatozoa?

Many publications state that the centrifugation method used during sperm preparation compresses the cells with the applied *g*-force, causing membrane and mitochondrial damage within the cells accumulated in the pellet and thus increasing the production of ROS [30]. It is unclear whether ROS production is caused directly by the sperm cells or other cell types in the semen [31]. It is suggested that the duration of centrifugation rather than the centrifugation speed leads to higher ROS production [32].

It is paradoxical that the centrifugation process, which is used to eliminate the existing ROS in the seminal plasma, is responsible for the production of ROS itself. However, since seminal plasma also contains antioxidant substances, it is known that its removal by centrifugation decreases the elimination of superoxide anion [33]. The current consensus includes choosing a sperm selection method in which the centrifuge step is minimalized or removed.

Sperm selection methods *in vitro*

Conventional sperm selection methods in IVF: Swim-up and density gradient centrifugation

Swim-up

The swim-up procedure imitates the natural process of spermatozoa migration through the cervical mucus. It is a commonly used technique in IVF laboratories to recover a pure fraction of highly motile sperm with tiny debris, leukocytes, or germ cells. A very rapid direct swim-up can be applied to the ejaculate, where the liquefied semen can be placed into the bottom of a centrifuge tube and overlaid directly with a sperm wash medium. As the sperm swim out of the seminal plasma, motile sperm are collected in the upper-layer culture medium, which leaves the lower layer containing debris, seminal plasma, and immotile sperm (Figure 7.2). The culture media is then pipetted out carefully to gather motile sperm in the final sperm preparation in this direct method [34].

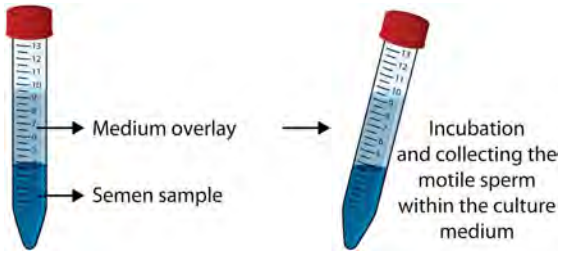

FIGURE 7.2 Rapid isolation of motile sperm using the direct swim-up technique.

Another method of the swim-up procedure is to wash the semen sample, remove the supernatant after centrifugation, and gently overlay the pellet with a fresh sperm wash medium. The centrifuge tube can be incubated at 37°C at an angle of 45° (to increase the surface area of migration) for 45–60 minutes. Still, the optimum period should preferably be adapted according to the initial sperm concentration and motility. Following incubation, the top portion of the overlay is used for the desired insemination method (Figure 7.3).

Swim-ups are unsuitable for oligo- and asthenozoospermic samples as they may yield a very low number of motile sperm. Highly viscous samples can also respond poorly to the swim-up technique.

Density gradient centrifugation (DGC)

Gradient separation techniques are simple and rapid methods to remove spermatozoa from the seminal plasma and are routinely used for sperm selection for IUI, IVF, and ICSI [35]. Density gradient centrifugation (DGC) and swim-up (SU), which are accepted as the basic sperm preparation methods, have been compared in many studies. Their superiority over each other has been reported in various publications. DGC consists of centrifugation of semen overlaid on the density gradient layer(s) containing silane-coated colloidal silica. Centrifuge forces migrate the cells and accumulate them in different gradient levels by density. Normal sperm have a greater density (1.10 g/mL) than abnormal sperm due to

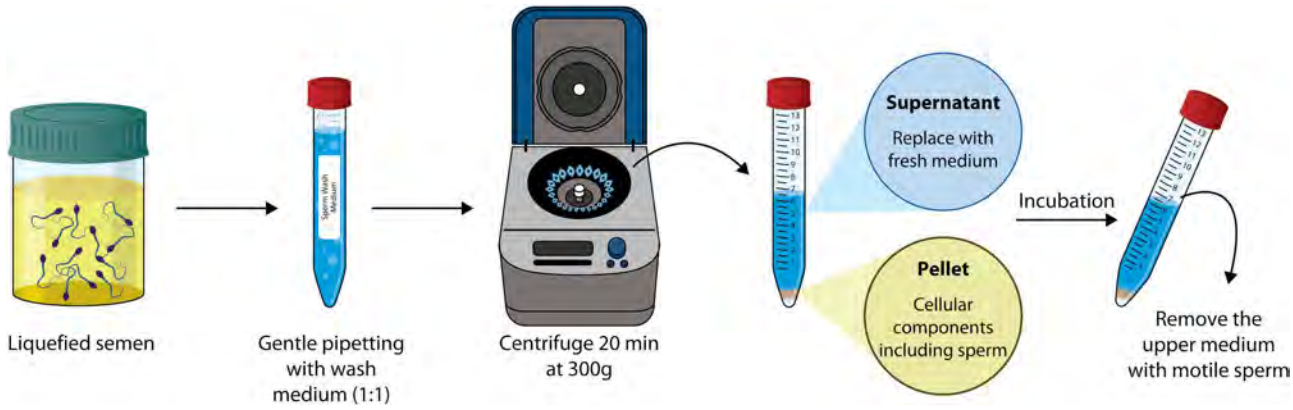

FIGURE 7.3 Swim-up methodology.

their highly condensed DNA. This is known as isopycnic centrifugation, as cells are accumulated at the point where their density is identical to the gradient media [36]. Sperm preparation using DGC usually results in a fraction of highly motile spermatozoa, a cohort free from debris, contaminating leukocytes, non-germ cells, and degenerating germ cells. A simple two-step discontinuous density-gradient preparation method is widely applied, usually following a washing step and with or without a further swim-up step session. Briefly, two different concentrations of gradient media (mostly 45%–90% or 40%–80%) are gently layered as a column to the bottom of a conical centrifuge tube, and the same volume of raw semen is overlaid on the top of them. Solutions can be prepared in desired concentrations, but commercial ready-to-use products are widely available. After centrifugation, a clean sperm suspension, free of seminal plasma, is obtained as a pellet which needs to be washed using a sperm wash medium to remove any trace of the gradient solution (Figure 7.4). In case of a very low number of sperm in the semen, a single layer (continuous) or reduced-volume discontinuous DGC (mini-DGC) can be used to increase the number of recovered sperm in the pellet. It is uncertain if this approach has an additional benefit in semen with abnormal parameters [37, 38].

Comparison of assisted reproductive technique (ART) outcomes after DGC and SU are insufficient due to the limited number of studies with limited sample sizes. A recent Cochrane meta-analysis included four RCTs to compare clinical pregnancy rates (CPR) after artificial insemination with the semen sample prepared using DGC or SU. There was no significant difference in CPR between the SU and DGC groups [39]. Similarly, a recent study compared cumulative live birth rates (CLBR) after using DGC- or swim-up-prepared sperm and found no significant difference between the groups [40]. Density gradients can be used for normozoospermic samples and those with sub-optimal parameters. They will generally result in high yields of motile sperm, even when samples have an initial low sperm concentration [41].

There is currently insufficient evidence to demonstrate improved results of IVF and ICSI cycles using different sperm preparation methods [40]. However, DGC and SU methods are established as the gold standard for sperm selection/preparation for insemination. To determine the benefits of the new and advanced sperm selection methods mentioned in this chapter, comparative studies should be examined meticulously against these routine methods.

Preparation of surgically aspirated/extracted samples

Epididymal or testicular aspirate fluid can be obtained from the male genital tract by microsurgical epididymal sperm aspiration (MESA), percutaneous epididymal sperm aspiration (PESA), testicular sperm aspiration (TESA), and testicular sperm extraction

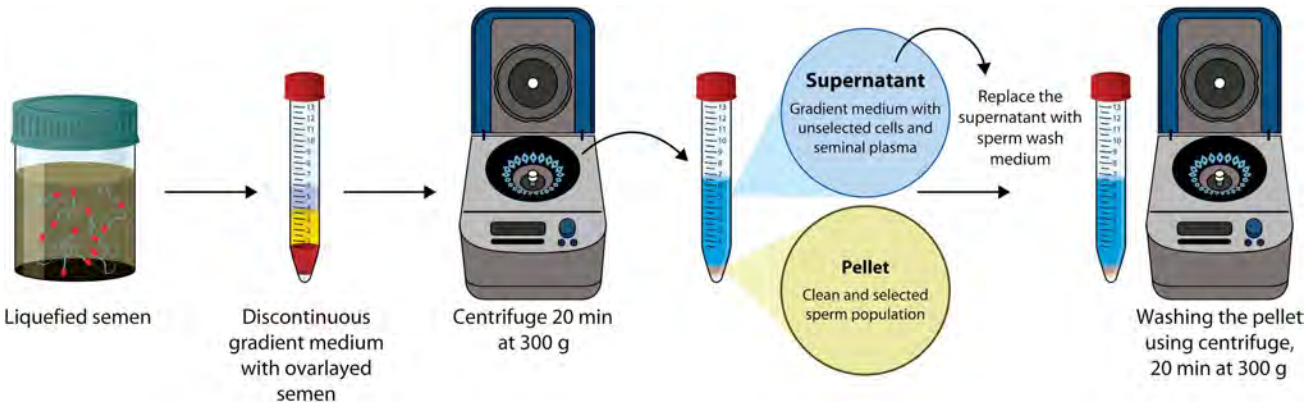

FIGURE 7.4 Steps for discontinuous density gradient centrifugation procedure.

(TESE). If the seminiferous tubules are collected by examining them under high magnification using a stereomicroscope during the TESE operation, it is called a micro-TESE (mTESE) procedure. It is generally acceptable to perform a diagnostic aspiration/extraction before ovarian stimulation and perform freezing to ensure that spermatozoa will be available on the day of oocyte collection for an ICSI treatment cycle. Alternatively, surgical sperm retrieval may be performed on the day of the oocyte retrieval procedure due to poorer freeze–thaw rates of the lower-quality sperm in cases of non-obstructive azoospermia [42].

Epididymal aspirates containing large numbers of motile spermatozoa, with minimal red blood cell and non-germ cell contamination, can be subjected to a DGC as a preparation method for subsequent use. However, a simple wash may also be preferable if a very low number of motile spermatozoa are expected [35].

To free the seminiferous tubule-bound elongated spermatids ("testicular spermatozoa"), enzymatic or mechanical methods are required. The enzymatic method involves incubating the testicular tissue with collagenase for 1.5–2 hours at 37°C and vortexing the suspension every 30 minutes. Centrifugation follows the incubation at 100 *g* for 10 minutes and a microscopic examination of the pellet [43–45].

The mechanical method involves maceration of the testicular tissue in a culture medium using either glass coverslips or fine needles (attached to disposable tuberculin syringes) that are bent parallel to the base of the culture dish until a fine slurry of dissociated tissue is produced [46]. It is recommended to use the DGC method to obtain clean preparations after the dissection of testicular extraction samples as they contain highly heterogeneous cells and connective tissue components.

Preparation of samples with potential viral load

Using new and improved anti-viral medications has increased life expectancy and quality of life for patients with blood-borne viruses (BBVs). The management of patients with a viral infection/disease in a fertility clinic aims to provide appropriate treatment options that will result in an increased chance of pregnancy whilst reducing the risk of horizontal (person to person) and vertical (mother to the baby) viral transmission.

In patients with a viral infection/disease, it is recommended that in serodiscordant couples (where one partner is BBV-positive and the other partner is uninfected) and when both partners test positive for hepatitis B virus (HBV), hepatitis C virus (HCV) or human immunodeficiency virus (HIV), that the cause of infertility should dictate the specific technique (IUI/IVF/ICSI) used for ART [47–53].

Specific semen preparation procedures have been recommended to reduce or eradicate viral load in treating infected individuals [47, 49, 54, 55]. Patients with BBV who seek fertility treatment must consult an infectious disease specialist before commencing any treatment, as the theoretical risk of vertical transmission remains a possibility.

Currently, no semen preparation techniques can select HBV DNA-free spermatozoa, and current evidence does not recommend HBV DNA testing on seminal fluid or sperm [47, 56–59]. A discontinuous DGC, washing and then swim-up are recommended for semen processing in patients positive for HCV [51, 60–68], especially with a double washing step for patients positive for HIV [69–74]. Regardless of the semen processing technique used, the post-preparation sample that will be used in ART from males who tested positive for HIV should be tested using polymerase chain reaction (PCR). In serodiscordant couples with the male testing positive for HIV, only an HIV-negative tested sperm sample should be used for the treatments. However, after advanced semen processing, PCR testing for HCV is not necessary. No studies were identified comparing routine semen preparation with advanced semen processing in males testing positive for Human T-lymphotropic virus I/II or Zika virus. For further reading, refer to ESHRE's guideline for medically assisted reproduction in patients with a viral infection or disease [47].

Sperm yield in patients with retrograde ejaculation

Retrograde ejaculation occurs when semen is ejected into the bladder during ejaculation, resulting in aspermia or hypospermia. This can be caused by uncontrolled diabetes mellitus; neurological conditions; side effects of certain drugs; or following prostate, abdominal, pelvic surgery, and radiotherapy. Urine is cytotoxic to spermatozoa due to its high osmolarity and low pH, and as such, in treatment cycles, spermatozoa should be rapidly retrieved from the urine [75–77]. Although alpha-adrenergic agonists, anticholinergic and antihistamine drugs have been described among the medical treatment options in retrograde ejaculation, the preferred method of obtaining spermatozoa is retrieving them from the patient's post-ejaculatory urine. Patients are pre-medicated to make the urine alkaline, using oral sodium bicarbonate or sodium chloride, 1 to 2 hours before attempting to collect an ejaculate. The pre-medication neutralises the highly acidic pH that spermatozoa are exposed to in urine samples, which increases the chance that any spermatozoa will retain their viability and motility characteristics [78]. When the patient is admitted to the andrology laboratory for sample collection, he is given a container for semen production. A second container with 9 mL of sperm washing medium is kept ready at 37°C. In case of ejaculation-with-aspermia, a urine sample is requested into the container containing the sperm washing medium. This sample is immediately distributed to several tubes and centrifuged at 300 *g* for 10 minutes. After centrifugation, all the supernatant fractions are discarded, and the pellets are collected into a sterile tube with a fresh sperm washing medium. The final suspension is evaluated under the microscope for the presence of viable spermatozoa. For the cells to be used in the ART setting, a double-layered DGC is also recommended [79]. A centrifuge speed of 700 *g* is suggested by Jarupoonpol et al., yielding a higher number of total motile spermatozoa without an increased risk of DNA fragmentation evaluated by the TUNEL assay [80].

Sperm selection in samples with no motility

Total asthenozoospermia is a rare condition but is still seen in low-quality semen parameters, systemic diseases and after freezing and thawing of the semen. If all sperm are immotile in the ejaculate, the possibility of inappropriate semen collection must first be considered. Using detergent-based lubricants, soap, etc., during masturbation is the most common cause of total asthenozoospermia in clinics. If this is the case, it is advisable to consult the patient and provide clear instructions for semen sample production. Some lubricants in the market are shown to be non-toxic to spermatozoa; thus, their usage in need is reported to be safe during infertility treatments [81].

It is recommended to perform vitality tests if the sperm motility is lower than 40% in repetitive semen analysis, according to the World Health Organization's laboratory manual for the examination and processing of human semen [35]. A motility stimulation test using phosphodiesterase inhibitors (PDEI) on a wet preparation might help diagnose and decide further interventions

during the insemination. In some rare genetic mutations, like in Kartegener's syndrome, structural defects in the ciliary axoneme cause total asthenozoospermia. A definitive diagnosis can be made by extensive history taking, genetic mutation screening, and examination of the ciliary structures under a transmission electron microscope. The only treatment option for these patients is through the use of ICSI, and different methods are suggested to allow the identification of viable sperm. In the presence of PDEI-resistant cases, the use of electrical stimulation [82], hypoosmotic swelling [83], laser-assisted viability assessment [84], and evaluating sperm head birefringence properties [85] are suggested.

Magnetic-activated cell sorting (MACS)

Phosphatidylserine (PS) is a phospholipid found extensively in eukaryotic plasma membranes, and the enzyme flippase typically stabilises PS head regions on the inner layer of the cell membrane. As an early response in biological processes such as apoptosis and necroptosis, the cell surface enzyme scramblase releases the PS and flips its head region to face outside the membrane [86]. The caspase-mediated apoptotic PS exposure is irreversible [87]. Externalized PS (EPS) can be detected by annexin-V immunolabeling, which has a high affinity to PS. The magnetic-activated cell sorting (MACS) technique was designed on the principle of selecting out apoptotic sperm (annexin-V positive cells) (Figure 7.5).

Studies evaluating the efficacy of MACS as a sperm selection method reveal mixed results. In addition to studies comparing MACS versus routine sperm selection techniques, there are studies examining in which order it should be applied. It is reported that when MACS is utilized with DGC, a mean reduction of 70% in EPS-positive sperm is achieved, with an improvement in the viability and motility of the suspension [88]. Other studies have found it more efficient when MACS is performed before DGC [89–91]. Bucar et al. demonstrated that a combination of MACS followed by DGC and swim-up yielded a final sperm preparation with a low level of DNA fragmentation [92]. Some studies have evaluated the use of MACS in a group of selected patients and have reported favourable clinical outcomes when used in patients with high DNA fragmentation [93–95], teratozoospermia [94, 96, 97], varicocele, and couples with unexplained fertility [98]. A recent randomized controlled trial revealed significantly higher ongoing pregnancy rates using MACS-selected sperm compared to DGC. However, the miscarriage rates were comparable [99]. Sánchez-Martín et al. reported that irrespective of the sperm DNA fragmentation rates in males, MACS significantly decreased the miscarriage rates in ICSI cycles with autologous or donor oocytes [100]. There are studies where no difference is found when sperm are selected with MACS vs routine sperm preparation methods in terms of motility, morphology and DNA fragmentation rates [101], live birth [102], and miscarriage rates [100]. A controlled randomized trial including oocyte donation cycles and unselected males revealed that reproductive outcomes did not improve when MACS was conducted before ICSI [103].

To conclude, there is insufficient evidence to recommend the MACS method to be used alone or in combination with routine procedures in sperm selection. A recent Cochrane database systemic review reported that the beneficial potential of MACS on clinical pregnancy, live birth, and miscarriage rates is uncertain, and the quality of evidence is very low [105]. In a randomized controlled trial, where the obstetric and perinatal outcomes of MACS were evaluated, it was stated that no perinatal adverse side effects were observed [106]. In light of these results, more clinical studies in different indications are needed for sperm selection with MACS, as short-term side effects seem relatively safe.

Microfluidic-based methods for sperm selection

Microfluidic systems have been developed to imitate the sperm movements in the female reproductive tract, the cervical canal, uterine tube lumen, and the complex epithelium of the uterine tubes to isolate a cohort of sperm that have high

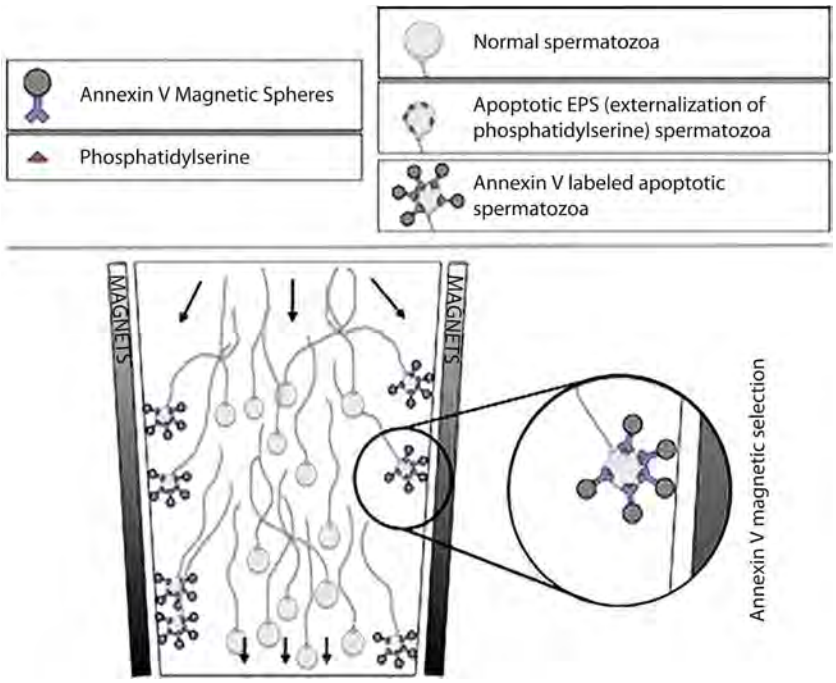

FIGURE 7.5 The principal of sperm selection using magnetic-activated cell sorting. (Modified from [104], with permission.)

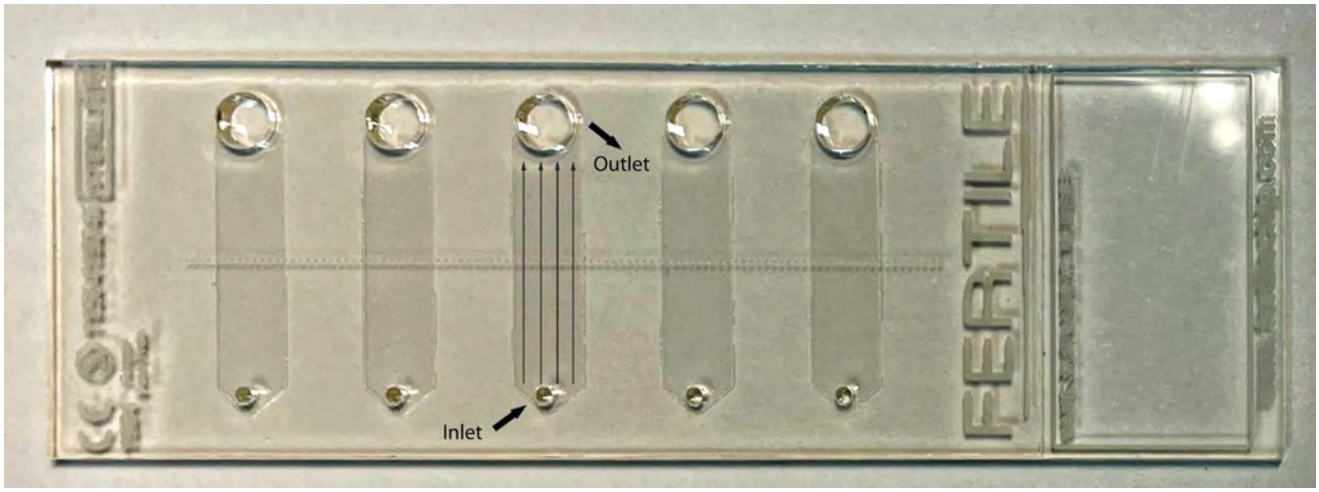

FIGURE 7.6 A commercial product introduced for sperm selection based on the motility of sperm. After the injection of unselected sperm suspension into inlets, by swimming through the microchannels (thin arrows), selected sperm can be collected from the outlet.

fertilization potential. Some of these systems create an actual fluidic environment and support sperm selection over different flow gradients and biophysical forces. However, most devices currently in the market aim to create static microchannel pathways, making selections based on the ability of sperm to pass through labyrinth-like paths depending on their motility (Figure 7.6).

Another strategy used in microfluidic sperm selection is taxis-mediated applications based on the sperm's ability to sense and respond to some external physical stimuli. While the female reproductive tract unites the sperm with the cumulus–oocyte complex (COC) and selects the sperm population with the highest capacity and motility, it has become a fascinating topic for developing novel microfluidic devices [107]. Some mediators, such as progesterone, may increase human sperm capacitation and also have chemotactic properties [108]. Progesterone's ability to activate CatSper channels and to increase Ca^{2+} influx in humans has been shown [107]. Some factors released from the ovulated COC have brought up chemotaxis in developing new microfluidic devices [108–114].

Liquid current forces created in a microfluidic environment also reveal the tendency of the sperm to swim against resistance towards the opposite direction of the current. This phenomenon, called rheotaxis, aims to simulate the indistinctive flow that emerges with ciliary action, muscle contractions, and fluid secretion within the lumen of the uterine tubes. When studies on dynamic microfluidic devices are examined, it can be observed that rheotaxis has been the most commonly studied topic. A recently published model by Jeon et al. used a multidimensional-double spiral microfluidic platform with inertial channels. The fluid flow was run by a syringe pump creating a recirculating flow within the microchannels called Dean flow. Compared to DGC-derived cells, a higher number of sperm were isolated, but the motility parameters were comparable with the control groups [115]. Using similar physical dynamics, Vasilescu et al. published striking data on the successful isolation of testicular-derived sperm with low motility and the elimination of the cancer cells. Separating sperm cells from cancer cells could be beneficial for fertility preservation applications [116].

The third physical property used in microfluidic devices is thermotaxis. The temperature difference of 1°C–2°C between the two ends of the uterine track may create an attraction for sperm that may lead to the expression of some sensory receptors with the capacity to detect temperature gradients. DeToni et al. localized the expression of TRPV1, known as the heat-sensing receptor, in the male gonads and the sperm cells. They determined that the receptor was effective in sensing temperature gradients, and the sensitivity was increased after sperm capacitation [117]. Despite promising results, studies examining sperm selection by thermotaxis are limited, and further data to support the use of this mechanism is required.

Most clinical studies on microfluidic sperm selection are based on the swimming capacity of the sperm throughout microchannels filled with a static fluid. One study published by Gode et al., where IUI cases were inseminated with sperm prepared using a microfluidic device or DGC, highlighted improved pregnancy rates [118]. A study evaluating the ICSI cycle results stated that there was no significant difference in any clinical outcomes among the included couples; however, when a subgroup analysis was performed, more favourable results were evident in cases of advanced maternal age and severe oligozoospermia [119]. Yildiz et al. stated that when couples' first IVF trials were considered, there was no significant difference between the reproductive outcomes when the microfluidic technique was used. Still, a higher pregnancy rate was obtained in the couples who underwent their second IVF cycles [120]. Similarly, it was reported in two different RCTs that there was no significant difference with the control group in terms of selected sperm parameters or the clinical outcomes in couples with unexplained infertility or unselected males when microfluidic sperm selection is performed [121, 122]. In another study, the results on sibling oocytes were evaluated, with no significant difference between the experimental groups [123].

Suffice it to say that there is insufficient data regarding the superiority of microfluidic sperm selection methods in ART cycles. More randomized controlled trials on the use and clinical outcomes of rheo-, thermo-, and chemo-attractants are required.

High-resolution selection of sperm for ICSI (IMSI/MSOME)

Sperm morphology is associated with chromatin maturation defects, DNA fragmentation, aneuploidies, fertilization and embryo development rates, and obstetric outcomes [124]. The association between the long-term well-being status of the offspring with paternal sperm quality has also been shown [125].

Although many methods discussed in this chapter allow the selection of a representative sperm population from raw semen, during the ICSI procedure, the final selection of sperm to be injected into the oocyte is made by the embryologists. In contrast to routine ICSI, where sperm for injection is selected under low-power magnification (200×–400×), Intracytoplasmic Morphologically Selected Sperm Injection (IMSI) combines ICSI and Motile Sperm Organelle Morphology Examination (MSOME) with high-power (>6000×) optical and digital methods to aid selection of the best available spermatozoa [126]. MSOME detects subtle organellar malformations in sperm by examining six subcellular organelles: acrosome, post-acrosomal lamina, neck, mitochondria, tail, and nucleus of the spermatozoa [126–128]. With this technology, many studies have focused on the clear visualization of vacuoles in the sperm head (Figure 7.7) and instant deselection. Vacuoles in the sperm head may originate from the nucleus, acrosome, or post-acrosomal region, and the small ones cannot be detected under conventional magnifications. Publications suggesting MSOME as a diagnostic tool in identifying sperm head vacuoles revealed various predisposing factors such as advanced male age [129], sperm DNA fragmentation [129], abnormal chromatin packing [130–133], and diminished outcomes like poor fertilization [129], suboptimal embryo development [134], low pregnancy rates [129, 135–137], implantation failure [129, 136, 137], and low live birth rates [136, 137]. Severe male infertility is the primary indication suggested for the selected group of patients [138–144], while no benefit was revealed for the cases of repeated implantation failure [145–148]. Data from randomized clinical trials, evaluated in a recently published meta-analysis, show that IMSI does not provide superiority in live birth or miscarriage rates over conventional sperm selection methods [147]. The data obtained from studies comparing the use of IMSI and conventional ICSI in a Cochrane systematic review reported a significant improvement in the clinical pregnancy rates in the presence of very-low-quality evidence but with no apparent significant difference in the miscarriage rates [149]. It is stated that the potential disadvantages of the technique are the increased average duration of the procedures and the cost of essential microscopic equipment [150].

In light of current data, and since it has been shown that the IMSI technique does not have any side effects on neonatal outcomes [152], it is possible that IMSI may be considered as a sperm selection method in cases of severe male factor infertility. However, randomized controlled studies are required to confirm this potential indication.

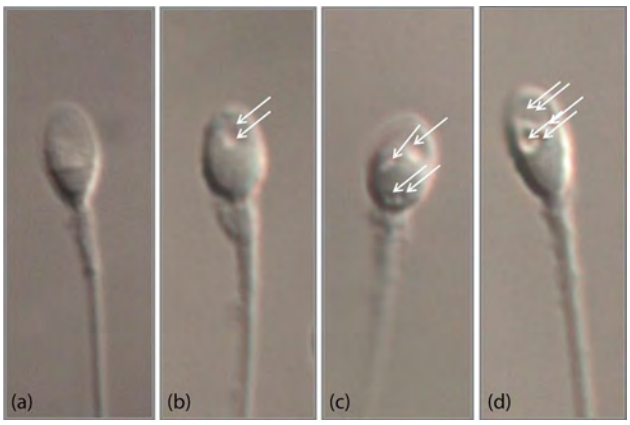

FIGURE 7.7 By using optical and digital high magnifications, sperm morphology (a) can be evaluated for selection during ICSI according to acrosomal (b), nuclear (c), or mixed (d) vacuoles in the sperm head. (From [151], with permission.)

Sperm selection based on hyaluronic acid binding capacity (PICSI)

The binding capacity of mature sperm to hyaluronic acid (HA, hyaluronan) was introduced as a commercial diagnostic kit in the early 2000s as the Hyaluronic acid Binding Assay (HBA). According to this principle, spermatozoa with higher capacity to bind to the zona pellucida, higher fertilization ability, advanced chromatin maturation, lower DNA fragmentation, lower aneuploidy rate, and superior morphology express HA binding sites which attach to HA-coated surfaces from their head region. Thus, with the help of HBA, in addition to the basic semen parameters of the patients, a new diagnostic test is proposed to improve the diagnosis of male factor infertility, particularly in the determination of ICSI indication [153–155].

Although the use of HBA as a screening test for the prediction of ICSI indication is not supported by subsequent studies [156], it has been recommended as a sperm selection method during the ICSI procedure. The method is based on the principle of building solid-state HA-coated areas on the surface of a classical ICSI dish. The sperm with higher potential adhere to these surfaces whilst passing through these areas. The ICSI practitioner collects these sperm prospectively with a microinjection needle and injects them into the oocyte (Figure 7.8).

Physiological selection (or namely "picking") of spermatozoa for ICSI (P-ICSI) has found a wide area of interest and has been the subject of many clinical studies. P-ICSI-selected sperm have been compared with conventional ICSI groups in couples with recurrent pregnancy loss [157, 158], high DNA fragmentation index [95, 99] and high teratozoospermia [159, 160], and conflicting results have been reported. The largest multicentral randomized clinical trial included 2772 couples in 16 different centres in the UK, was called the HABSelect study. The couples were randomly allocated into conventional ICSI and P-ICSI groups, where the primary outcome was full-term live birth. Miller et al. reported that sperm selection using HA binding provided no difference between the groups regarding live birth rates. As a secondary outcome in the study, miscarriage rates were significantly decreased in the P-ICSI group [161]. A predictive model based on the same database and re-analysis of the frozen samples has been published recently, stating that the use of HA-based selection during ICSI may be beneficial in the treatment of couples with older maternal age [162]. A sibling oocyte study which included 45 IVF cycles of patients with previous fertilization failure, poor embryonic development, implantation failure, or miscarriage, reported significantly improved fertilization and embryo utilization rates when oocytes were fertilized using P-ICSI dishes [163].

The results of studies examining the benefits of HA-based sperm selection during ICSI are highly contradictory. A systematic review found no statistically significant difference between

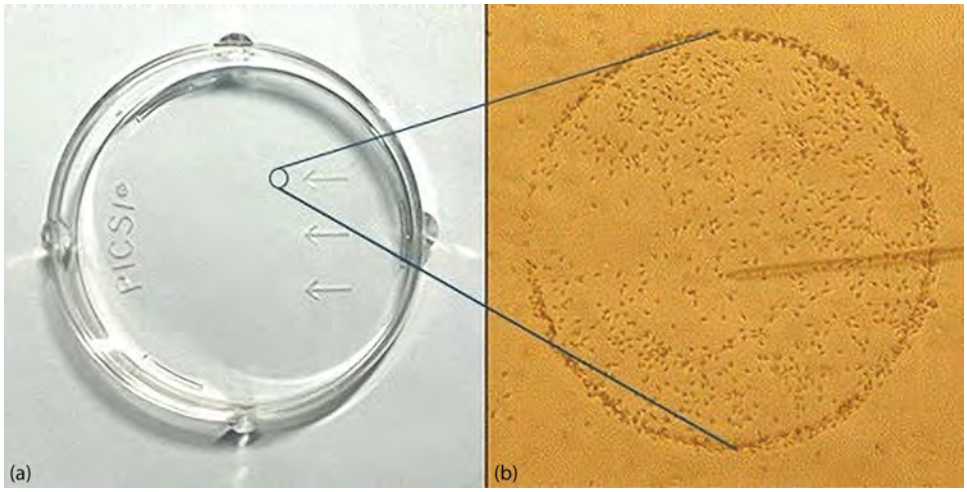

FIGURE 7.8 P-ICSI dish with solid HA-coated areas pointed by arrows (a) allows picking of bounded spermatozoa by the microinjection pipette during ICSI (b). (From [164], with permission.)

P-ICSI and ICSI groups regarding any analysed outcomes [165]. Finally, a meta-analysis reported improved embryo quality and implantation rate, whereas if solely prospective studies were considered, the only improved parameter remains as the embryo quality. Thus, they concluded that the evidence does not support the routine use of hyaluronic acid binding assays in all ICSI cycles, and further studies are required to establish which patients may benefit from this technique [166].

Other sperm selection methods

Sperm selection based on the electrostatic charge

The sperm membrane undergoes many chemical changes during sperm maturation, which are fundamental in maintaining the fertilization potential of the sperm. The maintenance of this physiological function is provided by the membrane-coating glycocalyx containing sialic acid so that the sperm outer membrane carries a negative charge of –16 to –20 mV, which decreases with the process of capacitation. In mammalian species, CD52 is a bipolar glycopeptide of epididymal origin that forms the main component of the sperm glycocalyx and is responsible for the net negative charge when transferred to the sperm membrane. It was reported that sperm with a negative charge on their membrane is significantly higher in fertile men when compared to ones who are sub-fertile [167]. A positively charged tube isolates sperm with a net negative charge in the zeta potential method. In contrast, electrophoresis attracts sperm with a negative charge to a positive electrode when suspended in an electrophoretic buffer. In this suspension, sperm move according to their net charge on the membrane and can pass through separating barriers with 5-µm pore size so that larger cells, such as immature germ cells and leukocytes, are eliminated in the final preparation [168]. There is a need to determine its effectiveness with detailed comparative clinical studies.

Birefringence

Birefringence is a polarized microscopy method based on the principle that light is refracted when passing through objects with an anisotropic structure; the refracted light delays and creates artificial brightness on the object. Studies show its benefit in selecting spermatozoa in patients with severe oligoasthenozoospermia and immotile sperm by examining the sperm head with a polarization microscope during ICSI [169]. It has also been reported that it can be used to select testicular sperm, sperm that have undergone acrosome reaction [170], and sperm with total asthenozoospermia [85], but more studies are needed on the technique.

Raman spectroscopy

Although the sperm selection method using Raman spectroscopy is still experimental, it has been the subject of intense research in recent years. By applying lasers and collecting the reflected Raman light spectrum, it is possible to distinguish the composition, crystal symmetry, crystal quality or the amount of supplemented ingredients in a given sample. This data can be used as a non-invasive detection of the sperm head to evaluate nuclear DNA status, identify chromatin damage, and construct maps showing the area where the fragmented DNA is found [171].

Summary

In assisted reproduction techniques, whether with ICSI or cIVF insemination, *in vitro* sperm selection methods are used to increase the chance that a functional spermatozoon is involved in fertilization. Several publications have presented data on which method is more advantageous for this selection; nevertheless, density gradient centrifugation, and swim-up remain as the routine and gold-standard sperm selection methods due to their ease of application and cost. One should be selective in introducing new techniques into the laboratory until basic and clinical studies have demonstrated the method's definitive superiority. Because of the potential commercial value of new techniques, the methodology and results of published studies need to be carefully evaluated Furthermore, the usefulness of these methods in patients with different diagnoses should be investigated, and specific indications should be determined.

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8

SPERM CHROMATIN ASSESSMENT

Ashok Agarwal and Rakesh Sharma

Introduction

Semen analysis is used routinely to evaluate infertile men. Attempts to introduce quality control within and between laboratories have highlighted the subjectivity and variability of traditional semen parameters. A significant overlap in sperm concentration, motility, and morphology between fertile and infertile men has been demonstrated [1]. In addition, standard measurements may not reveal subtle sperm defects such as DNA damage, and these defects can affect fertility. New markers are needed to better discriminate infertile men from fertile ones, predict pregnancy outcomes in the female partner, and calculate the risk of adverse reproductive events. In this context, sperm chromatin abnormalities have been studied extensively in past decades as a cause of male infertility [2, 3]. Focus on the genomic integrity of the male gamete has been intensified due to growing concerns about transmission of damaged DNA through assisted reproduction technologies (ARTs), especially intracytoplasmic sperm injection (ICSI). It is a particular concern if the amount of sperm DNA damage exceeds the repair capacity of oocytes. There are concerns related to potential chromosomal abnormalities, congenital malformations, and developmental abnormalities in ICSI-born progeny [4, 5].

Accumulating evidence suggests that a negative relationship exists between disturbances in the organization of the genomic material in sperm nuclei and the fertility potential of spermatozoa, whether *in vivo* or *in vitro* [6–14]. Abnormalities in the male genome characterized by damaged sperm DNA may be indicative of male subfertility regardless of normal semen parameters [15, 16]. Sperm chromatin structure evaluation is an independent measure of sperm quality that provides good diagnostic and prognostic capabilities. Therefore, it may be considered a reliable predictor of a couple's inability to become pregnant [17]. This may have an impact on the offspring, resulting in trans-generational infertility [18].

Poor intrauterine insemination (IUI) outcomes have been reported with elevated levels of sperm DNA fragmentation (SDF) in the semen [12]. A number of studies has shown the deleterious effect of SDF on IVF and ICSI outcomes, such as clinical pregnancies, miscarriages, and recurrent pregnancy loss [19–23]. These studies provide supporting evidence of the clinical utility of SDF testing in situations of repeat ART failure. SDF testing has emerged as a simple tool complementary to the conventional semen analysis that may enable clinicians to better manage infertile couples. The reproductive outcome data (live birth rate) is still limited [24–26] and American Society for Reproductive Medicine (ASRM), the American Urological Association (AUA), the European Association of Urology (EAU), and the National Institute of Clinical Excellence (NICE) still do not officially recommend SDF tests as an adjunct to male infertility assessment [27–29]. The clinical value of SDF testing has been confirmed in recent studies [30–36]. The guidelines of the Society for Translational Medicine recommend SDF testing as it may influence the clinical management of infertile patients with varicocele, since men with high-grade varicocele often present with normal semen parameters, while impaired semen parameters often correspond with low-grade varicocele patients with high levels of sperm DNA damage [37–40]. The WHO sixth edition (2021) now includes SDF testing [41].

Many techniques have been described to evaluate the sperm chromatin status. In this chapter, we describe the normal sperm chromatin architecture and the causative factors leading to its aberrations. We also provide the rationale for sperm chromatin assessment and discuss the different methods used to analyse sperm DNA integrity.

Human sperm chromatin structure

In many mammals, spermatogenesis leads to the production of highly homogenous spermatozoa. For example, more than 95% of the nucleoprotein in mouse sperm nuclei is composed of protamines [42]. This allows mature sperm nuclei to adopt a volume 40 times less than that of normal somatic nuclei [43]. The final, highly compact packaging of the primary sperm DNA filament is produced by DNA–protamine complexes. Contrary to nucleosomal organization in somatic cells, which is provided by histones, these DNA–protamine complexes approach the physical limits of molecular compaction [44, 45]. Human sperm nuclei, on the other hand, contain considerably fewer protamines (around 85%) than sperm nuclei of the bull, stallion, hamster, and mouse [46, 47]. Mature human spermatozoa contain some levels of nucleosomes, which are believed to be necessary for organizing higher-order genomic structure through interactions with the nuclear matrix. These regions are non-randomly distributed throughout the sperm genome [48]. Human sperm chromatin is therefore less regularly compacted and frequently contains DNA strand breaks [49].

To achieve this uniquely condensed state, sperm DNA must be organized in a specific manner that differs substantially from that of somatic cells [50]. The fundamental packaging unit of mammalian sperm chromatin is a toroid containing 50–60 kilobases of DNA. Individual toroids represent the DNA loop domains that are highly condensed by protamines and fixed at the nuclear matrix. Toroids are cross-linked by disulphide bonds formed by oxidation of sulfhydryl groups of cysteine present in the protamines [50]. Thus, each chromosome represents a garland of toroids, while all 23 chromosomes are clustered by centromeres into a compact chromocenter positioned well inside the nucleus; the telomere ends are united into dimers exposed to the nuclear periphery [51, 52]. This condensed, insoluble, and highly organized nature of sperm chromatin acts to protect the genetic integrity during transport of the paternal genome through the male and female reproductive tracts. It also ensures that the paternal DNA is delivered in the form that sterically allows the proper fusion of

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two gametic genomes and enables the developing embryo to correctly express the genetic information [52–53].

In comparison with other species [54], human sperm chromatin packaging is exceptionally variable both within and between men. This variability has been mostly attributed to its basic protein component. The retention of 15% histones, which are less basic than protamines, leads to the formation of a less compact chromatin structure [47]. Moreover, in contrast to the bull, cat, boar, and ram—whose spermatozoa contain only one type of protamine (P1)—human and mouse spermatozoa contain a second type of protamine called P2, which is deficient in cysteine residues [55]. Consequently, the disulphide cross-linking that is responsible for more stable packaging is diminished in human sperm as compared with species containing P1 alone [56]. The relative proportion of P1 to P2 is regulated at approximately 1:1 ratio at both mRNA and protein levels [57, 58]. This protamine ratio is unaltered in fertile men, but altered P1/P2 ratios and the absence of P2 are associated with male fertility problems [59–65]. Aberrant P1/P2 ratio is also associated with low fertilization rate and poor embryo quality [66]. A recent study reported that poor sperm protamination was associated with the development of low-quality embryos after *in vitro* fertilization [67]. The P1/P2 ratio has been shown to correlate with SDF, and significant differences were detected between fertile and infertile men [68]. The reference range reported for P1/P2 in a fertile, normozoospermic population ranges from 0.54 to 1.43. Such a wide range of P1/P2 shows that abnormal protamination can be an indicator of other disturbances that occur during spermatogenesis that can cause infertility [69]. Altered mRNA P1/P2 ratio has been shown to be a valuable indicator of sperm maturity and fertilization ability and, in these cases, patients can benefit from the use of testicular sperm [64].

Origin of sperm chromatin abnormalities

The susceptibility of male germ cells to DNA damage stems partly from the down-regulation of DNA repair systems during late spermatogenesis. In addition, the cellular machinery that allows these cells to undergo complete apoptosis is progressively lost during spermatogenesis. As a result, the advanced stages of germ cell differentiation cannot be deleted, even though they may have proceeded some way down the apoptotic pathway. As a consequence, the ejaculated gamete may exhibit genetic damage. Such DNA damage will be carried into the zygote by the fertilizing spermatozoon and must be then repaired, preferably prior to the first cleavage division. Several studies have shown that oocytes and early embryos can repair sperm DNA damage [70, 71]. Consequently, the biological effect of abnormal sperm chromatin structure depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it. Any errors that may occur during this post-fertilization period of DNA repair have the potential to create mutations that can affect fetal development and, ultimately, the health of the child [18, 72]. DNA damage can be a result of mismatch of bases, loss of base (abasic site), base modifications, DNA adducts and cross link, pyrimidine dimers and single-strand breaks (SSB), and double-strand breaks (DSB) [31] (Figure 8.1). Any of these alterations can induce SDF and compromise natural conception or ART outcomes.

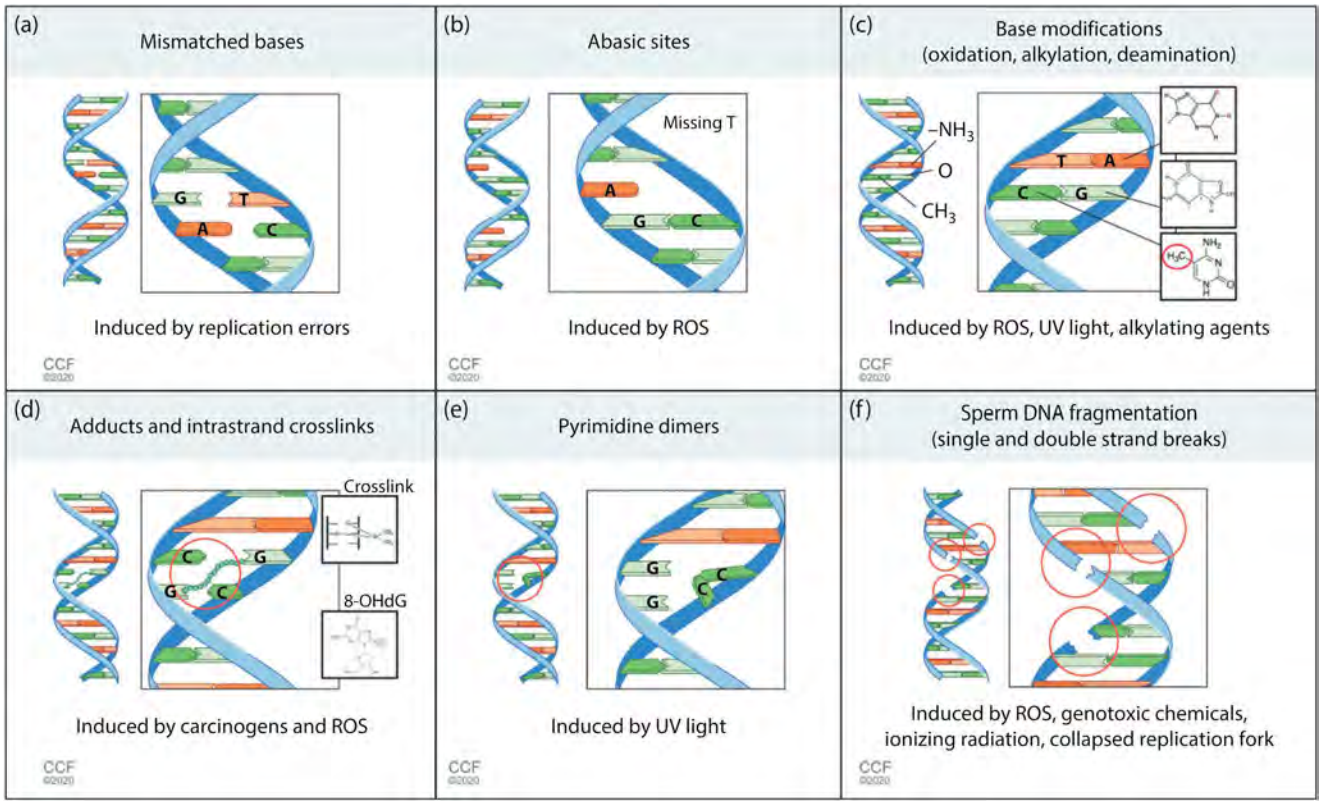

FIGURE 8.1 Different types of DNA damage. (a) Mismatched bases, (b) abasic sites, (c) base modifications (oxidation, alkylation, deamination), (d) adducts and intrastrand cross links, (e) pyrimidine dimers, and (f) single- and double-strand fragmentation. *Abbreviations*: ROS, reactive oxygen species; UV, ultraviolet.

The exact mechanisms by which chromatin abnormalities/DNA damage arise in human spermatozoa are not completely understood. Three main theories have been proposed: defective sperm chromatin packaging, abortive apoptosis, and oxidative stress (OS) [73]. Deficiencies in recombination may also play a role.

Defective sperm chromatin packaging

Stage-specific introduction of transient DNA strand breaks during spermiogenesis has been described [73–76]. DNA breaks have been found in round and elongating spermatids. Such breaks are necessary for transient relief of torsional stress. During maturation, the nucleosome histone cores in elongating spermatids are cast off and replaced with transitional proteins and protamines [73, 77, 78]. Thus, chromatin repackaging includes a sensitive step necessitating endogenous nuclease activity, which is evidently fulfilled by coordinated loosening of the chromatin by histone hyperacetylation and by topoisomerase II, which can create and ligate breaks [77, 78]. Although there is little evidence to suggest that spermatid maturation-associated DNA breaks are fully ligated, unrepaired DNA breaks are not allowed [79].

Ligation of DNA breaks is necessary not only to preserve the integrity of the primary DNA structure but also for reassembly of the important unit of genome expression—the DNA loop domain. Interaction of sperm DNA with protamines results in the coiling of sperm DNA into toroidal subunits known as doughnut loops [80]. If these temporary breaks are not repaired because of excessive topoisomerase II activity or a deficiency of topoisomerase II inhibitors [81, 82], then DNA fragmentation in ejaculated spermatozoa may result. Similarly, if appropriate disulphide bridge formation does not occur because of inadequate oxidation of thiols during epididymal transit, the DNA will be more vulnerable to damage caused by suboptimal compaction. Recent studies have postulated the hypothesis that large nuclear vacuoles could be an indicator of abnormal chromatin packaging [83, 84].

Further, the ratio of P1 to P2 maintained by P2 precursor (pre-P2) has a crucial role in sperm fertilization. Abnormal sperm morphogenesis with reduced motility can also result due to defective pre-P2 mRNA translation [69, 85–88].

Abortive apoptosis

The incidence of apoptosis in ejaculated sperm is still a contentious issue. Until recently, the inability of a mature spermatozoon to synthesize new proteins was believed to make it impossible for such cells to respond to any of the signals that lead to the programmed death cascade. However, a number of recent observations have raised the possibility that abortive apoptosis may contribute to DNA damage in human spermatozoa: (1) the detection of Fas on ejaculated spermatozoa [89]; (2) the high proportion of spermatozoa with potentially apoptotic mitochondria [90]; and (3) the finding that potential mediators of apoptosis, including endonuclease activity, are present in spermatozoa [91]. It has been postulated that OS can interfere with sperm chromatin remodelling. Cells with altered chromatin structure can enter the apoptotic pathway, which is characterized by loss of motility, caspase activation, phosphatidylserine externalization, and the activation of reactive oxygen species (ROS) generation by the mitochondria. ROS causes lipid peroxidation and oxidative DNA damage, which, in turn, leads to DNA fragmentation and eventually cell death [92].

It has been suggested that an early apoptotic pathway, initiated in spermatogonia and spermatocytes, is mediated by Fas protein. Fas is a type I membrane protein that belongs to the tumour necrosis factor–nerve growth factor receptor family [93]. It has been shown that Sertoli cells express Fas ligand, which by binding to Fas leads to cell death via apoptosis [93]. This in turn limits the size of the germ cell population to a number that Sertoli cells can support [94]. Ligation of Fas ligand to Fas in the cellular membrane triggers the activation of caspases and therefore this pathway is also characterized as a caspase-induced apoptosis [95].

Men exhibiting deficiencies in their semen profile often possess a large number of spermatozoa that bear Fas. This fact prompts the suggestion that these dysfunctional cells are the product of an incomplete apoptotic cascade [49]. However, a contribution of aborted apoptosis in the DNA damage seen in the ejaculated spermatozoa is doubtful in cases where this process is initiated at the early stages of spermatogenesis. This is because at the stage of DNA fragmentation, apoptosis is an irreversible process [96], and these cells should be digested by Sertoli cells and removed from the pool of ejaculated sperm. Some studies have not found correlations between DNA damage and Fas expression [97] or, in contrast, have not revealed ultrastructural evidence for the association of apoptosis with DNA damage in sperm [98]. Alternatively, if the apoptotic cascade is initiated at the round spermatid phase, where transcription (and mitochondria) is still active, abortive apoptosis might be an origin of the DNA breaks. A Bcl2 anti-apoptotic family gene member called Bclw has been shown to suppress apoptosis in elongating spermatids [99]. Although many apoptotic biomarkers have been found in the mature male gamete, particularly in infertile men, their definitive association with DNA fragmentation remains elusive [100–108].

Oxidative stress

Normal levels of ROS play an important physiological role, modulating gene and protein activities that are vital for sperm proliferation, differentiation, and function. In semen, the amount of ROS generation is controlled by seminal antioxidants that ensure a balance between ROS and antioxidant capacity. Any imbalance that occurs either by high ROS production or low antioxidant levels leads to OS [109–114]. The human spermatozoon is highly susceptible to OS [115]. This process induces peroxidative damage in the sperm plasma membrane and DNA fragmentation. A number of pro-inflammatory cytokines at physiological levels are responsible for the lipid peroxidation of sperm membrane, which is considered important for the fecundation capacity of the spermatozoa. However, OS may lead to abnormal production of certain interleukin/cytokines such as IL-8 and TNF-α, either alone or in combination with any infection, which may be able to drive the lipid peroxidation to a level that can affect the sperm fertilizing capacity [116]. Such stress may arise from a variety of sources. Morphologically abnormal spermatozoa (with residual cytoplasm, in particular) and leukocytes are the main sources of excessive ROS generation in semen [109]. Also, a lack of antioxidant protection and the presence of redox cycling xenobiotics may be the cause of OS. Antioxidant supplementation in these infertile men has been reported to be beneficial [117]. Whenever levels of OS in the male germ line are high, the peroxidation of unsaturated fatty acids in the sperm plasma membrane leads to the depressed fertilization rates associated with DNA damage [18, 37, 39, 40, 118].

Single- and double-strand DNA breaks

DNA fragmentation may be due to single-strand breaks (SSBs) or double-strand breaks (DSBs) (Figure 8.2). Single-strand breaks (SSBs) are a result of abortive topoisomerase or DNA ligase activity adjacent to a lesion and can be easily repaired [119]. The most common SSBs are base and sugar modifications and SSBs following oxidation, alkylation, deamination, and spontaneous hydrolysis. OS, lipid peroxidation, and protein alteration may also lead to SSBs (Figure 8.2a). When these lesions are not repaired, they can compromise the integrity of the genome. Endogenous sources during the DNA replication process, collapsed replication forks, or increased levels of free radicals all result in increased DSBs (Figure 8.2b).

In addition, exogenous sources such as ionizing radiation, genotoxic chemicals, and radiomimetic drugs can also lead to DSBs. Both SSBs and DSBs can affect the overall fertility and reproductive outcomes. Higher levels of SDBs are inversely related to the natural pregnancy outcome [120]. DSBs are more damaging to the genomic integrity and have been associated with recurrent miscarriages in couples without a female factor [120].

Deficiencies in recombination

Meiotic crossing-over is associated with the genetically programmed introduction of DNA double-strand breaks (DSBs) by specific nucleases of the SPO11 family [121]. These DNA DSBs should be ligated until the end of meiosis I. Normally, a recombination checkpoint in meiotic prophase does not allow meiotic division I to proceed until DNA is fully repaired or defective spermatocytes are ablated [121]. A defective checkpoint may lead to persistent SDF in ejaculated spermatozoa, although direct data for this hypothesis in humans is lacking.

The processes leading to DNA damage in ejaculated sperm are inter-related. For example, a defective spermatid protamination and disulphide bridge formation caused by inadequate oxidation of thiols during epididymal transit, resulting in diminished sperm chromatin packaging, makes sperm cells more vulnerable to ROS-induced DNA fragmentation. A two-step hypothesis has been proposed, suggesting that OS acts on poorly protaminated cells that are generated as a result of defective spermiogenesis [122].

Contributing factors

Advanced paternal age, smoking, obesity, radiofrequency, electromagnetic radiation, and xenobiotics are the common factors attributed to sperm DNA damage [30, 37, 123–127]. Advancing age has been associated with an increased percentage of ejaculated spermatozoa with DNA damage [11, 128–131]. Young men with cancer typically have poor semen quality and sperm DNA damage even before starting the therapy. Further damage from radiation or chemotherapy is dependent on both the duration and dose of radiation [132, 133]. Spermatogenesis may not occur months to years after therapy, but evidence of sperm DNA damage often persists beyond that period [134, 135]. Data on men with testicular cancer showed that radiation therapy induced transient sperm DNA damage and that this damage was present three to five years later, but three or more cycles of chemotherapy, in turn, decreased the percentage of sperm with DNA damage [135].

Cigarette smoking is associated with a decrease in sperm count and motility and an increase in abnormal sperm forms and sperm DNA damage [126]. It is suggested that smoking increases production of leukocyte-derived ROS; the OS may be the underlying reason why sperm DNA from smokers contains more strand breaks than that from non-smokers [136]. Also, genital tract infections and inflammation result in leukocytospermia and have been associated with OS and subsequent sperm DNA damage [137]. Exposure to pesticides (organophosphates), persistent organochlorine pollutants, and air pollution have also been associated with sperm DNA damage [11, 138]. Varicocele has been associated with seminal OS and sperm DNA damage. Clinical varicoceles cause both single-strand and double-strand breaks. In addition, in normozoospermic men, clinically significant varicoceles can also cause sperm DNA damage without alterations in the conventional semen parameters result in sperm [39, 139–148].

Various contributing factors for sperm DNA damage are shown in Figure 8.3. These may originate from defective sperm maturation, abortive apoptosis, and oxidative stress, along with other clinical and environmental risk factors [31].

Sperm DNA integrity has been shown to improve after varicocele repair [40, 149–154].

A deficiency in gonadotropic hormones such as follicle stimulating hormone (FSH) can cause sperm chromatin defects. FSH

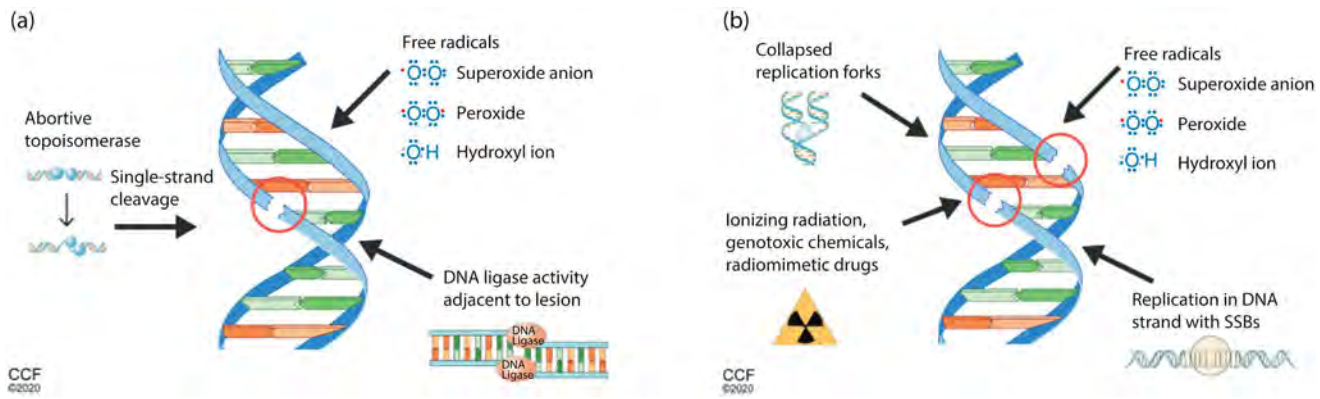

FIGURE 8.2 (a) Single-strand breaks in DNA are a result of abortive topoisomerase, free radicals, and DNA ligase activity adjacent to lesion. (b) Double-strand DNA breaks are caused by free radicals; collapsed replication forks; replication in DNA strand with single-strand breaks; and ionizing radiation, genotoxic chemicals, and radiomimetic drugs.

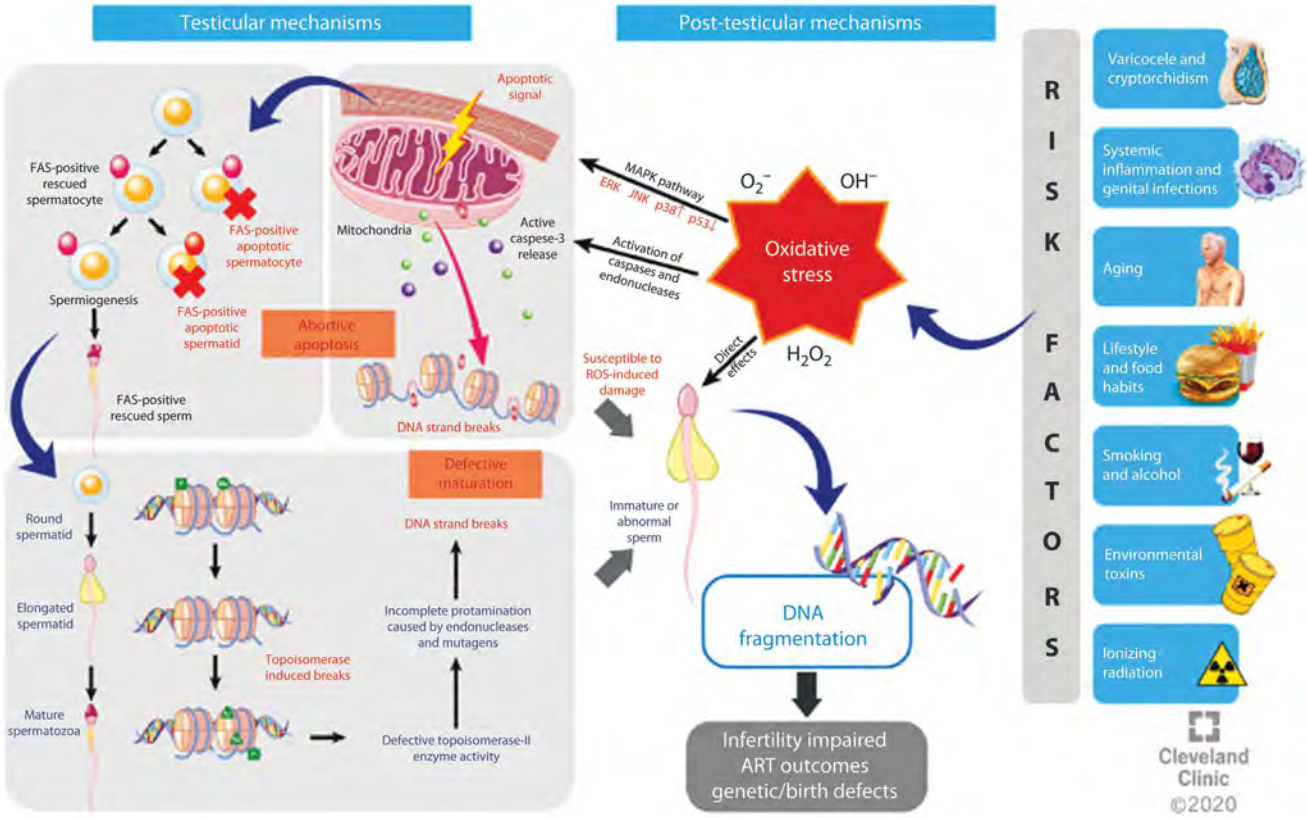

FIGURE 8.3 Overview of the origins of sperm DNA fragmentation (SDF). Underlying mechanisms such as defective maturation, abortive apoptosis, and oxidative stress can result in SDF. Clinical (age, infection, cancer, hormonal imbalances, obesity, diabetes) and environmental (heat exposure, environmental toxins, radiation, smoking, drug abuse, diet) risk factors also result in SDF. *Abbreviations*: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-JUN N-terminal kinase; ROS, reactive oxygen species; ART, assisted reproductive techniques.

receptor-knockout mice have been found to have higher levels of DNA damage in sperm [155]. Febrile illness has been shown to cause an increase in the histone/protamine ratio and DNA damage in ejaculated sperm [156]. Direct mild testicular and epididymal hyperthermia has also been shown to cause these effects [157]. Finally, sperm preparation techniques involving repeated high-speed centrifugation and the isolation of spermatozoa from the seminal plasma, which is a protective antioxidant environment, may contribute to increased sperm DNA damage via mechanisms that are mediated by the enhanced generation of ROS [14].

Indications for sperm chromatin assessment

Evaluating sperm chromatin can be challenging for several reasons: it can be difficult to link the results of chromatin integrity tests to known physiological mechanisms; the role that sperm chromatin structure assessment plays in clinical practice (especially in ART) is still controversial; and there is no one standardized method for measuring sperm chromatin integrity [17, 39, 158–162]. On the other hand, sperm chromatin structure is complex, and several methods may be necessary in order to assess this. In addition, a number of confounding factors can complicate the interpretation of the results, including heterogeneity in the sperm population and the fact that not all DNA damage is lethal (most DNA contains non-coding regions or introns, and oocytes can repair sperm DNA damage). Nevertheless, at the present time, it is clear that sperm chromatin assessment provides good diagnostic and prognostic capabilities for fertility/infertility [27–29].

It must be stressed that among all methods employed for sperm chromatin assessment, clinical thresholds so far have been demonstrated only for the sperm chromatin structure assay (SCSA) and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay, and these thresholds have been confirmed by different laboratories for SCSA only. However, a recent study published a detailed step-by-step approach for measuring SDF by TUNEL using a benchtop flow cytometer, which is user friendly and facilitates data interpretation [163]. A cross-sectional survey across 19 countries showed that 30.6% of SDF measurements are done using TUNEL and SCSA, whereas 20.4% and 6.1% use sperm chromatin dispersion (SCD) and single-cell gel electrophoresis (Comet), respectively [162]. Also, the reported biological variability of sperm DNA damage within men over time should be considered, although it is more stable than standard semen parameters [164–166]. Indications for sperm DNA evaluation include male infertility diagnosis (varicocele, idiopathic infertility) recurrent pregnancy loss, unexplained infertility, use of ARTs, and follow-up after oncological treatment such as radiotherapy or chemotherapy.

Diagnosis of male infertility

Although a spermatozoon with damaged DNA can fertilize an egg, future embryonic growth is compromised, which may ultimately lead to miscarriage or childhood deformities. Many studies have shown, using a variety of techniques, significant differences in sperm DNA damage levels between fertile and infertile men [167–171]. Moreover, spermatozoa from infertile patients are generally more susceptible to the effects of DNA-damaging agents such as H_2O_2, smoking, obesity, and radiation [172]. The probability of fertilization *in vivo* reduces drastically if the proportion of sperm cells with DNA damage exceeds 30% as detected by the SCSA [17, 173] or 20% as detected by TUNEL [174]. However, the latest commentary on the utilization of SDF testing in fertility outcomes suggests avoiding this test. The debate argues that several couples have become pregnant even though the threshold of DNA damage was higher than what we consider normal; in addition, some studies failed to find any difference in outcome in men who differ in SDF levels [175]. In continuation of such investigations, some people support the diagnostic value of sperm DNA integrity and suggest that it may be considered an objective marker of sperm function that serves as a significant prognostic factor for male infertility [7, 17, 29]. A significant increase in SCSA-defined DNA damage in sperm from infertile men with normal sperm parameters has been demonstrated [170], indicating that analysis of sperm DNA damage may reveal a hidden sperm abnormality in infertile men classified with idiopathic infertility based on apparently normal standard semen parameters.

Assisted reproduction technologies

The probability of fertilization by IUI or IVF is reduced in cases where the proportion of sperm cells with DNA damage exceeds 30% by means of SCSA [12, 176, 177]. As described in the previous section, the controversy as to whether sperm DNA damage negatively affects the results of IVF and ICSI has yet to be resolved [178–180]. Although no association between sperm DNA damage and IVF/ICSI outcome has been demonstrated in some studies [181], most show a significant negative correlation between sperm DNA damage and embryo quality in IVF cycles [23, 182], blastocyst development following IVF [183], and fertilization rates following IVF [179, 184] and ICSI, even though sperm DNA damage may not necessarily preclude fertilization and pronucleus formation during ICSI [185, 186]. In addition to other studies, two meta-analyses concluded that sperm DNA damage is predictive for reduced pregnancy success using routine IVF but has no significant effect on ICSI outcome [9, 20, 23, 25, 159, 187, 188]. Thus, assessment of sperm chromatin may help predict the success rates of IUI and IVF. It has been also suggested that in patients with a high proportion of DNA-damaged sperm who are seeking to use ART, ICSI should be the method of choice [12, 189–191].

Embryonic loss

Data on miscarriages as a possible consequence of sperm DNA damage are rather scarce. It has been shown that the proportion of sperm with DNA damage is significantly higher in men from couples with recurrent pregnancy loss than in the general population or fertile donors [20, 22, 160, 192]. It has also been reported that 39% of miscarriages could be predicted using a combination of selected cut-off values for percentage spermatozoa with denatured (likely fragmented) DNA and/or abnormal chromatin packaging as assessed by SCSA [17]. The percentage of spontaneous abortions following IVF/ICSI was increased when sperm with high levels of DNA damage were used [19, 20, 192, 193], which highlights the need to assess sperm DNA damage in order to predict possible future miscarriage [22].

Cancer patients

Sperm DNA evaluation in patients with cancer requires special attention when future fertility and the health of the baby are considered [194]. The stressful micro-environment that develops during cancer can cause OS, which indirectly can damage sperm DNA. Patients with cancer are often referred to sperm banks before chemotherapy, radiation therapy, or surgery is initiated. Data suggest there is compromised semen quality, including DNA integrity, before the commencement of treatment [195, 196] and increased chromosomal aneuploidy after chemotherapy [197–201]. The extent of DNA damage may help to determine how semen should be cryopreserved before therapy begins. Specimens with high sperm concentrations and motility and low levels of DNA damage should be preserved in relatively large aliquots that are suitable for IUI [202]. If a single specimen of good quality is available, then it should be preserved in multiple small aliquots suitable for IVF or ICSI [198, 203, 204].

Methods used in the evaluation of sperm chromatin/DNA integrity

Different methods can be used to evaluate the status of the sperm chromatin/DNA for the presence of abnormalities or simply immaturity (Table 8.1). These methods include simple staining techniques such as the acidic aniline blue (AAB) and basic toluidine blue (TB), fluorescent staining techniques such as the sperm chromatin dispersion (SCD) test, chromomycin A_3 (CMA_3), DNA breakage detection–fluorescence *in situ* hybridization (DBD–FISH), *in situ* nick translation (NT), and flow cytometric-based SCSA. Some techniques employ more than one method for the analysis of their results. Examples of these include the acridine orange (AO) and TUNEL assays. Other methods less frequently used include measurement of 8-hydroxy-2-deoxyguanosine (8-OHdG) by high-performance liquid chromatography (HPLC).

AAB staining

Principle

Aniline blue is an acidic dye that has more binding affinity with the proteins in decondensed or loose chromatin due to the residual histones. AAB staining differentiates between lysine-rich histones and arginine/cysteine-rich protamines. This technique provides a specific positive reaction for lysine and reveals differences in the basic nuclear protein composition of ejaculated human spermatozoa. Histone-rich nuclei of immature spermatozoa are rich in lysine and will consequently take up the blue stain. On the other hand, protamine-rich nuclei of mature spermatozoa are rich in arginine and cysteine and contain relatively low levels of lysine, which means they will not take up the stain [219].

Technique

Slides are prepared by smearing 5 µL of either a raw or washed semen sample, which is air-dried and fixed for 30 minutes in 3% glutaraldehyde in phosphate-buffered saline (PBS). The fixed smear is dried and stained in 5% aqueous aniline blue solution (pH 3.5) for five minutes. The staining characteristics depict the

TABLE 8.1 Various Methods for Assessing Sperm Chromatin Abnormalities

Assay	Parameter	Method of Analysis
Acidic aniline blue [205]	Nuclear maturity (DNA protein composition)	Optical microscopy
Toluidine blue staining [206]	Nuclear maturity (DNA protein composition)	Optical microscopy
Chromomycin A_3 [207]	Nuclear maturity (DNA protein composition)	Fluorescence microscopy
DNA breakage detection–fluorescence *in situ* hybridization [208]	DNA fragmentation (ssDNA)	Fluorescence microscopy
In situ nick translation [209]	DNA fragmentation (ssDNA)	Fluorescence microscopy Flow cytometry
Acridine orange [210]	DNA denaturation (acid)	Fluorescence microscopy Flow cytometry
Sperm chromatin dispersion [119, 211]	DNA fragmentation	Fluorescence microscopy
Comet (neutral) [148, 212]	DNA fragmentation (dsDNA)	Fluorescence microscopy
Comet (alkaline) [22, 148, 213]	DNA fragmentation (ssDNA/dsDNA)	
TUNEL [163, 164, 174, 214–216]	DNA fragmentation	Fluorescence microscopy Flow cytometry
Sperm chromatin structure assay [17]	DNA denaturation (acid/heat)	Flow cytometry
8-OHdG measurement [217, 218]	8-OHdG	High-performance lipid chromatography

Abbreviations: 8-OHdG, 8-hydroxy-2-deoxyguanosine; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling.

status of nuclear maturity. Sperm heads containing immature nuclear chromatin stain blue and those with mature nuclei do not. A total of 200 spermatozoa per slide are counted using bright field microscopy, and the percentage of spermatozoa stained with aniline blue is determined [205].

Modification of the AAB assay with eosin

One of the limitations of AAB staining is poor visualization of unstained sperm cells under ordinary light microscopy. To overcome this issue, counterstaining using eosin-Y is recommended. Sperm smears are fixed in 4% formalin solution for five minutes and rinsed in water. Slides are stained in 5% aniline blue prepared in 4% acetic acid (pH 3.5) solution for five minutes, rinsed in water, and counterstained in 0.5% eosin for one minute followed by rinsing and air drying [220].

Clinical significance

AAB staining has shown a linkage between chromatin immaturity and male infertility. In patients with varicocele, unilateral cryptorchidism, and idiopathic infertility, high sperm nuclear instability with a higher number of AAB-stained spermatozoa was observed [221]. However, the correlation between the percentage of aniline blue-stained spermatozoa and other sperm parameters remains controversial. AAB-stained spermatozoa showed normal conventional parameters such as count, motility, and morphology [222]. Immature sperm chromatin may or may not correlate with asthenozoospermic samples and abnormal morphology patterns [219]. Most important is the finding that chromatin condensation as visualized by aniline blue staining is a good predictor for IVF outcome, although it cannot determine the fertilization potential and the cleavage and pregnancy rates following ICSI [220]. Evaluation of sperm chromatin using AAB staining could be considered as one of the complementary tests of semen analysis for assessment of male factor infertility [223, 224]. Counterstaining with eosin can facilitate interpretation of sperm chromatin integrity [220].

Advantages and limitations

The AAB technique is simple and inexpensive and requires only bright field microscopy for analysis. The only drawback is the heterogeneous slide staining.

TB staining

Principle

Toluidine Blue (TB), or tolonium chloride, is a basic thiazine metachromatic dye that selectively binds the acidic components of the tissue. It partially dissolves in water and alcohol. Alternatively known as methylamine or aminotoluene, the dye represents three isoforms: ortho-toluidine, para-toluidine, and meta-toluidine. It has high binding affinity for phosphate residues of sperm DNA in immature nuclei and provides a metachromatic shift from light blue to a purple–violet colour [225]. This stain is a sensitive structural probe for DNA structure and packaging.

Technique

TB staining follows the principle of metachromasia in which a dye can absorb light at different wavelengths and can change colour without changing chemical structure. Sperm smears are air-dried, fixed in freshly made 96% ethanol–acetone (1:1) at 4°C for at least 30 minutes, hydrolysed in 0.1 N HCl at 4°C for five minutes, and rinsed three times in distilled water for two minutes each. Smears are stained with 0.05% TB for five minutes. The staining buffer consists of 50% citrate phosphate (McIlvain buffer, pH 3.5). Permanent preparations are dehydrated in tertiary butanol twice for three minutes each at 37°C and in xylene twice for three minutes each; the preparations are embedded in DPX (a mixture of distyrene, a plasticizer, and xylene). Sperm heads with good chromatin integrity stain light blue and those of compromised integrity stain violet (purple) [226]. The results of the TB test are visualized using light microscopy. Based on the different optical densities of cells stained with TB, the image analysis cytometry test is elaborated (Figure 8.4a–c) [205].

Clinical significance

TB staining may be considered a fairly reliable method for assessing sperm chromatin. Abnormal nuclei (purple–violet sperm heads) have been shown to be correlated with counts of red–orange sperm heads as revealed by the AO method [225]. Significant correlations between the results of the TB, SCSA, and TUNEL tests have been demonstrated [226]. Clinical applicability of the TB test for male fertility potential assessment has also been demonstrated, with specificity for infertility diagnosis as high as

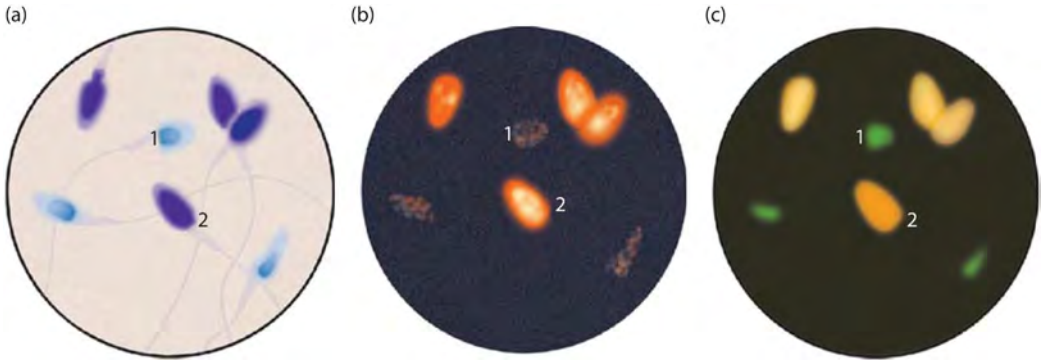

FIGURE 8.4 (a) Human ejaculate stained with toluidine blue: (1) sperm heads with normal chromatin conformation are light blue; (2) sperm heads with abnormal chromatin conformation are violet. (b) DNA breakage detection–fluorescence *in situ* hybridization labelling with a whole-genome probe (red fluorescence), demonstrating extensive DNA breakage in those nuclei that are intensely labelled. (c) Acridine orange stain to native DNA fluoresces green (1), whereas relaxed/denatured DNA fluoresces red (2).

92% and sensitivity reaching 42% when the threshold of 45% is used for sperm cells with abnormal nuclei [227]. TB staining has been used in several studies for evaluating sperm chromatin quality [228–231], alone and in conjunction with other tests, proving it to be an effective tool for evaluation of chromatin status.

Advantages and limitations

The TB method is simple and inexpensive and has the advantage of providing permanent preparations for use on an ordinary microscope. The stained smears can also be used for morphological assessment of the cells. Also, with the threshold for infertility diagnostics using TB staining having been established, the TB staining method is more advantageous. However, this method may have the inherent limitations of reproducibility dictated by the limited number of cells that can be reasonably scored.

CMA_3 assay

Principle

CMA_3 is a guanine–cytosine-specific fluorochrome that reveals poorly packaged chromatin in spermatozoa and is the indirect measure of protamine deficiency in sperm DNA [232]. CMA_3 is specific for GC-rich sequences and is believed to compete with protamines for binding to the minor groove of DNA. Therefore, high CMA_3 fluorescence is a strong indicator of a low protamination state in spermatozoa [206].

Technique

Sperm smears are fixed in methanol–glacial acetic acid 3:1 at 4°C for 20 minutes and are then allowed to air-dry at room temperature for 20 minutes. The slides are treated for 20 minutes with 100 µL CMA_3 solution. The CMA_3 solution consists of 0.25 mg/mL CMA_3 in McIlvain's buffer (pH 7.0) supplemented with 10 mmol/L $MgCl_2$. The slides are rinsed in buffer and mounted with 1:1 v/v PBS–glycerol. The slides are then kept at 4°C overnight. Fluorescence is evaluated using a fluorescent microscope. A total of 200 spermatozoa are randomly evaluated on each slide. CMA_3 staining is evaluated by distinguishing spermatozoa that stain bright yellow (CMA_3 positive) from those that stain a dull yellow (CMA_3 negative) [207, 210].

Clinical significance

As a discriminator of IVF success (>50% oocytes fertilized), CMA_3 staining has a sensitivity of 73% and a specificity of 75%. Therefore, it can distinguish between IVF success and failure [233]. In cases of ICSI, Sakkas et al. [234] reported that the percentage of CMA_3 positivity does not indicate failure of fertilization entirely and suggested that poor chromatin packaging contributes to a failure in the decondensation process and probably reduced fertility. It appears that semen samples with high CMA_3 positivity (>30%) may have significantly lower fertilization rates if used for ICSI [235], but this observation is not seen in studies [236].

Advantages and limitations

The CMA_3 assay yields reliable results as it is strongly correlated with other assays used in the evaluation of sperm chromatin [206, 237]. CMA_3 staining results have been reported to have a strong negative correlation with sperm concentration, motility, and especially normal morphology. Men with low scores of morphologically normal spermatozoa tend to have a greater degree of protamine deficiency and DNA damage [84, 237]. The number of CMA_3-positive sperm was significantly higher in globozoopermic patients than in controls, which indicates high levels of DNA damage [232]. In addition, the sensitivity and specificity of the CMA_3 stain are comparable with those of the AAB stain (75% and 82% vs. 60% and 91%, respectively) if used to evaluate the chromatin status in infertile men [207]. However, the CMA_3 assay is limited by observer subjectivity.

DBD–FISH assay

Principle

The DBD–FISH is a technique that can detect DNA breaks in single cells, not only in the whole genome but also in specific sequences of DNA. Cells embedded within an agarose matrix on a slide are exposed to an alkaline unwinding solution, which transforms DNA strand breaks into single-stranded DNA motifs. After neutralization and protein removal, single-stranded DNA becomes accessible to hybridization with whole-genome or specific DNA probes that highlight the chromatin area to be analysed. As the number of DNA breaks increase, so does production of single-stranded DNA by the alkaline solution, resulting in an increase in fluorescence intensity and the surface area of the FISH signal. Abnormal chromatin packaging in sperm cells greatly increases the accessibility of DNA ligands and the sensitivity of DNA to denaturation by alkali, and this relates to the presence of intense labelling (red fluorescence) by DBD–FISH. Therefore,

DBD–FISH allows *in situ* detection and quantification of DNA breaks and reveals structural features in the sperm chromatin [207, 238].

Technique

Sperm cells are mixed with 1% low-melting point agarose to a final concentration of 0.7% at 37°C. A volume of 300 µL of the mixture is pipetted onto polystyrene slides and allowed to solidify at 4°C. The slides are immersed into a freshly prepared alkaline denaturation solution (0.03 mol/L NaOH, 1 mol/L NaCl) for five minutes at 22°C in the dark to generate single-stranded DNA from DNA breaks. The denaturation is then stopped, and proteins are removed by transferring the slides to a tray with neutralizing and lysing solution 1 (0.4 mol/L Tris, 0.8 mol/L dithiothreitol [DTT], 1% sodium dodecyl sulphate [SDS], and 50 mmol/L ethylenediaminetetraacetic acid [EDTA], pH 7.5) for 10 minutes at room temperature, which is followed by incubation in a neutralizing and lysing solution 2 (0.4 mol/L Tris, 2 mol/L NaCl, and 1% SDS, pH 7.5) for 20 minutes at room temperature. The slides are thoroughly washed in Tris–borate–EDTA buffer (0.09 mol/L Tris–borate and 0.002 mol/L EDTA, pH 7.5) for 15 minutes, dehydrated in sequential 70%, 90%, and 100% ethanol baths (two minutes each), and air-dried. A human whole-genome probe is hybridized overnight (4.3 ng/µL in 50% formamide/2 × standard saline citrate [SSC], 10% dextran sulphate, and 100 mmol/L calcium phosphate, pH 7.0; 1 × SSC is 0.015 mol/L sodium citrate and 0.15 mol/L sodium chloride, pH 7.0). It is then washed twice in 50% formamide/2 × SSC (pH 7.0) for five minutes and twice in 2 × SSC (pH 7.0) for three minutes at room temperature. The hybridized probe is detected with streptavidin indocarbocyamine (1:200) (Sigma Chemical Co., St Louis, MO), and cells are counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1 µg/mL) and visualized using fluorescence microscopy [208].

Advantages and limitations

DBD–FISH is used to detect *in situ* DNA breaks and to reveal structural features of chromatin. Its major advantage is the possibility to simultaneously detect and discriminate single- and double-strand DNA breaks [239]. Nevertheless, it is expensive and time-consuming and involves sophisticated laboratory procedures.

In situ NT assay

Principle

The NT assay is a modified version of the TUNEL assay; it quantifies the incorporation of biotinylated dUTP at single-strand DNA breaks in a reaction that is catalysed by the template-dependent (unlike TUNEL) enzyme DNA polymerase I.

It specifically stains spermatozoa that contain appreciable and variable levels of endogenous DNA damage. The NT assay indicates anomalies that have occurred during remodelling of the nuclear DNA in spermatozoa. In doing so, it is more likely to detect sperm anomalies that are not indicated by morphology.

Technique

To perform the assay, smears containing 500 sperm each should be prepared. The fluorescent staining solution is prepared by mixing 10 µL streptavidin–fluorescein–isothiocyanate, 90 µL Tris buffer, and 900 µL double-distilled water. A total of 100 µL of this solution is added to the slides. The slides are incubated in a moist chamber at 37°C for 30 minutes. After incubation, the slides are rinsed in PBS twice, washed with distilled water, and finally mounted with a 1:1 mixture of PBS and glycerol. The slides are examined using fluorescence microscopy. A total of 100–200 spermatozoa should be counted, and those fluorescing and hence incorporating the dye are classified as having endogenous nicks [209].

Clinical significance

Sperm nuclear integrity as assessed by the NT assay demonstrates a very clear relationship with sperm motility and morphology and, to a lesser extent, sperm concentration [182, 240, 241]. The results of the assay are supported by the strong positive correlations detected with the sensitivity of CMA_3 and TUNEL assays ($r = 0.86$, $p < 0.05$ and $r = 0.87$, $p < 0.05$, respectively) [206]. The NT assay can also indicate if there is damage arising from factors such as heat exposure [242] or the generation of ROS following exposure to leukocytes within the male reproductive tract [243].

Advantages and limitations

The advantage of the NT assay is that the reaction is based on direct labelling of the termini of DNA breaks. Thus, the lesions that are measured are identifiable at the molecular level. In addition, if flow cytometry is used to analyse the results, it may be performed on fixed cells, as the duration of cell storage in ethanol may vary [208]. However, the NT assay has a lower sensitivity than the other assays and does not correlate with fertilization in *in vivo* studies.

AO assay

Principle

AO is a dye that intercalates with DNA or RNA and fluoresces to emit different colours, making it easy to differentiate cellular organelles. The binding that occurs is the property of electrostatic interactions between acridine molecules and base pairs of nucleic acid. It measures the susceptibility of sperm nuclear DNA to acid-induced denaturation *in situ* by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured DNA) [244]. The fluorochrome AO intercalates into double-stranded DNA as a monomer and binds to single-stranded DNA as an aggregate. The monomeric AO bound to native DNA fluoresces green, whereas the aggregated AO on relaxed or denatured DNA fluoresces red (Figure 8.4c) [245].

Technique

The AO assay can be used for either fluorescence or flow cytometry. For fluorescence microscopy, thick semen smears are fixed in Carnoy's fixative (methanol:acetic acid 1:3) for at least two hours. The slides are stained in AO for five minutes and gently rinsed with deionized water. At least 200 cells should be counted so that the estimates of the numbers of sperm with green and red fluorescence are accurate. Spermatozoa that emit green fluorescence are considered to have normal DNA content, whereas those displaying a spectrum of yellow–orange to red fluorescence are considered to have damaged DNA. The DNA fragmentation index (DFI) can be calculated by the ratio of (yellow to red)/(green + yellow to red) fluorescence [244].

For flow cytometry, aliquots of semen (about 25–100 µL, containing one million spermatozoa) are suspended in 1 mL of ice-cold PBS (pH 7.4) and centrifuged at 600 *g* for five minutes. The pellet is resuspended in ice-cold TNE (0.01 mol/L Tris-HCl, 0.15 mol/L NaCl, and 1 mmol/L EDTA, pH 7.4) and again centrifuged at 600 *g* for five minutes. The pellet is then resuspended in 200 µL of ice-cold TNE with 10% glycerol and immediately fixed

in 70% ethanol for 30 minutes. The fixed samples are treated for 30 seconds with 400 µL of a solution of 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 N HCl (pH 1.2). After 30 seconds, 1.2 mL of staining buffer (6 µg/mL AO, 37 mmol/L citric acid, 126 mmol/L Na_2HPO_4, 1 mmol/L disodium EDTA, and 0.15 mol/L NaCl, pH 6.0) is added to the test tube and analysed by flow cytometry. After excitation by a 488-nm wavelength light source, AO bound to double-stranded DNA fluoresces green (515–530 nm) and AO bound to single-stranded DNA fluoresces red (630 nm or greater). A minimum of 5000 cells are analysed by fluorescent-activated cell sorting [209].

Clinical significance

The AO technique has shown significantly higher DNA damage in infertile men with and without varicocele as compared to controls [230]. Further, a decrease in AO-positive spermatozoa has also been documented after varicocelectomy, which shows its clinical utility in the evaluation of DNA integrity [148]. AO-positive cells are likely to have more structural abnormalities than AO-negative cells [246]. A negative correlation has been reported between AO staining results and conventional sperm parameters [247]. The "cut-off" value set to differentiate between fertile and infertile men varies between 20% and 50% [17, 210]. Studies show that single-stranded DNA that is detected by a low incidence (<50%) of green AO fluorescence negatively affects the fertilization process in a classical IVF program, resulting in lower fertilization and pregnancy rates and a lower proportion of grade A embryos [173, 248, 249]. However, no correlation was found with the pregnancy rate and live births achieved by ICSI except in patients having 0% of spermatozoa with single-stranded DNA, in whom the pregnancy rate was significantly higher [173, 248, 249].

Advantages and limitations

The AO assay is a biologically stable measure of sperm DNA quality. The intra-assay variability is less than 5%, rendering the technique highly reproducible [250]. A strong positive correlation exists between the AO assay and other techniques used to evaluate single-stranded DNA (e.g. the TUNEL assay [see the "TUNEL assay" section]) [250]. Limitations include inter-observer variability in case of fluorescence microscopic analysis and expensive instrumentation for flow cytometric analysis.

SCD test (Halosperm® assay)

Principle

The SCD test produces sperm nucleoids consisting of a central or core and peripheral halo caused by release of DNA loops, signifying the absence of DNA fragmentation. When sperm are treated with an acid solution prior to lysis buffer, a complete absence or a minimal halo is produced in spermatozoa with fragmented DNA. A distinct halo is seen in spermatozoa with intact DNA integrity [211]. When spermatozoa with non-fragmented DNA are immersed in an agarose matrix and directly exposed to lysing solutions, the resulting deproteinized nuclei (nucleoids) show extended halos of DNA dispersion, which can be observed either by bright field microscopy or fluorescence microscopy. The presence of DNA breaks promotes the expansion of the halo of the nucleoid [119, 251–256] (Figures 8.5 and 8.6).

Technique

Aliquots of sperm at a concentration of 5–10 million/mL are prepared by diluting in PBS. The samples are mixed with 1% low-melting point aqueous agarose (to obtain a 0.7% final agarose concentration) at 37°C. Aliquots of 50 µL of the mixture

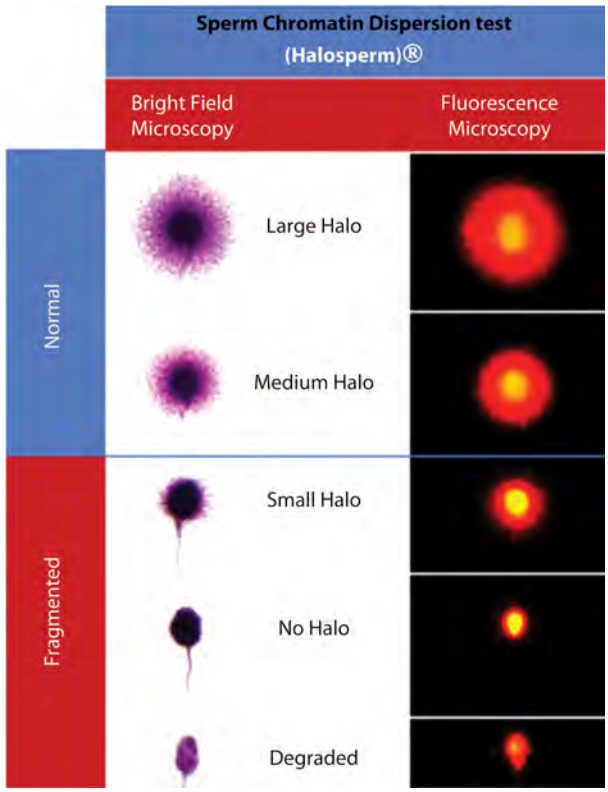

FIGURE 8.5 Classification of human SDF with respect to halo size and visualization under bright field or fluorescence microscopy using Halosperm®. *Abbreviation*: SDF, sperm DNA fragmentation.

are pipetted onto a glass slide precoated with 0.65% standard agarose dried at 80°C, covered with a coverslip, and left to solidify at 4°C for four minutes. The coverslips are then carefully removed, and the slides are immediately immersed horizontally in a tray of freshly prepared acid denaturation solution

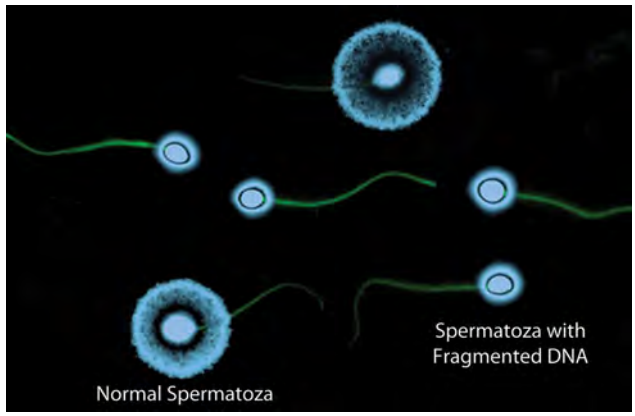

FIGURE 8.6 Visualization of SDF using Halosperm. Sperm was simultaneously stained for protein visualization (green) and DNA (blue). This image was electronically filtered for halo enhancement and discrimination between sperm containing fragmented DNA (no halo of dispersed chromatin) and non-fragmented DNA (large halo of dispersed chromatin). *Abbreviation*: SDF, sperm DNA fragmentation.

(0.08 N HCl) for seven minutes at 22°C in the dark, which generates restricted single-stranded DNA motifs from DNA breaks. Denaturation is then stopped, and the proteins are removed by transferring the slides to a tray with neutralizing and lysing solution 1 (0.4 mol/L Tris, 0.8 mol/L DTT, 1% SDS, and 50 mmol/L EDTA, pH 7.5) for 10 minutes at room temperature. The slides are then incubated in neutralizing and lysing solution 2 (0.4 mol/L Tris, 2 mol/L NaCl, and 1% SDS, pH 7.5) for five minutes at room temperature. The slides are thoroughly washed in Tris–borate EDTA buffer (0.09 mol/L Tris–borate and 0.002 mol/L EDTA, pH 7.5) for two minutes; dehydrated sequentially in 70%, 90%, and 100% ethanol baths (two minutes each); and air-dried. Cells are stained with DAPI (2 µg/mL) for fluorescence microscopy (Figures 8.5 and 8.6).

Clinical significance

Reports suggest that SDF as reported by the SCD test is negatively correlated with fertilization rates and embryo quality in IVF/ICSI, but not with clinical pregnancy rates or births [252–257]. A meta-analysis examining the outcomes of medically assisted reproduction failed to show any predictive value of SCD between IVF and ICSI [258]. Simon et al. [23] demonstrated a negative effect of sperm DNA damage on clinical pregnancy rate following IVF and ICSI, whereas Deng et al. [33], demonstrated the negative effect of lower pregnancy rate only in IVF. In another study by Ribas-Maynou [259], DNA damage by SCD adversely affected only pregnancy and live birth rate but not implantation rate, fertilization rate, embryo quality, or blastocyst formation in IVF; but none of these parameters were affected in ICSI.

Advantages and limitations

The SCD test is simple, fast, and reproducible, with comparable results to those of the SCSA [251, 254] and TUNEL assay [260]. The currently available protocol is suitable for bright field microscopy as it significantly reduces equipment cost. The test is successfully used in clinical studies to detect sperm DNA damage [261] and can be simultaneously combined with the FISH (SCD–FISH) assay for detection of aneuploidy in sperm cells [262]. This is the only test allowing assessment of SDF and chromosomal aneuploidy by FISH in the same cell. Oxidative DNA damage also can be simultaneously determined in the same sperm cell by combining the SCD test and incubation with an 8-oxoguanine DNA probe [263]. A commercially available Halosperm® kit has been recently developed [121, 264].

Comet assay

Principle

The comet assay (single-cell gel electrophoresis) was first introduced by Ostling and Johanson in 1984 [265] and is based on the principle of permeabilization and electrophoretic migration of cleaved fragments of DNA [266]. In the beginning, neutral electrophoresis buffer conditions were used to show that the migration of double-stranded DNA loops from a damaged cell in the form of a tail unwinding from the relaxed supercoiled nucleus was proportional to the extent of damage inflicted on the cell. This finding took on the appearance of a comet with a tail when viewed using a fluorescence microscope and DNA stains. Singh et al. modified the comet assay in 1988 [212] by using alkaline electrophoresis buffers to expose alkali-labile sites on the DNA; this modification increased the sensitivity of the assay to detect both single- and double-stranded DNA breaks [212]. The routine comet assay lacks the ability to differentiate between single- and double-stranded DNA breaks in the same sperm cell, but a modified two-tailed comet assay can simultaneously evaluate single- and double-stranded DNA breaks [267]. The chromosome comet assay is a new application that detects DNA damage by generating comets in sub-nuclear units, such as the chromosome, based on the chromosome isolation protocols currently used for whole-chromosome mounting in electron microscopy. It has not been used with sperm cells thus far [268].

In the comet assay, DNA damage is quantified by measuring the displacement between the genetic material of the nucleus "comet head" and the resulting tail. The tail lengths are used as an index for the damage. Also, the tail moment—the product of the tail length and intensity (fraction of total DNA in the tails)—has been used as a measuring parameter. The tail moment can be more precisely defined as being equivalent to the torsional moment of the tail [269].

Technique

Sperm cells are cast into miniature agarose gels on microscopic slides and lysed *in situ* to remove DNA-associated proteins in order to allow the compacted sperm DNA to relax. The lysis buffer (Tris 10 mmol/L, 0.5 mol/L EDTA, and 2.5 mol/L NaCl, pH 10) contains 1% Triton X-100, 40 mmol/L DTT, and 100 µg/mL proteinase K. The slide immersion time in alkaline lysis solution ranges between 1 and 20 minutes and does not affect assay results [270]. Micro-gels are then electrophoresed (20 minutes at 25 V/0.01 A) in neutral buffer (Tris 10 mmol/L containing 0.08 mol/L boric acid and 0.5 mol/L EDTA, pH 8.2), during which time the damaged DNA migrates from the nucleus towards the anode. The DNA is visualized by staining the slides with the fluorescent DNA binding dye SYBR Green I. Comet measurements are performed manually or by computerized image analysis using fluorescence microscopy (Figure 8.7) [211].

In the two-tailed comet technique, sperm cells are diluted in PBS to a concentration of 10×10^6 spermatozoa/mL. A 25-µL cell suspension is mixed with 50 µL of 1% low-melting point agarose in distilled water at 37°C. A total of 15 µL of the mixture is placed on the slide, covered with a coverslip, and transferred to an ice-cold plate. As soon as the gel solidifies, the coverslips are removed and the slides are rinsed in two lysing solutions: lysing solution 1 (0.4 mol/L Tris–HCl, 0.8 mol/L DTT, and 1% SDS, pH 7.5) for 30 minutes, followed by lysing solution 2 (0.4 mol/L Tris–HCl, 2 mol/L NaCl, 1% SDS, and 0.05 mol/L EDTA, pH 7.5) for 30 minutes. Then, the slides are rinsed in TBE buffer (0.09 mol/L Tris–borate and 0.002 mol/L EDTA, pH 7.5) for 10 minutes, transferred to an electrophoresis tank, and immersed in fresh TBE electrophoresis buffer. Electrophoresis is performed at 20 V (1 V/cm) and 12 mA for 12.5 minutes. After washing in 0.9% NaCl, nucleoids are unwound in an alkaline solution (0.03 mol/L NaOH and 1 mol/L NaCl) for 2.5 minutes, transferred to an electrophoresis chamber, and oriented at 90° to the first electrophoresis.

The second electrophoresis is performed at 20 V (1 V/cm), and 12 mA for four minutes in 0.03 mol/L NaOH. Then, the slides are rinsed in a neutralization buffer (0.4 mol/L Tris–HCl, pH 7.5) for five minutes, briefly washed in TBE buffer, dehydrated in increasing concentrations of ethanol, and air-dried. DNA is stained with SYBR Green I at a 1:3000 dilution in Vectashield® (Vector Laboratories, Burlingame, CA). Samples are assessed by visual scoring or digitalization and image processing. The frequency of sperm cells with fragmented DNA is established by measuring at least 500 sperm cells per slide. Cells are classified as undamaged

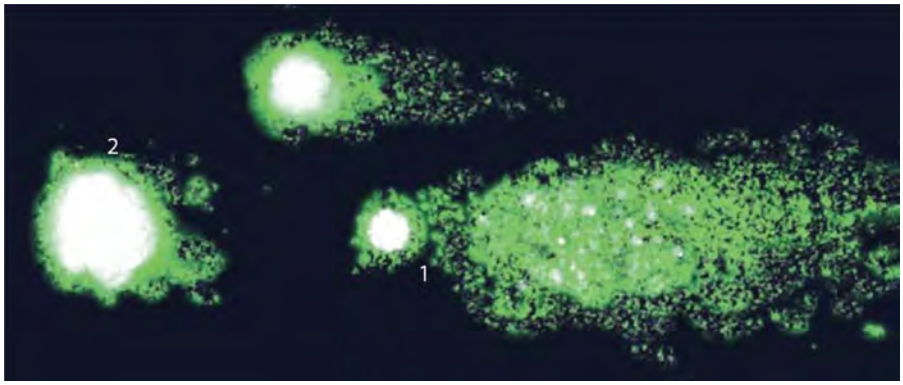

FIGURE 8.7 Comet image showing damaged DNA (1) and undamaged DNA (2).

or damaged based on the length of the tail, which contains DNA fragment single-stand breaks (up/down migration), DSBs (right/left migration), or both [267].

Clinical significance

The assay has been successfully used to evaluate DNA damage after cryopreservation [271]. Using the alkaline and neutral comet assay also showed that normozoospermic men with varicoceles have high single- and double-strand DNA damage [148]. ROS are directly responsible for damage to the DNA single and double strands. It may also predict embryo development after IVF and ICSI, especially in couples with unexplained infertility [272], and some clinical thresholds were set for infertility diagnostics and IVF outcome prediction [273–274], although some studies failed to demonstrate such an association [275]. DNA damage by alkaline comet assay was also shown to negatively affect pregnancy rate and/or live-birth rate in IVF but not in ICSI [20, 23, 33, 259, 276, 277]. A modified version of the comet assay protocol is capable of detecting different mutagen impacts on sperm DNA integrity [278]. Alkaline comet assay has been demonstrated as a robust biomarker for sporadic and recurrent miscarriages after spontaneous or assisted conception [22].

Advantages and limitations

The comet assay is a well-standardized, simple, versatile, sensitive, and rapid assay that correlates significantly with the TUNEL assay and SCSA [279]. It can assess DNA damage qualitatively as well as quantitatively with low intra-assay variation. Two-tailed comet assay can discriminate between single- and double-stranded DNA breaks; for example, the resistance of sperm DNA to oxidative damage can be specifically assessed [280]. Because it is based on fluorescence microscopy, the assay requires an experienced observer to analyse the slides and interpret the results.

TUNEL assay

Principle

This single-step staining method labels DNA breaks with fluorescein isothiocyanate (FITC)-dUTP followed by flow cytometric analysis. TUNEL utilizes a template-independent DNA polymerase called TdT, which non-preferentially adds deoxyribonucleotides to 3′-hydroxyl (OH) single- and double-stranded DNA. dUTP is the substrate that is added by the TdT enzyme to the free 3′-OH break-ends of DNA (Figure 8.8).

FIGURE 8.8 BD Accuri C6 flow cytometer.

Technique

Strand breaks can be quantified with conventional or the newly introduced benchtop flow cytometry or fluorescence microscopy in which DNA-damaged sperm fluoresce intensely [164, 281]. To assess the DNA fragmentation by TUNEL, an APO-DIRECT™ Kit (BD Pharmingen, CA, USA) is used. It contains the reaction buffer, TdT, FITC-dUTP, and propidium iodide/RNase stain. The assay kit also contains negative and positive controls, which are not sperm cells. About 5×10^6 sperm cells are fixed with 3.7% paraformaldehyde for a minimum of 30 minutes at 4°C. The sample is centrifuged at 300 *g* for seven minutes. Paraformaldehyde is removed by centrifuging the samples at 300 *g* for seven minutes. Supernatants are discarded and the pellets resuspended with 1 mL of ice-cold ethanol (70% v/v). The tubes are kept at –20°C for at least 30 minutes. To create negative sperm controls, the enzyme terminal transferase is omitted from the reaction mixture. To create positive sperm controls, the samples are pre-treated with 0.1 IU DNase I for 30 minutes at room temperature. A total of 50 µL of the stain is added and incubated for one hour. Following two washes with 1 mL of the "rinse buffer," propidium iodide/RNase stain is added and incubated for 30 minutes. For flow cytometry, two laser detectors are used: FL1 (488) with a standard 533/30 band-pass (BP) that detects green fluorescence, and FL2 with a standard 585/40 BP that detects red or propidium iodide fluorescence. The tubes are analysed for DNA fragmentation using the BD Accuri™ C6 flow cytometer (BD cytometers, USA) (Figure 8.9). A quality control assay is also run using the eight-peak beads as per the manufacturer's instructions. Although less accurate, the samples can also be assessed by scoring about 500 sperm cells under fluorescence microscopy [164, 281].

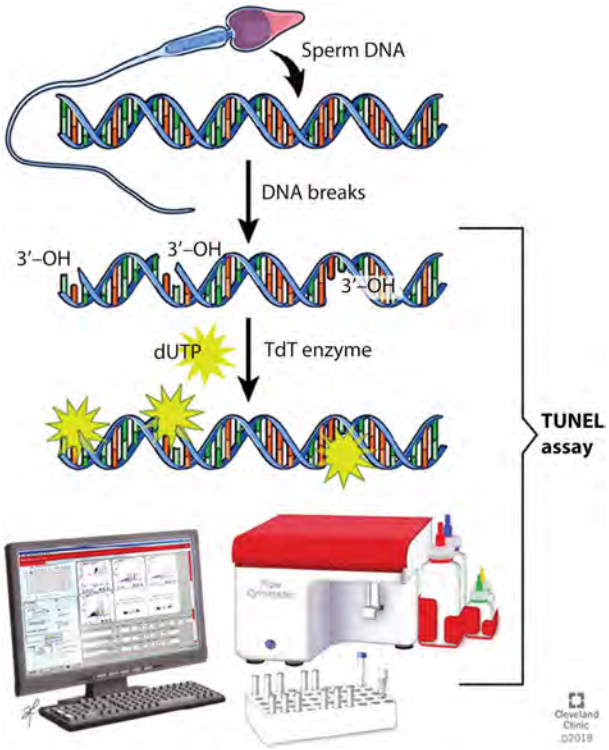

FIGURE 8.9 Schematic of DNA staining using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay.

The standard TUNEL assay can be improved to become more sensitive to DNA fragmentation by incubating sperm cells in 2 mm DTT solution for 45 minutes prior to fixation with formaldehyde. This modified version of the TUNEL assay was shown to significantly enhance its sensitivity. Mitchell et al. modified the TUNEL methodology by incubating spermatozoa for 30 minutes at 37°C with LIVE /DEAD™ Fixable Dead Cell Stain (far red) (Molecular Probes, Eugene, OR). The cells were then washed three times with Biggers–Whitten–Whittingham medium (BWW) before incubation with DTT; this allowed both DNA integrity and vitality to be simultaneously assessed [282].

Clinical significance

The TUNEL assay has been widely used in male infertility research related to SDF. A negative correlation was found between the percentage of DNA-fragmented sperm and motility, morphology, and concentration in the ejaculate. It also appears to be potentially useful as a predictor for IUI pregnancy rates, IVF embryo cleavage rates, and ICSI fertilization rates. In addition, it provides an explanation for recurrent pregnancy loss [18, 279, 283]. A cut-off value of 19.2% has been shown significant differentiation between fertile and infertile men with a sensitivity of 64.9% and a specificity of 100% [174, 279]. This is higher than that demonstrated for IUI procedures (12%) [284]. A very high specificity (91.6%) and positive predictive value (PPV) (1.40%) at a cut-off point of 16.8% [163]. The high specificity of the TUNEL assay is helpful in correctly identifying infertile patients who do not have SDF as a contributory factor [214]. Due to its high positive predictive value, the assay is able to confirm that a man who tests positive is likely to be infertile due to elevated SDF (Figure 8.10a and b) [285]. The calculated cut-off would be ideal as any value above this threshold will be strongly associated with infertility.

Advantages and limitations

The TUNEL assay is relatively expensive and time and labour consuming. Also, a number of factors can significantly affect assay results, including the type and concentration of fixative, fixed sample storage time, the fluorochrome used to label DNA breaks, and the method used to analyse flow cytometric data [286]. The flow cytometric method of assessment is generally more accurate and reliable than fluorescence microscopy, but it is also more sophisticated and expensive and it presents limitations in the accuracy and reproducibility of the measures of SDF [163, 287]. Fairly good-quality control parameters with minimal inter- and intra-observer variation (<8%) have been reported [163]. Similarly, the two flow cytometers showed very high precision (98%) and accuracy (>99%) along with interobserver agreement, establishing the robustness of both instruments [216].

Sperm chromatin structure assay

Principle

The SCSA measures *in situ* DNA susceptibility to the acid-induced conformational helix–coil transition by AO fluorescence staining. Acridine orange easily penetrates the dense chromatin and intercalates with the double-stranded DNA. This fluoresces green under blue laser light at 488 nm. The extent of conformational transition *in situ* following acid or heat treatment is determined by measuring the metachromatic shift of AO fluorescence from green (native DNA) at 515–530 nm under blue laser light to red (denatured or relaxed DNA) under red laser >630 nm. This protocol has been divided into $SCSA_{acid}$ and $SCSA_{heat}$ in order to distinguish the physical means of inducing conformational

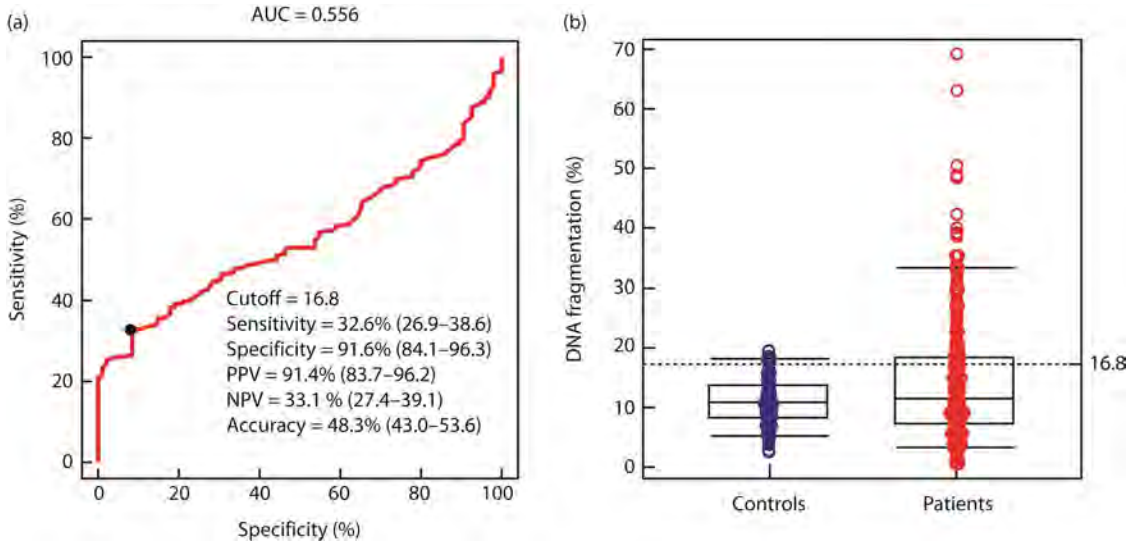

FIGURE 8.10 (a) Receiver operator characteristic curve showing terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) cut-off and the area under the curve (AUC). Values within the parentheses represent 95% confidence intervals. (b) Distribution of TUNEL values between controls and infertile men. *Abbreviations*: PPV, positive predictive value; NPV, negative predictive value.

transition. The two methods give essentially the same results, but the $SCSA_{acid}$ method is easier to use.

Technique

To perform SCSA, an aliquot of unprocessed semen (about 13–70 µL) is diluted to a concentration of 1–2 × 10^6 sperm/mL with TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, and 1 mM EDTA, pH 7.4). This cell suspension is treated with an acid detergent solution (pH 1.2) containing 0.1% Triton X-100, 0.15 mol/L NaCl, and 0.08 N HCl for 30 seconds and then stained with 6 mg/L purified AO in a phosphate–citrate buffer (pH 6.0). The stained sample is placed into the flow cytometer sample chamber [17]. The assay measures 5000 sperm by flow cytometry and DNA damage is indicated by the % DNA Fragmentation Index (DFI), which is a ratio of red fluorescence divided by total (red + green) fluorescence [17].

Clinical significance

Because the SCSA results are more constant over prolonged periods of time than routine World Health Organization (WHO) semen parameters, it may be used effectively in epidemiological studies of male infertility [288]. No significant male age-related increase in DFI has been demonstrated [289]. Currently, the SCSA is the only assay that has clearly established clinical thresholds for utility in the human infertility clinic [290]. In clinical applications, the SCSA parameters not only distinguish fertile and infertile men but also are able to classify men according to the level of *in vivo* fertility as high fertility (pregnancy initiated in less than three months), moderate fertility (pregnancy initiated within 4–12 months), and no proven fertility (no pregnancy by 12 months). In addition, a DFI threshold was established that identifies samples that are compatible with *in vivo* pregnancy (<30%) [12, 290–293].

The SCSA has been considered a gold standard as a robust assay for measuring DNA damage by flow cytometry and can predict various ART outcomes. However, the ability to predict ART outcomes, including fertilization and implantation rates, is true only for neat semen [9, 12, 187, 294]. An increased abortion rate in the high-DFI (>27%) group has been reported [275]. It has also been suggested that DFI can be used as an independent predictor of fertility in couples undergoing IUI [9], but the association between SCSA results and IVF and ICSI outcomes is not strong enough [295]. It has also been proposed that all infertile men should be tested with the SCSA in addition to standard semen analysis [296] and, if DFI is higher than 30%, ICSI should be recommended [12]. The SCSA also measures sperm with high DNA stainability, which is related to the nuclear histones retained in immature sperm. Sperm with high DNA stainability is reported to be predictive of pregnancy failure [297]. The current clinical threshold has changed from >30% to 25% DFI. DNA fragmentation above 25% categorizes a patient into the following statistical probabilities: (i) longer time to natural pregnancy; (ii) low odds of IUI pregnancy; (iii) more miscarriages; or (iv) no pregnancy [297]. The SCSA is considered to be a precise and repeatable DNA fragmentation test that can reliably identify a man who is at risk for infertility.

Advantages and limitations

The SCSA accurately estimates the percentage of DNA-damaged sperm and has a cut-off point (30% DFI) to differentiate between fertile and infertile samples. This has been recently revised to 25% [9, 290, 297]. However, it requires the presence of expensive instrumentation (flow cytometer) and highly skilled technicians. The SCSA DFI is significantly associated with TUNEL assay results when Spearman's rank correlation is used. However, regression and concordance correlation results showed that these methods are not comparable. The SCSA measures DNA damage in terms of susceptibility to DNA denaturation, whereas TUNEL measures "real" DNA damage [298].

Measurement of 8-OHdG

Principle

This assay measures levels of 8-OHdG, which is a by-product of oxidative DNA damage, in spermatozoa. It is the most commonly studied biomarker for oxidative DNA damage. Among various oxidative DNA adducts, 8-OHdG has been selected as a

representative of oxidative DNA damage owning to its high specificity, potent mutagenicity, and relative abundance in DNA [299].

Technique

Step I

DNA extraction is performed with chloroform–isoamyl alcohol (12:1 v/v) after the sperm cells are washed with sperm wash buffer (10 mmol/L Tris-HCl, 10 mmol/L EDTA, and 1 mol/L NaCl, pH 7.0) and lysed at 55°C for one hour with 0.9% SDS, 0.5 mg/mL proteinase K, and 0.04 mol/L DTT. After ribonuclease A treatment to remove RNA residue, the extracted DNA is dissolved in 10 mmol/L Tris–HCl (pH 7.0) for DNA digestion.

Step II

Enzymatic DNA digestion is performed with three enzymes: DNase I, nuclease P1, and alkaline phosphatase. The final solution is dried under reduced temperature and pressure and is redissolved in distilled and deionized water for HPLC.

Step III

The third step is HPLC analysis. The HPLC system used for 8-OHdG measurements consists of a pump, a Partisphere® 5 C18 column (Hichrom Limited, UK), an electrochemical detector, a UV detector, an autosampler, and an integrator. The mobile phase consists of 20 mmol/L $NH_4H_2PO_4$, 1 mmol/L EDTA, and 4% methanol (pH 4.7). The calibration curves for 8-OHdG are established with standard 8-OHdG, and the results are expressed as 8-OHdG/10^4 dG [218].

Clinical significance

The assay provides the most direct evidence suggesting that oxidative sperm DNA damage is involved in male infertility based on the finding that 8-OHdG levels in sperm are significantly higher in infertile patients than in fertile controls and are inversely correlated with sperm concentration [300]. 8-OHdG formation and DNA fragmentation as assessed by TUNEL are highly correlated with each other [301]. 8-OHdG levels also are highly correlated with the disruption of chromatin remodelling [122]. Levels of 8-OHdG in sperm DNA have been reported to be increased in smokers, and they are inversely correlated with the intake and seminal plasma concentration of vitamin C—the most important antioxidant in sperm. Infertile patients with varicocele have increased 8-OHdG expression in the testis, which is associated with deficient spermatogenesis [302]. If not repaired, 8-OHdG modifications in DNA are mutagenic and may cause embryo loss, fetal malformations, or childhood cancer. Moreover, these modifications could be a marker of OS in sperm, which may have negative effects on sperm function [303, 304].

Advantages and limitations

Although 8-OHdG is a potential marker for oxidative DNA damage, artificial oxidation of dG can occur during analysis, which can lead to inaccurate results. A fixed number of sperm cells should be analysed as a precaution. However, the DNA yield cannot be excluded as a potential confounder.

Clinical utility of sperm DNA fragmentation

In the past decade, there has been mounting evidence that supports the clinical utility of DNA tests in male fertility evaluation [30, 139, 148, 180, 297]. Due to distinct assay characteristics, the results obtained from one method do not necessarily match those provided by other tests. There seems to be a fairly good correlation among the three widely used tests, namely SCSA, TUNEL, and SCD, although the evidence is not unequivocal. Emerging evidence indicates that SDF has a clear influence on the reproductive outcomes, both naturally and via assisted reproductive techniques. A recently published practice recommendation by Agarwal et al. represents the first attempt to propose specific clinical indications for SDF testing. These indications were clinical varicocele, unexplained infertility/IUI failure or recurrent pregnancy loss, IVF/ICSI failure, and lastly borderline abnormal or normal semen parameters with risk factors [180]. This consensus statement was later endorsed by the Society for Translational Medicine and Clinical Practice Guidelines (CPG) for SDF testing in male fertility in 2017 [162]. Utilizing a Strengths-Weakness-Opportunity-Threat (SWOT) analysis to understand the perceived advantages and drawbacks of SFDF as a specialized function test in clinical practice [34], the analysis revealed that CPG provides reasonable evidence-based proposal for integration of SDF testing in routine daily practice and provides opportunity to further improve SDF testing.

The WHO manual provides a detailed description of different tests used in the evaluation of SDF [41] but does not provide a clinical description of the possible indications for SDF testing. On the other hand, the indications governing the clinical utility of SDF testing have been clearly defined in the recent EAU guidelines [36]. It clearly establishes SDF testing in (i) nonazoospermic men with unexplained infertility (strong recommendation) and (ii) couples with RPL (from natural conception and ARTs) (strong recommendation). In addition, two research groups have reported and published the guidelines on the indications of SDF testing [31, 32, 35]. These two reports also recommend indications for SDF testing in cases of unexplained, male infertility (Grade B and C), varicocele (Grade C), RPL (Grade B-C), RPL (Grade B-C), and ART before and after failure [35]. High SDF is also linked with recurrent pregnancy loss, elevated levels of DNA damage, and miscarriages [22]. In men with high SDF, the use of testicular sperm has also been recommended in cases of oligozoospermia or recurrent pregnancy loss [305, 306].

Men can also benefit from lifestyle modifications and SDF testing [38]. The results of SDF testing may change the management decision by selecting the most appropriate ART with the highest success rate for infertile couples.

SDF testing should be considered as one of the tests in a panel of male fertility assessment rather than a stand-alone test. A combination of a selected panel of tests, when appropriately applied, could offer additional complementary information for making a clinical diagnosis while considering various male and female factors in a clinical scenario. SDF assays have been adopted by many andrology laboratories worldwide, and gaps in our knowledge have been identified and recommendations made to further improve the clinical utility of SDF in clinical practice [143, 162].

Strategies to reduce sperm DNA damage

In view of the impact SDF has on reproductive outcomes, it is important to develop and implement appropriate treatment methods, preventive measures, and strategies to minimize DNA damage in the spermatozoa used in assisted reproduction [307]. Some of the strategies include:

1. *Appropriate sperm preparation methods*: Most of the commonly used methods such as density gradient centrifugation, swim up, and glass wool filtration yield sperm with better DNA integrity than native semen [14]. Sperm

preparation should be aimed at minimizing damage to the spermatozoa and can be accomplished by exercising some simple precautions, such as (i) slow dilution of the samples, especially when using cryopreserved spermatozoa; (ii) gradual changes in temperature and tests performed at 37°C; (iii) minimal use of centrifugation and, when necessary, it being performed at the lowest possible speed; and (iv) controlled exposure to potentially toxic materials. Plastic ware, glassware, media, and gloves should be checked for potential toxicity as the spermatozoa may be immobilized when in contact with any potential toxic substances in these materials. In patients who are unable to produce a semen sample by masturbation, use of non-toxic condoms is important, and when necessary, a second sample should be collected a few hours after the first.

2. *Electrophoretic separation of sperm*: This is based on the principle that high-quality spermatozoa tend to be viable and morphologically normal and have a low degree of DNA fragmentation as measured by TUNEL assay [308].
3. *Antioxidant treatments*: One of the causes of sperm DNA damage is OS. Studies have investigated the ability of antioxidant treatments to manage male subfertility, both *in vivo* and *in vitro*. It is generally accepted that antioxidants may be beneficial for reducing sperm DNA damage, but their exact mechanism of action is still not established, and some studies have reported adverse effects such as increased sperm chromatin decondensation [309, 310]. Significant improvement in clinical pregnancy and implantation rates have been shown in patients with high sperm DNA damage as assessed by TUNEL assay when treated with antioxidants before assisted reproduction [311, 312]. Therefore, in patients in whom OS is the cause of sperm DNA damage, adequate oral antioxidant supplementation appears to be a simple strategy to enhance sperm genome integrity and reproductive outcomes. Standard and reliable oral antioxidant treatment protocols and alternative treatment strategies for non-responders are needed [312].
4. *Magnetic cell separation*: Magnetic cell separation is a useful technique to separate apoptotic and non-apoptotic spermatozoa [313].
5. *High-magnification ICSI for patients with SDF*: It is possible to observe spermatozoa with apparently normal morphology and intranuclear vacuoles that appear to be associated with chromatin packaging by using inverted microscopes with Nomarski differential interference contrast optics combined with digitally enhanced secondary magnification [314, 315].
6. *PICSI*: Hyaluronan-bound sperm are thought to reflect higher maturity, acrosome activity, along with lower aneuploidies and SDF [316, 317]. PICSI is highly specific and thought to improve successful pre-implantation embryogenesis [318].
7. *Microfluidics:* By controlling fluid dynamics, it is possible to mimic the physiological conditions of pH and temperature of the female genital tract [319]. This allows the selection of spermatozoa with high motility, while debris and dead spermatozoa can be easily separated [320]. This results in significant improvement in sperm motility, morphology, and increased pregnancy rates in couples undergoing ICSI [321] and Chapter 28.
8. *Use of testicular sperm*: Spermatozoa retrieved from the testis in men with high SDF in neat semen tend to have better DNA quality compared to ejaculated sperm. These men may benefit from testis-ICSI if male partners have confirmed high SDF in the ejaculate [4, 143, 305, 322].
9. *Lifestyle modifications*: Infertile men with modifiable lifestyle risk factors such as smoking and obesity and high SDF can benefit from lifestyle modifications [38, 323].

Conclusion

The importance of assessing sperm chromatin integrity is well established, and the results provide useful information in cases of male idiopathic infertility and in couples pursuing assisted reproduction. Pathologically increased SDF is one paternal-derived cause of repeated assisted reproduction failures in the ICSI era. Several studies have demonstrated that sperm DNA integrity correlates with pregnancy outcome in IVF. Therefore, SDF should be included in the evaluation of the infertile male especially in infertile men as well as normozoospermic men with clinical varicoceles, and men with idiopathic infertility. Assessment of sperm DNA damage appears to be a potential tool for evaluating semen samples prior to their use in assisted reproduction. It allows for the selection of spermatozoa with intact DNA or with the least amount of DNA damage for use in assisted conception. It provides better diagnostic and prognostic capabilities than standard sperm parameters for assessing male fertility potential.

There are multiple assays that can be used to evaluate sperm chromatin. Most of these assays have advantages along with limitations. Choosing the right assay depends on many factors, such as the expense, the available laboratory facilities, and the presence of experienced technicians. The establishment of a cut-off point between normal levels in the average fertile population and minimal levels of sperm DNA integrity required for achieving pregnancy is still debated and a single cut-off may not be ideal as different tests measure different aspects of DNA fragmentation. Further research is needed on the clinical SDF thresholds to be used with each SDF test on IUI, IVF, and ICSI, using different endpoints (e.g. live birth, miscarriage). Given the importance of sperm DNA integrity, it is important to determine the real cause of DNA damage and provide proper therapeutic treatments. Methods for selecting sperm with undamaged DNA should be designed, especially in cases where ICSI is strongly recommended. With further refinements in SDF testing and emerging supporting evidence, SDF testing will help improve ART success and the health of both fathers to be and the resulting offspring.

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9

OOCYTE RETRIEVAL AND SELECTION

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Introduction

Today, assisted reproductive technologies (ART) refer not only to *in vitro* fertilization (IVF) but to all the approaches tailored to patients' specific conditions. The technology transfer in ART resulted in advanced practices that improved patients' journeys and IVF outcomes.

The reproductive axis is regulated by the pulsatile release of the hypothalamic gonadotropins-releasing hormone (GnRH). It determines the pattern of secretion of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), which then regulate both the endocrine function and gamete maturation in the gonads [1]. Therefore, in IVF, novel protocols regarding ovarian stimulation have been theorized based on the use of gonadotrophins to prompt the growth of antral follicles, to improve the effectiveness and efficacy of IVF treatment in specific patient populations [2].

Controlled ovarian stimulation (COS) entails the administration of exogenous gonadotrophins to stimulate multi-follicular development, a co-treatment with either GnRH agonist or antagonists to suppress pituitary function and prevent premature ovulation, and the trigger of final oocyte maturation 36–38 hours prior to oocyte retrieval [3].

After ovulation triggering, oocyte meiosis (blocked at the prophase of the first meiotic division) is reinitiated, going through germinal vesicle (GV) breakdown and the formation and extrusion of the first polar body (PB1). After entering the second meiotic division, a second arrest occurs at metaphase stage II (MII). The presence of MII spindle together with the PB indicates completion of oocyte maturation, which is required before performing IVF/intracytoplasmic sperm injection (ICSI) procedures. The evaluation of oocyte quality in the laboratory is based on the appearance of the cumulus-oocyte complex (COC) and, after denudation, also on the morphology of the ooplasm and on the aspect of the extra-cytoplasmic structures. However, currently the role of oocyte assessment is controversial; the main aspects evaluated over time to characterize the oocyte developmental competence are summarized in this chapter.

Ovarian stimulation protocols

Prediction of ovarian response

Currently, different stimulants of multi-follicular growth have been suggested, including recombinant, biosimilar or urinary gonadotropins, GnRH analogues (agonists or antagonists), steroid hormones, and other drugs like aromatase inhibitors or growth hormones. The choice of the most suitable COS regimen is based on the prediction of ovarian response so to properly tailor COS [4]. Several predictors have been identified, including maternal age, hormones (FSH and anti-Müllerian hormone [AMH]), morphological parameters (antral follicle count [AFC]), clinical conditions like polycystic ovary syndrome (PCOS), and BMI [5].

Basal serum FSH was adopted as one of the most widespread markers to this end. Yet, its accuracy is limited, and it is only suggested for counselling purposes [6]. The correlation between different groups of patients and basal FSH values is, in fact, statistically not significant, and it does not allow exact classification.

AMH is produced by preantral follicles and small antral follicles of up to 7–8 mm in size. It functions as an inhibitor of FSH-mediated granulosa cell (GC) proliferation, follicular growth, and aromatase activity. AMH level remains stable during menstrual cycles; hence, it is a strong candidate marker for ovarian reserve in women and, in addition, is a strong predictor for the number of oocytes retrieved in patients undergoing IVF treatment.

AFC is conducted through transvaginal ultrasound; it is associated with the ovarian reserve and may predict IVF outcomes. A linear relationship, in fact, exists between AFC and the number of oocytes retrieved [7]. Recently, the combination of AFC and AMH level has been supported as the favourite method to predict the ovarian reserve with a reasonable precision. Single centre receiver operating characteristic curve analyses and meta-analyses showed that both AFC and AMH can identify patients more likely to respond to exogenous gonadotrophins with poor, normal, or hyper-response [7].

Individualized stimulating regimens

Over the past 30 years, particular attention has been paid to the development of simplified ovarian stimulation regimens to identify novel and more convenient approaches to maximize the IVF result. The number of oocytes retrieved is a key factor to improve IVF outcomes [4]. In fact, a large multicentre study reported a significant progressive increase of the cumulative live birth rate (CLBR) with the number of oocytes, thus suggesting that ovarian stimulation may not have a detrimental effect on oocyte/embryo quality [8]. Moreover, it has been assessed that aneuploidy rates do not increase with ovarian response or gonadotropin doses. Also, the number of euploid embryos available for embryo transfer increases as the number of oocytes obtained increases [9]. Different stimulation protocols have been adopted over time to fully exploit the ovarian reserve. In the 1990s, the administration of short-term treatments with GnRH agonist (GnRHa) was reduced in favour of long-term GnRHa stimulation protocols [10]. About 20 years ago, GnRH antagonists were introduced in IVF [11]. These GnRH analogues induce an immediate suppression of the pituitary function, which allows the administration of gonadotropins without pituitary suppression, resulting in shorter and more feasible stimulation protocols [12]. This approach provides similar live birth rates compared with the standard long GnRHa protocols, while minimizing the risk of ovarian hyperstimulation syndrome (OHSS).

Different types of gonadotropins have been adopted to optimize COS. Among them, corifollitropin alfa is a recent therapeutic option developed as an injectable, long-acting FSH agonist. It is a chimeric recombinant molecule composed of

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FSH and the carboxy-terminal peptide of human chorionic gonadotropin (hCG). Corifollitropin alfa has a longer half-life compared to recombinant FSH (rFSH) and thus requires less frequent administrations [13]. The results achieved with corifollitropin alfa treatment in terms of clinical pregnancy rate, ongoing pregnancy rate, multiple pregnancy rate, miscarriage rate, ectopic pregnancy rate, and congenital malformation rate (major or minor) was like that obtained with daily treatment of rFSH [14].

Additionally, an unconventional stimulation protocol was introduced that allowed the assessment of the clinical contribution of luteal-phase stimulation (LPS) to follicular-phase stimulation (FPS) in a single ovarian cycle. This protocol is known as DuoStim and it is indicated to poor prognosis patients. It has been reported that a second stimulation in about 15 days resulted in an increased CLBR per ovarian cycle, especially since all embryological, chromosomal, clinical, gestational, and perinatal outcomes are similar between LPS-derived oocytes as FPS-derived ones [15].

Oocyte growth and selection

Oocyte developmental competence is the capacity of a female gamete to reach maturation, be fertilized, and sustain the initial phases of embryonic development until the blastocyst stage [16]. Many factors contribute to the production of a good quality oocyte, and abnormalities in this process lead to infertility and recurrent ART failure. Oocyte quality is not defined only based on its euploid chromosomal constitution but also on all other aspects that concur to its capacity to reach the blastocyst stage and implant. For instance, limited non-invasive tools are available to permit a proper classification of human oocyte quality prior to fertilization. However, slight deviations from morphological normality should not be considered abnormal. The main factors that could be related to the oocyte quality are summarized in Figure 9.1, and thoroughly described in the following sections.

Perifollicular vascularization evaluation

Oocyte growth, selection, and acquisition of developmental competence is influenced by a dynamic network of blood vessels. This perifollicular vascularization is required to convoy oxygen, growth factors, gonadotrophins, and steroid precursors, required to sustain follicle-oocyte maturation [17]. Although several studies demonstrated a strong correlation between a correct vasculature and a healthy follicle development, the cause-effect relation remains poorly understood. Ovarian perifollicular blood flow assessment during IVF using power doppler ultrasound (PDU) has been documented as a good marker of oocyte competence, embryo viability, and implantation potential [18]. Conversely, other studies were not able to confirm the clinical value of the association between perifollicular vascularization and oocyte competence to improve the reproductive outcomes [19, 20]. For these reasons, further prospective randomized studies may clarify the effectiveness of perifollicular vascularization assessment in order to incorporate PDU as a protocol for assessing the gamete/embryo quality.

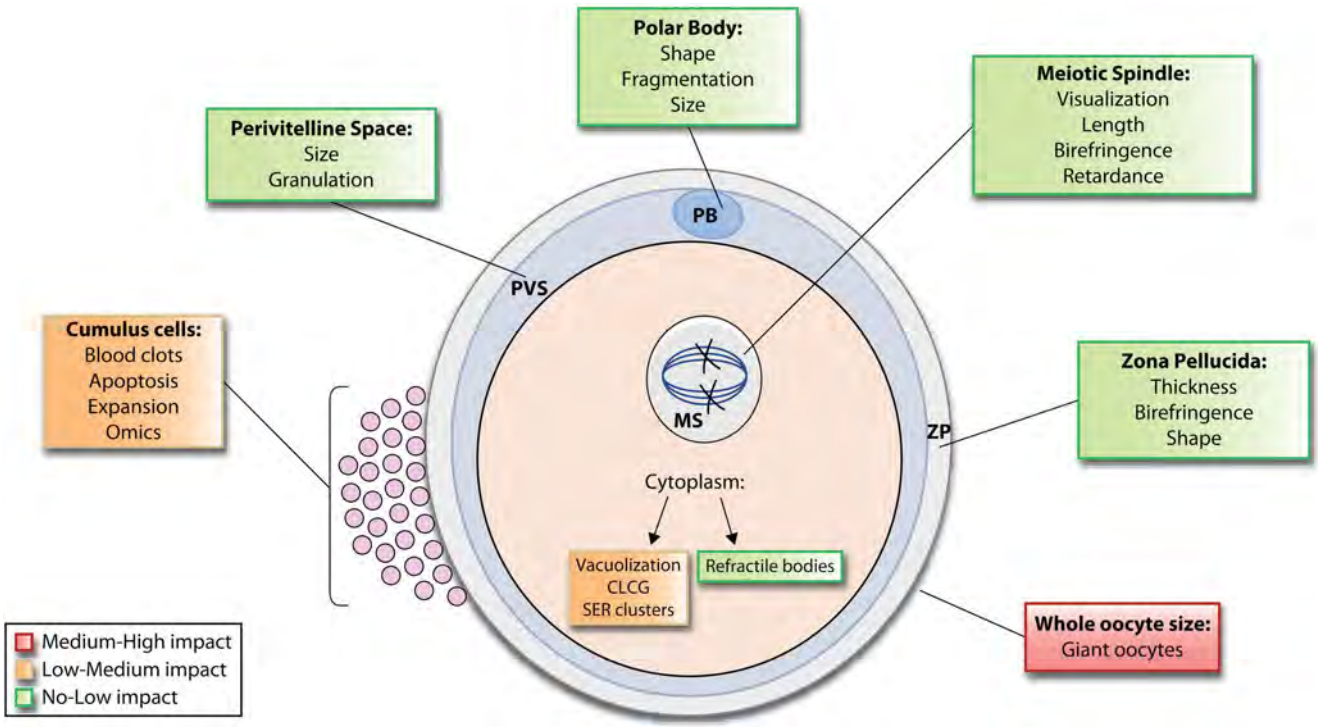

FIGURE 9.1 Extracytoplasmic and intracytoplasmic morphological properties used to assess oocyte competence. The red, orange, and green boxes represent the oocyte dysmorphism with high, medium, and low predictive value of oocyte quality, respectively. *Abbreviations*: CLCG, centrally located cytoplasmic granulation; SER, smooth endoplasmic reticulum.

Cumulus-oocyte complex evaluation

The mature cumulus-oocyte complex (COC) consists of the secondary oocyte at MII stage, following extrusion of the PB1, surrounded by cumulus cells (CCs). The latter are somatic cells surrounding the oocyte that originate from undifferentiated GCs and promote oocyte growth and maturation. A bi-directional paracrine communication between oocytes and CCs, mediated by the delivery and reception of paracrine factors at the oocyte–GCs interface, regulate the early ovarian follicle development. As CC function is involved in the completion of oocyte meiotic maturation and ovulation, fertilization, and subsequent early embryo development, several authors investigated the morphological features of the COC for the evaluation of oocyte quality.

It was noted that the presence of blood clots in CCs was associated with dense central granulation of oocytes and may adversely affect fertilization, cleavage, and blastulation rates. Blood clots also lead to fewer retrieved oocytes compared with the number of oocytes whose CCs exhibit normal appearance [21]. The adverse effects of blood clots seem to originate from the production of reactive oxygen species (ROS) at high levels from existing blood components [22].

The apoptotic index of CCs was analysed to predict the nuclear maturity of oocytes. The rate of apoptosis in the CCs of immature oocytes at GV or metaphase I (MI) stage is at higher levels than in the CCs from mature oocytes. Moreover, a higher apoptotic rate in CCs of mature oocytes correlates with a lower fertilization rate [23]. Consistently, Faramarzi et al. suggest that oocytes showing a lower apoptotic level independent of cumulus expansion have a better developmental potential [24].

Oocyte developmental potential was also correlated with cumulus expansion. Some studies revealed a positive correlation between cumulus expansion and fertilization rate and blastocyst quality [25, 26]. Although other groups found no difference in the fertilization rate among various COC expansion grades, they found a positive correlation with implantation and pregnancy rates of the relative oocytes [27]. On the contrary, other studies found a negative correlation between the cellular density of the corona radiata and the rate of mature oocytes but not between COC morphology and fertilization, cleavage, and clinical pregnancy rates [21, 28].

Overall, the COC might indicate oocyte quality, and Alpha and ESHRE scientific societies stated that it is reasonable to adopt a binary score to grade compacted CCs (score 0) or expanded CCs and corona radiata (score 1) [29].

Recently, interesting approaches have been developed to better understand oocyte quality based on CCs and GCs RNA/protein content and metabolite production (the so-called-omics technology; e.g. genomic, transcriptomic, proteomic, metabolomic). Thus, it is conceivable that the study of GCs and CCs, which are usually discarded in ICSI treatments, offer a totally non-invasive tool to predict oocyte competence [30, 31].

Current transcriptomics enable qualitative and quantitative characterization of gene expression in cells or tissues. It allows the creation of a list of genes differentially expressed in GCs and CCs surrounding healthy oocytes versus non-viable ones. The data produced to date are still controversial and inconclusive, though, and there is limited consensus on the markers identified [32]. A very recent study from Tiegs and collaborators investigated the presence of differentially expressed genes among CCs of euploid versus embryos affected from trisomy 21, supporting that CCs gene expression may be adopted to identify biomarkers of oocyte quality [33]. Other studies adopted proteomic and metabolomic approaches to build additional information about the functional status of a certain biological system (i.e. COCs function) [34].

In summary, -omic technologies are still evolving and at present are considered too expensive and time-consuming to be efficiently implemented in the clinical setting. However, soon they might be integrated with morphological analyses to constitute a more efficient platform to assess oocyte/embryo viability [35].

Oocyte nuclear maturity evaluation

Oocyte nuclear maturity is determined by the presence of an extruded PB1 in the perivitelline space (PVS) and by the absence of a GV. The presence of PB1 provides information on the nuclear maturation stage but its does not provide any information on the degree of cytoplasmic maturity. In fact, nuclear and cytoplasmic maturation should be completed in a coordinated manner to ensure optimal conditions for subsequent fertilization. Non-synchronous oocyte maturation is often observed after ovarian stimulation [36]. At the MII stage, the oocyte chromosomes are aligned at the equatorial region of the meiotic spindle (MS). This structure plays a crucial role in the sequence of events leading to the correct completion of meiosis and fertilization and thus is a key determinant of oocyte developmental potential. It has been shown that the MS is highly sensitive to the chemical and physical changes that may occur during oocyte retrieval and handling [37, 38]. Other parameters, such as advanced maternal age [39] and oocyte *in vitro* aging [40], are also associated with the disruption of MS architecture therefore leading to the formation of aneuploid embryos.

The introduction of an orientation-independent polarized light microscopy system allowed the visualization of MS in living oocytes [41]. The absence of a detectable MS and the consequent oocyte developmental impairment may be primarily ascribed to oocyte immaturity. A positive correlation between MS visualization, fertilization rate, and/or embryo development and/or blastocyst progression was described in several studies [42–45]. In contrast, Chamayou et al. reported no correlation between spindle visualization, embryo quality, and clinical pregnancy rates [46]. Interestingly, time-lapse morphokinetic events in conjunction with zona pellucida birefringence (ZPB) and meiotic spindle visualization (MSV) have been assessed for predicting pregnancy outcome by Tabibnejad and collaborators. Implantation, live birth, chemical, and clinical pregnancy rate were reported higher in the transferred embryos deriving from oocytes showing visible spindles [47].

Besides its role in chromosome segregation, the MS is also involved in the extrusion of the PB1. Its position at the very periphery of the cell, attached to the oolemma cortex, dictates the orientation of the cleavage furrow and thus the PB1 extrusion site. However, PB1 has been found frequently dislocated from the MS location after denudation. A moderate degree of PB1/MS deviation does not seem to involve a significant relationship with embryo implantation. However, another possible drawback of PB1 displacement is the potential damage to the MS during ICSI. Consequently, the correct orientation of the oocyte with the MS (and not the PB1) as far as possible from the injection needle allowed ICSI to be safely performed [48].

A possible correlation between MS birefringence, oocyte quality, and embryo development has been suggested [49, 50]. Nevertheless, MS mean retardance, area, and length are not significantly associated with the achievement of a live birth.

Thus, it seems that polarization microscopy cannot be used as a non-invasive marker to predict IVF outcomes [51].

MII oocyte morphological evaluation

An ideal mature human oocyte, based on morphological characteristics, should have a moderately granular cytoplasm; a round and clear ZP, appropriately thick and containing a single, non-fragmented PB1; and a normal PVS [52]. However, many of the oocytes retrieved after ovarian stimulation exhibit one or more morphological abnormalities of the cytoplasm aspect and/or of the extra-cytoplasmic structures [53].

A systematic review of all papers published between 1990 and 2019 aiming at evaluating the predictive value of oocyte morphology suggested that the influence of oocyte dysmorphisms in terms of IVF success is still controversial [54]. The adoption of different criteria for oocyte evaluation may be responsible for the discrepancies across different studies. Alpha and ESHRE scientific societies suggested a common terminology for oocyte morphology assessment to simplify inter-laboratory comparison [29]. Morphological oocyte abnormalities are mainly classified as extra-cytoplasmic features (altered COC morphology, ZP thickness, large PVS, PVS granularity, fragmented or irregular PB1, shape and dimension of the oocyte) or intra-cytoplasmic features (vacuolization, increased cytoplasmic granularity and viscosity, presence of cytoplasmic inclusions, refractile bodies, and smooth endoplasmic reticulum clusters). These morphological properties used to assess oocyte viability are graphically shown in Figure 9.2, along with their predictive value on oocyte quality.

Extra-cytoplasmic abnormalities

Cumulus-oocyte complex

COC morphological evaluation has been discussed in the "Oocyte–corona–cumulus complex evaluation" paragraph.

Zona pellucida

The ZP is a specialized extracellular matrix layer surrounding the oocyte. Beyond its involvement in oocyte–somatic cell interactions, the ZP also plays an essential role at fertilization, permitting sperm–egg interactions, the acrosome reaction, and an adequate block to polyspermy. Many ZP variants (colour, appearance, thickness, irregularities, composition, and organization) have been described, and abnormal ZP morphology can be observed in 2%–5% of all oocytes [55]. In the past, several studies reported an association between a thicker ZP and decreased fertilization rates, implantation, and pregnancy rates [56, 57]. Oocytes with an oval-shaped ZP have been associated with a high risk of abnormal embryo cleavage and are associated with lower rates of implantation and pregnancy after IVF [58]. However, other analyses, including a randomized controlled trial [59], showed no benefits of ZPs thickness measurements [60–62]. The Istanbul consensus claimed no benefit in measuring zona thickness due to insufficient evidence of any effect on either biological or clinical outcomes. Different colour or thickness of the ZP could be related to patient-specific effects [63]. Additionally, oocytes with heterogeneous ZP (HZP) have a bright vitreous appearance with an irregular outer edge. A study with a limited sample size found that HZP is associated with reduced oocyte maturity, reduced fertilization rates, and lower high-quality embryo rates [64]. Further investigation into the outcome of oocytes with HZP is required. Apparently,

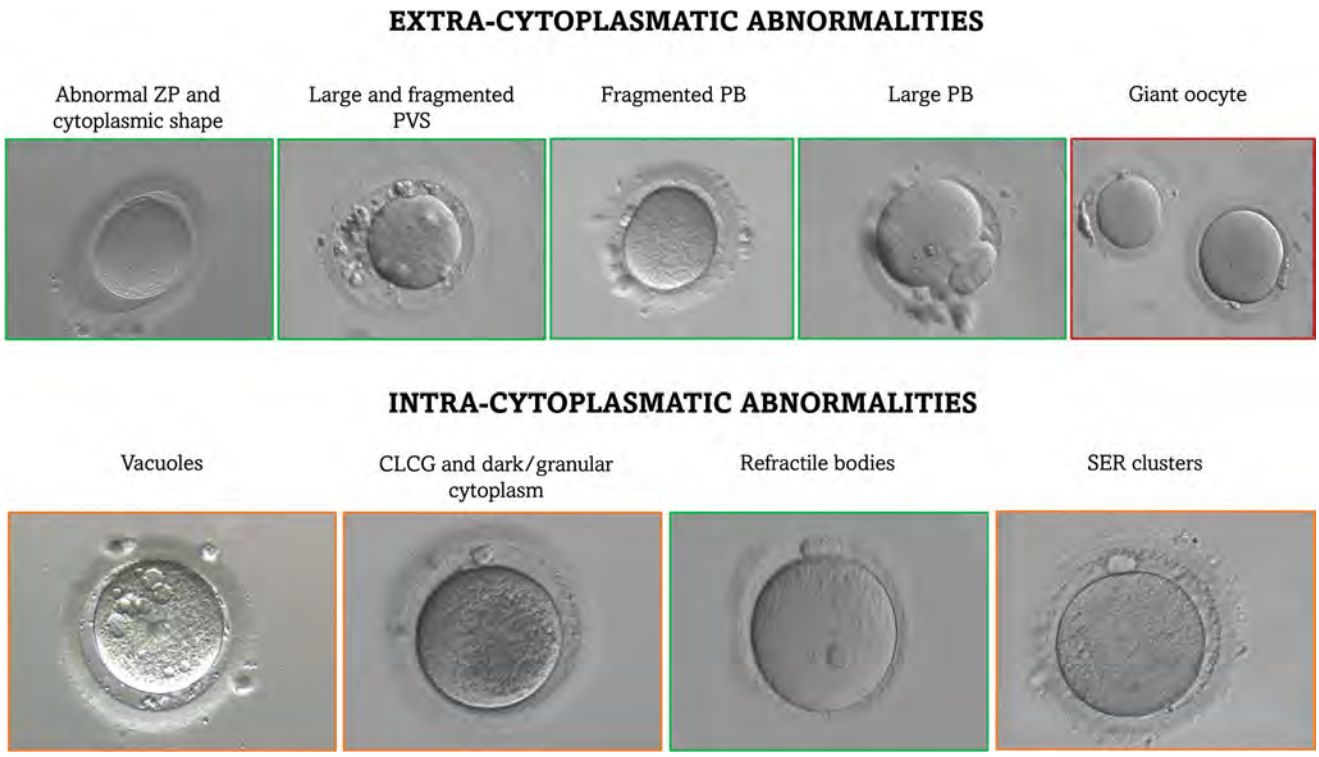

FIGURE 9.2 Examples of extracytoplasmic and intracytoplasmic oocytes abnormalities. The abnormalities with no to low impact on oocytes competence are highlighted in green, in orange if involving low to medium impact, and in red if involving medium to high impact. *Abbreviations*: PVS, perivitelline space; PB, polar body; CLCG, centrally located cytoplasmic granularity; SER, smooth endoplasmic reticulum.

only drastic morphological alterations (broken or empty ZP) were regarded unsuitable for ICSI.

ZP birefringence, a refractive index obtained from polarization and propagation direction of light, can be analysed to predict the developmental potential of oocytes and early embryos. Oocytes that show a high birefringence of the inner layer of ZP resulted in higher implantation, pregnancy, and live birth rates than that of ones with low birefringence of the inner layer of ZP [44, 50, 65]. The miscarriage rate was also higher in the transferred embryos obtained from oocytes showing low birefringence [44]. On the other hand, another study revealed no differences among high and low birefringence of the inner layer of ZP [47]. In summary, the definition of a potential relationship between ZP characteristics and oocyte competence still requires more studies.

Perivitelline space

PVS represents the acellular compartment in between the plasma membrane of the oocyte and its ZP. It becomes clearly visible in a mature oocyte with the extruded PB located in its most prominent portion. Generally, normal MII oocytes have a small PVS including the single polar body (PB) and no granulated dispersed material [66]. A large PVS seems to reflect an over-maturity of the oocytes at the time of ICSI [67]. Several researchers found that a large PVS may affect oocyte survival [68, 69] and fertilization rate [62] after ICSI. Rienzi and co-authors [62] found that a large PVS is correlated with low fertilization rates and compromised pronuclear morphology, but not with a compromised embryo quality. Moreover, a correlation exists between large PVS and the presence of granulated material in the embryo, but not with implantation and other clinical outcomes [46, 70]. Other different studies failed to report a correlation between the size and shape of the PVS, fertilization rate, and embryo development [60, 71, 72]. However, there was insufficient evidence to support any specific prognosis associated with this observation. In conclusion, an abnormal PVS is insufficient to predict oocyte quality.

First polar body morphology

First polar body (PB1) extrusion indicates completion of the first meiotic division in human oocytes. It has been assumed that PB1 extrusion determines the correct progression of oocyte meiosis in MII and therefore it may mark an important transition into the acquisition of meiotic competence [73]. For this reason, the morphology of the PB could represent a marker of quality. The studies available are distributed between pros and cons, and as a result this assumption is still in debate. Some of these studies showed a correlation between PB1 morphology, such as smooth surface, rough surface, and fragmented with fertilization rate and embryo quality [74, 75] along with implantation and pregnancy rates [76]. According to Rienzi and co-authors [62], abnormal (large or degenerated) PB1 was related to decreased fertilization rates, but no correlation with pronuclear morphology or embryo quality, while fragmentation was not associated with any of these outcomes. Moreover, embryos derived from oocytes with an intact PB1 were more prone to develop into a blastocyst than embryos derived from oocytes with fragmented PB1 [76]. Navarro et al. [77] found a correlation between large PB1 and decreased fertilization, cleavage rates, and compromised embryo quality.

Surprisingly, fragmentation or degeneration of PB1 was found to be related to higher fertilization rates and lower levels of fragmentation of embryos by Fancsovits and co-authors [75], while large PB1 were associated with compromised fertilization and low embryo quality. Conversely, several other studies failed to demonstrate a relationship between PB1 fragmentation and embryo development [49, 71, 78–80], quality, implantation, or aneuploidies [49, 78, 81]. This discrepancy in the literature seems due to methodological variations in the published studies, sample size, and timing of PB morphology evaluation [82]. Thus, this morphological trait seems a marker of post-ovulatory *in vitro* oocyte aging rather than a proper marker of oocyte quality.

Shape

Good-quality mature human oocytes have a round and clear ZP. Approximately 7% of the oocytes show a deformation of the cytoplasm, which can vary from slightly oval to substantially elongated. An oocyte shape alteration may occur due to mechanical pressure during follicle aspiration or due to the physiological process of folliculogenesis [83]. Oocyte deformation is often associated with ZP abnormalities, and it was thought to be associated with altered embryo cleavage and intercellular blastomeres contacts, in turn affecting compaction and blastocyst formation [84, 85]. Conversely, other studies did not find any correlations between oocyte shape anomalies and fertilization rate, embryo development or embryo quality [86], aneuploidy [87], cryo-survival [62, 79], implantation, and pregnancy [46]. Overall, oocyte shape does not seem to affect IVF outcomes.

Giant oocyte

A giant oocyte is an uncommon abnormality with the incidence around 0.3% among retrieved oocytes [88]. They show about twice the volume of normal oocytes and, presumably, derived from cytoplasmic fusion of two oogonia or nuclear division without cytoplasmic division of an oogonium, therefore originating a tetraploid gamete [89]. It is known that embryos deriving from giant oocytes are at higher risk of digynic triploidy, thus the transfer of embryos derived from them may increase the miscarriage rate [90]. Based on this evidence, giant oocytes should not be used for clinical practice.

Intracytoplasmic abnormalities

Vacuolization

Vacuoles are dynamic cytoplasmic dysmorphisms formed by membrane-bound cytoplasmic inclusions filled with fluid. Vacuoles arise either spontaneously or by fusion of vesicles derived from the smooth endoplasmic reticulum (SER) and/or Golgi apparatus. Intracytoplasmic vacuoles have been associated with severe oocyte degeneration, displacement of the MII spindle from its polar position [91], abnormal cytokinesis pattern [92], compromised embryo development [71], and impaired blastocyst formation [93]. Also, oocytes with vacuoles in the cytoplasm or central granulation exhibited declined cryo-survival and developed into good quality blastocysts less frequently [87]. Rienzi et al. reported a slight but significant decrease in fertilization rates among vacuolated oocytes, but no effect on pronuclear or embryo morphology [62]. The size of the vacuoles might be relevant [94], yet the evidence to date is insufficient to claim that their presence indicates lower oocyte competence.

Cytoplasmic granularity

The presence of centrally located cytoplasmic granulation (CLCG) is considered abnormal and it was originally reported as an indication of cytoplasmic immaturity and lower implantation rate [95]. Merviel et al. found that patients with high ratios (>75%) of CLCG are characterized by lower cleavage, pregnancy, and live birth rates [96]. Rienzi et al. reported that diffuse peripheral

granulation in the MII oocytes resulted in compromised pronuclear morphology [62], as confirmed by several other studies [60, 85]. Ongoing pregnancy rates are seriously compromised in case of CLCG [97–99], perhaps due to allegedly higher embryonic aneuploidies [100]. However, also in this case, the evidence is contrasting, with most of the authors reporting that oocytes with or without CLCG show comparable developmental and reproductive potential [101, 102]. In summary, CLCG is a feature poorly prognostic of oocyte competence.

Dark/granular cytoplasm

The colour variation of the ooplasm is rarely observed and is often described as "dark–granular cytoplasm." Also, for this feature, the reports are controversial, with some studies reporting an association with a reduced embryo quality [79, 103], and other studies showing no negative correlation [60, 61, 72, 87]. Overall, ooplasm darkness, too, shall be considered a feature with a very limited predictive value.

Cytoplasmic viscosity and refractile bodies

In a limited number of studies, other cytoplasmic changes, such as cytoplasmic viscosity and refractile bodies, were explored. High cytoplasmic viscosity in the oocytes adversely affected fertilization, embryo quality, and blastocyst formation rates [104]. Also, the presence of refractile bodies derived from lipofuscin inclusions correlated with the decreased fertilization rate and defective blastocyst formation [105]. However, other researchers reported that refractile bodies and cytoplasmic viscosity in oocytes do not impact on embryological (fertilization and embryo quality) and clinical (implantation and pregnancy) outcomes [60, 72].

Smooth endoplasmic reticulum aggregates

SER aggregates (SER-a) look like fat disks in the oocyte cytoplasm corresponding to large tubular SER clusters surrounded by mitochondria. SER is a structure dedicated to calcium storage and release, necessary for oocyte activation at fertilization. Moreover, it plays a crucial role in energy accumulation, synthesis of proteins, lipids, and nuclear membrane throughout early embryo development [106]. SER-a were associated with lower oocyte maturation and embryo quality; lower fertilization, implantation, and pregnancy rates; and increased miscarriage rates when compared with control oocytes [84, 106–108]. SER-a presence has been associated with cytoskeleton alterations including increased spindle length and cortical actin disorganization [109, 110]. The potential association between SER-a and certain imprinting disorders [106, 111–113] led embryologists to discard these oocytes for several years, as initially suggested also by the main international scientific societies in the field of ART. However, more recent evidence suggests that embryos derived from oocytes with SER-a undergo a normal development, resulting in healthy newborns [114]. Considering all these controversial data and the lack of a real link between oocytes with SER-a and a higher prevalence of aneuploidies and/or malformations, discarding them cannot be justified clinically. Indeed, they can be valuable especially for poor prognosis patients. It is clearly advisable to monitor the embryos and babies born deriving from oocytes with SER-a in their cytoplasm.

Cumulative effect of multiple abnormalities

Regarding the cumulative effect of multiple morphological features, Xia [115] showed that oocyte grading based on PB1 morphology, size of PVS, and cytoplasmic inclusions was correlated with its developmental potential after ICSI. In the study of Chamayou et al. [46], the cumulative effect of morphological features, including cytoplasmic texture, inclusions, vacuoles, refractile bodies, and central granulation, was found related to impaired embryo quality but did not influence pregnancy rates. A completely different conclusion has been obtained by Serhal and co-authors [95], who found that similar features did not influence *in vitro* developmental parameters, but implantation and pregnancy rates were lower when embryos were derived from oocytes with cytoplasmic abnormalities.

Conclusions

Full oocyte maturation requires nuclear and cytoplasmic changes that must be completed in a timely manner to ensure optimal cellular conditions. An altered nuclear or cytoplasmic maturation or their asynchrony may compromise oocyte quality, resulting in various oocyte dysmorphisms. Nevertheless, most of the oocytes retrieved after oocyte stimulation exhibit one or more of these abnormal morphologic characteristics. To date, all these MII oocytes morphological parameters resulted in a very limited predictive value because of contradicting results, therefore their clinical use to foresee oocyte competence cannot be supported. The only morphological anomaly with clear clinical consequences is oocyte size (giant oocytes); all the remaining abnormalities are ascribable to phenotypic variance. Assessing chromosomal abnormalities at the blastocyst stage remains the most powerful and effective approach to predict embryo competence. Further relevant information could be obtained from blastocyst morphological assessment and perhaps "-omics technologies" in the future, while the predictive power of oocyte morphology with respect to embryo competence remains very limited.

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10

PREPARATION AND EVALUATION OF OOCYTES FOR INTRACYTOPLASMIC SPERM INJECTION

Irit Granot and Nava Dekel

Introduction

Resumption of meiosis in the oocyte is an essential prelude to successful fertilization. The meiotic division of the mammalian oocyte is initiated during fetal life, proceeds up to the diplotene stage of the first prophase, and arrests at birth. Meiotic arrest persists throughout childhood until the onset of puberty. In a sexually mature female, at each cycle one or more oocytes, according to the species, re-initiate the meiotic division. The chromatin in the meiotically arrested oocytes is encapsulated by a nuclear structure known as the germinal vesicle (GV; Figure 10.1a). The GV in oocytes resuming meiosis disappears (Figure 10.1b), the condensed chromosomes align on the newly formed meiotic spindle, and the pairs of homologous chromosomes segregate between the oocyte and the first polar body (Figure 10.1c). Emission of the first polar body, which represents the completion of the first round of meiotic division, is immediately followed by the formation of the second meiotic spindle with the remaining set of homologous chromosomes aligned on its equatorial plate. The whole series of events, initiated by GV breakdown (GVB) and completed at the metaphase of the second round of meiosis (MII), leads to the production of a mature fertilizable oocyte, also known as an egg. The egg is arrested at MII and will complete the meiotic division only after the penetration of the spermatozoon [1]. The physiological stimulus for re-initiation of meiosis is provided by the pre-ovulatory surge of luteinizing hormone (LH) [2]. Once oocyte maturation is completed, LH further induces ovulation, during which the follicle releases the mature oocyte that is picked up by the infundibular fimbria of the oviduct.

The egg released from the ovarian follicle is accompanied by the cumulus cells. Prior to ovulation, in concomitance with oocyte maturation, the cumulus undergoes characteristic transformations that are also stimulated by LH. In response to this gonadotropin, the cumulus cells produce specific glycosaminoglycans, the secretion of which results in cumulus mucification and its expansion. The major component of the extracellular matrix secreted by the cumulus cells is hyaluronic acid [3–7]. The mucified cumulus mass that encapsulates the ovulated egg is penetrated by the spermatozoon that uses enzymes localized on its surface membrane to accomplish this mission. Sperm membrane protein PH-20, which is present on the plasma membrane of sperm cells of many species, such as guinea pigs, mice, macaques, and humans, exhibits hyaluronidase-like activity that facilitates this action [8–11]. Furthermore, a later study has demonstrated that a plasma membrane-associated hyaluronidase is localized at the posterior acrosomal region of equine sperm [12].

Having traversed the cumulus, the spermatozoon undergoes acrosome reaction and binds to the zona pellucida. Sperm–zona pellucida binding is mediated by specific sperm surface receptors. The primary ligand on the zona pellucida, ZP3, specifically binds to the plasma membrane of the acrosomal cap of the intact sperm. The secondary zona ligand, ZP2, binds to the inner acrosomal membrane of the spermatozoon [13–15]. One of the inner acrosomal membrane sperm receptors was identified as acrosin [16–18]. In order to penetrate the zona pellucida, the spermatozoon utilizes enzymatic as well as mechanical mechanisms. Specific enzymes that are released by the acrosome-reacted spermatozoon allow the invasion of the zona pellucida by local degradation of its components [19, 20]. This enzymatic action is assisted by mechanical force generated by vigorous tail beatings that facilitate the penetration of the sharp sperm head [18–22].

Having penetrated the zona pellucida, the sperm crosses the perivitelline space and its head attaches to the egg's plasma membrane (oolemma). The key molecules that are crucial for the direct sperm–oolemma interaction in mammalian fertilization have been identified. The essential player on the sperms' side is known as the protein IZUMO1 and its prime binding partner on the oolemma is a folate receptor 4 named Juno [23].

Sperm head attachment to the oolemma is followed by its incorporation into the egg cytoplasm (ooplasm). Sperm incorporation is initiated by phagocytosis of the anterior region of its head followed by fusion of the head's posterior region along with the tail with the egg membrane [24–26]. The scientific efforts that have been invested by reproductive biologists in studying the process of gametogenesis and fertilization in animal models laid the groundwork for the design of *in vitro* procedures for assisted reproduction. These procedures that are successfully practiced at present in human patients essentially attempted to mimic the biological processes *in vivo*.

In vitro fertilization (IVF) regimens of treatment, which are continuously being improved, have allowed the birth of more than eight million babies all over the world, since the first IVF birth in 1978 of Louise Brown. One such improvement, which represents a major breakthrough in this area, is intracytoplasmic sperm injection (ICSI). Until 1992, most infertility failures originating from a severe male factor were untreatable. Micromanipulation techniques such as partial zona dissection [27–30] and subzonal sperm injection [29, 31–35, 48], designed to overcome the poor performance of sperm cells, did not result in a substantial improvement of the rate of success of *in vivo* fertilization. However, ICSI, which was established by the team led by Professor Van Steirteghen at the Free University in Brussels, Belgium, and initially reported by Palermo et al. [35], has generated dramatic progress [36–39]. The ICSI procedure involves the injection of a single sperm cell intracytoplasmically into an egg. Fertility failures associated with an extremely low sperm count were found to be successfully treated by this technique. Furthermore, as the sperm is microinjected into the ooplasm, it bypasses the passage through the zona pellucida and is not required to interact with the oolemma. Therefore, infertility problems that originate from faulty sperm–egg interaction may also be resolved by this IVF protocol of treatment.

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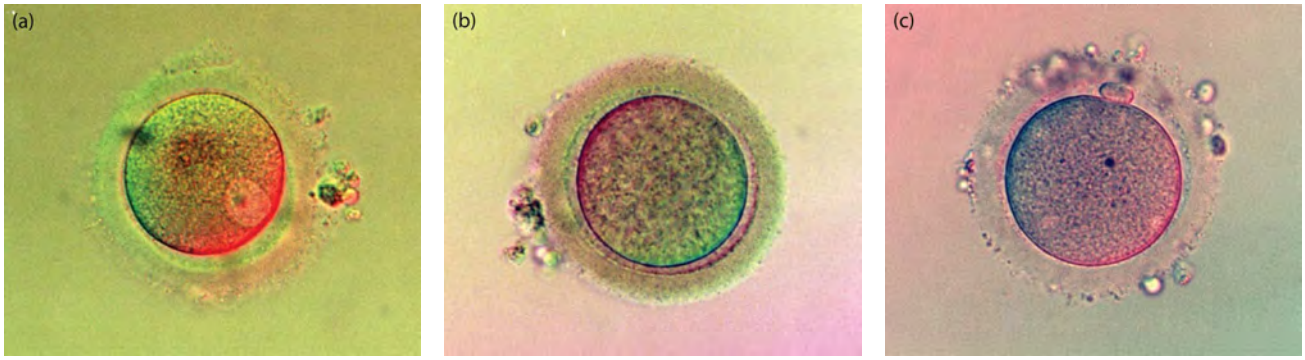

FIGURE 10.1 Morphological markers characterizing the meiotic status of oocytes. (a) Immature germinal vesicle (GV) oocyte: meiosis has not been re-initiated and the typical nuclear structure is visible. (b) Immature GV breakdown oocyte (metaphase I [MI]): meiosis has been re-initiated, the GV has disappeared, but the first polar body is still absent. (c) Mature oocyte (MII): the GV has disappeared and the first polar body has been extruded.

Handling of oocytes

Similar to conventional IVF, patients for ICSI undergo programmed induction of superovulation followed by scheduled oocyte retrieval (Chapter 9). Under all protocols of treatment, identification of the cumulus–oocyte complexes and evaluation of their maturity are carried out immediately after follicle aspiration, as described in Chapter 9. However, unlike conventional IVF, in which intact mature cumulus–oocyte complexes are inseminated, cumulus cells that surround the eggs are removed before microinjection.

Denudation of the mature oocytes is an essential prerequisite for ICSI. Cumulus cells may block the injecting needle, thus interfering with oocyte microinjection. Furthermore, in the presence of the cumulus, visualization of the egg is very limited. Since only mature oocytes that have reached MII are suitable for ICSI, optimal optical conditions that allow the accurate assessment of the meiotic status of the oocytes are required. Oocyte maturation is determined morphologically by the absence of the GV and the presence of the first polar body. Good optical conditions are also necessary for the positioning of the mature oocyte in the right orientation for injection (Chapter 13). Preparation of the retrieved mature oocytes for ICSI should be carried out under conditions of constant pH of 7.3 and a stable temperature of 37°C. In order to maintain the appropriate pH, 4-(2-hydroxyet4hyl)-1-piperazineethane sulfonic acid (HEPES)-buffered, or 3-(*N*-morpholino) propanesulfonic acid) (MOPS)-buffered culture media are used. The correct temperature is maintained during egg handling by the use of a microscope equipped with a heated stage. Most of the procedures are performed under paraffin/mineral oil that prevents evaporation of the medium and minimizes the fluctuations of both the pH and the temperature.

Temperature fluctuations that are likely to accompany the handling of eggs have been shown to be specifically detrimental for the microtubular system. Changes in spindle organization were observed in human mature oocytes cooled to room temperature for only 10 minutes. These changes included a reduction in spindle size, disorganization of microtubules within the spindle, and, in some cases, even a complete absence of microtubules [40, 41]. The susceptibility of the microtubules to temperature variations has been also shown in mature mouse oocytes [42]. Interference with spindle organization can disturb the faithful segregation of the chromosomes, resulting in aneuploidy.

Laboratory procedures

Removal of the surrounding cumulus cells is accomplished by combined enzymatic and mechanical treatment carried out under a stereoscopic dissecting microscope. Different studies have tried to determine the pre-incubation time that is needed between egg retrieval and egg denudation and ICSI performance, however, no conclusive results were obtained.

A pre-incubation period of at least three hours between oocyte retrieval and removal of the cumulus cells to allow immature oocyte to resume meiosis *in vitro* was recommended by one study [43]. This recommendation was challenged by other studies which did not demonstrate differences in ICSI outcomes that correlated with the time interval between egg aspiration and microinjection [44–46]. On the other hand, pre-incubation time that exceeded nine hours resulted in embryos of lower quality [44]. This maximal time window was also challenged by a later study that showed no effect of OPU–ICSI time between 1 and 17 hours on fertilization rate, embryo quality, as well as pregnancy and live birth rates [46]. Garor et al. [47] demonstrated that it is not the time of oocyte denudation and injection that determines the ICSI outcome but, in fact, the time interval between human chorionic gonadotropin (hCG) administration and oocyte pickup (OPU). Specifically, fertilization along with pregnancy rates were significantly higher in IVF cycles in which the hCG–OPU interval exceeded 36 hours, regardless of the time of oocyte handling [47]. Since oocyte denudation cannot be carried out before some preliminary laboratory preparations that are described next are completed, a pre-incubation period of about 30 minutes is unavoidable. During this period, the retrieved mature cumulus–oocyte complexes are kept in the incubator at 37°C with 5%–6% CO_2 according to the recommendations of the culture media manufacturer.

Preliminary preparations for oocyte denudation

Injecting dish

A special shallow Falcon dish (type 1006) is used for placing the denuded eggs. Nine small droplets of MOPS (3-(*N*-morpholino) propanesulfonic acid)-HEPES-buffered human tubal fluid culture

media (MHM; Irvine Scientific, CA, USA), containing 10% synthetic serum, 5–10 µL each, are arranged in a square of 3 × 3 within this dish. Two droplets of 5 µL of 10% polyvinylpyrrolidone (PVP), in which sperm will be placed, are added above the MHM droplets. This viscos solution slows sperm motility, facilitating sperm collection and control of the injecting pipette. It is recommended that one of the PVP droplets be elongated and flattened to ease sperm capture and visualization under the microscope. All droplets are then covered with either paraffin or mineral oil, and the dish is placed on the heated area in the hood to warm up before removal of the cumulus cells.

In cases of low sperm count and/or motility, sperm is placed in MHM droplets to allow better motility. In cases of very low sperm motility, the use of SpermMobile (GM501; Hamilton Thorne) is recommended. This "caffeine-like" solution increases cAMP levels in the sperm cells, stimulating their motility. This solution is added into one or more of the MHM droplets, to a final dilution of 1:20, according to the manufacturer's protocol.

Enzymatic solution

Since hyaluronic acid is a major component of the mucified cumulus mass that surrounds the mature oocyte, hyaluronidase is employed for enzymatic removal of these cells (80 IU/mL; Sage In-Vitro Fertilization, Inc., Trumbull, CT). The high concentration of 760 IU/mL of hyaluronidase that was used initially [49] was found to induce parthenogenetic activation of the mature oocytes. A lower concentration of the enzyme, such as 80 IU/mL, which is commonly used, significantly decreased the rate of parthenogenesis [50]. According to our experience, hyaluronidase at a concentration of 60 IU/mL effectively denudes the oocytes. Further reduction of the enzyme concentration to as low as 10 IU/mL was also found to be sufficient [51].

Denuding dish

Two drops of ~100 µL of hyaluronidase solution and five drops of ~100 µL of MHM containing 10% serum covered with oil are placed in a 60-mm culture dish and covered with oil. The dish is then placed on the heated area in the hood to warm up.

Removal of the cumulus cells

Cumulus–oocyte complexes are transferred into the drop of hyaluronidase solution and repeatedly aspirated through a Pasteur pipette for up to 30–40 seconds. At this time, dissociation of the cells is initially observed. Further mechanical denudation is carried out in the enzyme-free MHM drops by repeated aspiration through commercially prepared stripper tips with decreasing inner diameters of 275, 170, and 140 µM and, when necessary, 135 µM. The oocytes are transferred through the drops of medium, until all coronal cells have been finally removed and all traces of enzyme have been washed off. This procedure is carried out very gently in order to avoid mechanical damage to the oocytes. Pricking of the oocyte has been shown to induce parthenogenetic activation [51, 52]. Finally, the denuded oocytes are placed in the droplets of the injecting dish and their morphology and meiotic status are evaluated. These procedures are performed on the heated area in the hood.

In order to maintain a temperature of 37°C during preparations and egg handling, the heated working area in the hood and microscope stage must be calibrated to a higher temperature (around 38°C).

In cases of extremely low sperm count or testicular sperm injection, oocytes must be kept in the incubator in CO_2-equilibrated culture medium until a sufficient number of sperm cells have been collected.

Evaluation of denuded oocytes for ICSI

Oocytes are assessed for their maturation and for their morphology under an inverted microscope equipped with Nomarski differential interference contrast optics at 200× magnification. Only mature oocytes that resume their first meiotic division, reaching MII, are appropriate for ICSI. Evaluation of the meiotic status of the oocyte is based on morphological markers. In mature oocytes, the GV has disappeared and the first polar body is present and localized in the perivitelline space (Figure 10.1c).

Different studies have reported that in patients who undergo controlled ovarian stimulation (COS) 10%–20% of the retrieved oocytes have not resumed their meiotic division [53–57]. These oocytes can be divided into two categories: GV oocytes in which meiosis has not been re-initiated and the typical nuclear structure is visible (Figure 10.1a) and GVB oocytes in which meiosis has been re-initiated but did not proceed beyond the first metaphase (MI). In these oocytes, the GV has disappeared but the first polar body has not been extruded (Figure 10.1b). Oocytes in both of these categories are separated from the MII oocytes. MI oocytes are further incubated and those that extrude the first polar body within two to four hours are inseminated by ICSI [57, 58]. It has been reported that 74% of the MI oocytes completed meiosis *in vitro* within 20 hours after retrieval. This report did not find differences in the rates of fertilization and embryo development between these oocytes and other oocytes retrieved at MII. However, only sporadic pregnancies were achieved following the transfer of embryos obtained from fertilized MI oocytes that had matured *in vitro* [58, 59]. Another study demonstrated that 26.7% of MI oocytes extruded the first polar body *in vitro* within four hours. These oocytes were injected on the same day of follicle aspiration in parallel to the oocytes retrieved at MII. In this study, however, the MI oocytes that completed their maturation *in vitro* exhibited a lower fertilization rate, with no differences in embryo quality between oocytes that underwent maturation in vitro and those retrieved at MII. Similar to the previous study, only sporadic pregnancies were obtained following transfer of embryos developed from MI oocytes that had matured *in vitro* [60, 61]. Other studies support these observations, showing that although *in vitro*-matured (IVM) MI oocytes can be normally fertilized, the embryos derived from these oocytes rarely provide pregnancies [62, 63]. This is compatible with the findings that these embryos exhibit low morphological quality and a high rate of chromosomal abnormalities [56], along with the recent demonstration that blastulation rate is directly associated with oocyte maturation [57].

In patients with few MII oocytes, rescue of MI oocytes may increase the number of embryos for transfer; however, the chance of improving pregnancy rates by this procedure is minimal. Oocytes with GV require an overnight (30-hour) incubation in order to reach the MII stage. Only very sporadic pregnancies were reported from oocytes that were at the GV stage when retrieved during standard IVF treatment with COS [61, 62, 64]. Because of the very poor results, these GV oocytes are usually discarded. It has been demonstrated that GV oocytes that are retrieved after COS exhibit damaged DNA [65]. Therefore, only in cases in which very few or no MII oocytes are retrieved are the GV oocytes rescued for fertilization, provided they complete their maturation.

Incubation of the GV oocytes for 24 hours after OPU, to allow their spontaneous maturation, indeed resulted in a fertilization

rate of 64%, however, the embryos developed from these oocytes were of a very poor quality and usually aneuploid [66].

Different COS protocols may result in follicular asynchrony and variations in oocyte number and quality [67]. Furthermore, during aspiration, oocytes are collected from heterogeneous groups of follicles, leading to aspiration of mature MII oocytes or meiotically immature MI or GV oocytes [68]. As shown in Figure 10.3a, using a modified polarized light microscope, the "PolScope," it is possible to detect oocytes with PB in which the second meiotic spindle has not yet been formed and the first meiotic spindle (mid-body) is still observed. This indicates that the presence of a PB does not necessarily represent a fully mature fertilizable egg. A high proportion of immature oocytes during OPU, may reflect lower quality of the retrieved MII oocytes. Such MII oocytes result in a lower success rate as compared to MII oocytes in cycles with no GV/MI oocytes [57, 65].

Immature GV oocytes can also be retrieved from the small (3–13 mm) ovarian follicles present in unstimulated patients [69–72]. Although these oocytes were not exposed to LH *in vivo*, they are apparently meiotically competent and can be expected to mature spontaneously *in vitro* and produce normal eggs. In 1998, Goud et al. showed a fertilization rate of 46% by ICSI of such IVM GV oocytes [72] resulting in a few pregnancies. However, as more experience is gained in handling immature oocytes, success rates are increasing worldwide [73, 74]. Later studies demonstrated that hCG administration before oocyte retrieval from the small follicles accelerates their *in vitro* maturation, resulting in better embryonic development and leading to higher pregnancy rates. It was further demonstrated that administration of low doses of follicle-stimulating hormone (FSH) before hCG priming enables the retrieval of *in vivo*-matured oocytes (MII) from the small follicles (<10 mm). Such oocytes have a higher potential to develop into good-quality embryos than IVM oocytes, achieving even higher pregnancy rates [75].

Nowadays, IVM is characterized by minimal administration of FSH or human menopausal gonadotropin (hMG) prior to oocyte retrieval, without triggering of ovulation by hCG. Using new IVM culture systems the efficacy of IVM has significantly improved, reaching a live birth rate of 40% in different clinics worldwide (reviewed by Voung et al. [76]). Advantages of IVM over *in vitro* fertilization (IVF) include mild or no hyperstimulation, lower medication costs, and less patient burden. It is clinically beneficial for patients who suffer previously from ovarian hyperstimulation syndrome and patients who must undergo immediate chemotherapy and need fertility preservation.

In addition to the meiotic status, the morphology of the oocytes is also evaluated before ICSI. The various morphological defects may be manifested by an amorphic shape of the oocyte, enlargement of or granularity in the perivitelline space, inclusions, vacuolization, granularity, and dark colour of the cytoplasm, changes in the colour and construction of the zona pellucida, and changes in the shape and size of the polar body (Figure 10.2). Most defective oocytes exhibit more than one of the aforementioned abnormalities. All these observations should be recorded and may help in later analysis of the fertilization rate, embryo development, and pregnancy outcomes after ICSI. The correlations between egg morphology and the rates of fertilization, embryo quality, and pregnancy after ICSI have been extensively studied. Most of the studies reported that abnormal egg morphologies of patients undergoing ICSI are associated with a lower rate of fertilization,

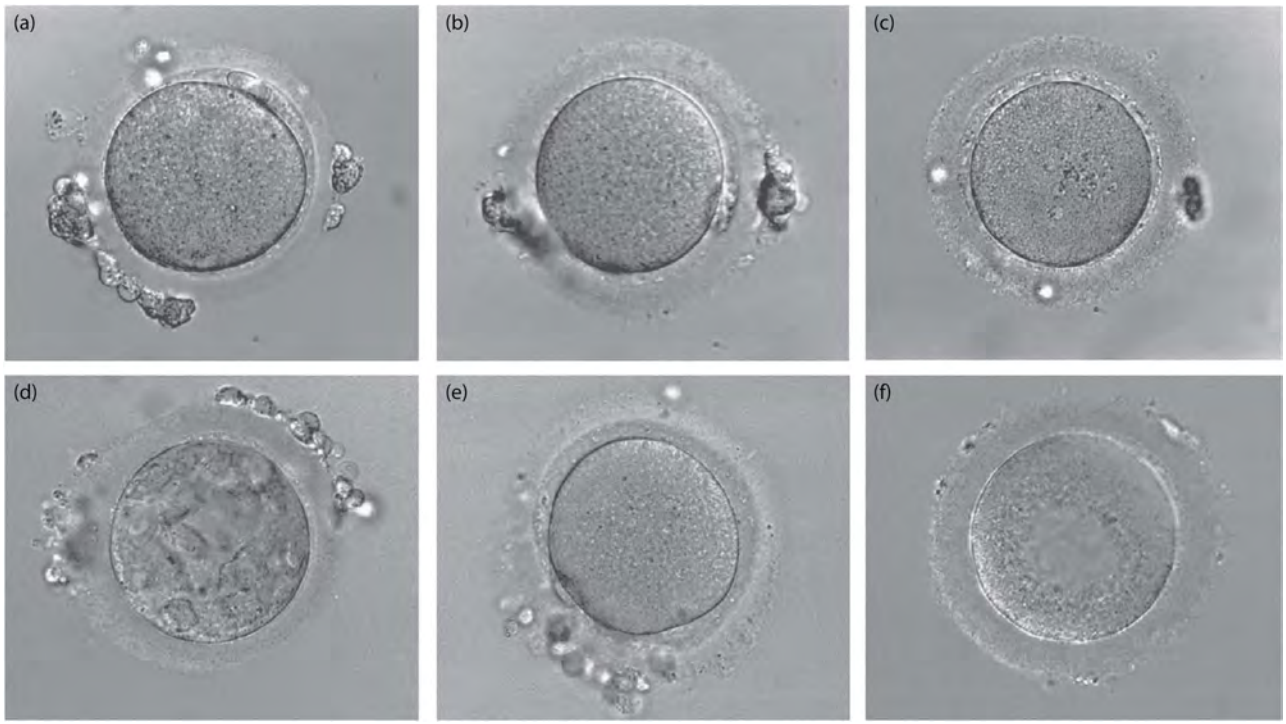

FIGURE 10.2 Various morphological abnormalities exhibited by oocytes. (a) Granulated perivitelline space; (b) a fragmented polar body; (c) thickened and dark-coloured zona pellucida; (d) cytoplasmic inclusions; (e) enlarged and granulated perivitelline space; and (f) a large cytoplasmic vacuole.

embryos of poor quality, and, consequently, lower pregnancy rates [77–79]. Other studies demonstrated successful fertilization and normal early embryo development in microinjected eggs with defective morphologies, such as large perivitelline space, cytoplasmic vacuoles, or a fragmented polar body [80–83]. However, the transfer of these seemingly normal embryos resulted in a poor implantation rate [79] and a high incidence of early pregnancy loss [81]. The use of a triple marker model of polar body shape, size of the perivitelline space, and cytoplasmic inclusions has been suggested by Xia for human oocyte grading [78]. This laboratory reported that the evaluation of oocyte quality based on these criteria correlated well with the rate of fertilization and with embryo quality after ICSI.

As mentioned previously in this chapter, the integrity of the meiotic spindle in MII oocytes is crucial for fertilization capacity and embryo development. Therefore, in addition to the aforementioned features of the oocyte, the morphology of the spindle may serve as a reliable marker for predicting its potential for normal fertilization and embryonic development [84]. The "PolScope" mentioned earlier, which is a modification of the polarized light microscope, equipped with novel image-processing software [85], has emerged as a non-invasive tool to view the meiotic spindle in living oocytes. The image of the spindle is based on the highly birefringent characteristic of the microtubule filaments under a polarization microscope. Meiotic spindle in the cytoplasm of living oocytes cannot be visualized with the standard light microscope that is routinely used in IVF laboratories. Due to this obvious advantage, the use of PolScope has been implemented in several IVF units worldwide [41, 84, 86–88]. Examination of human oocytes by PolScope has indeed demonstrated that the absence or abnormal morphology of the spindle is highly correlated with lower fertilization rates and impaired embryonic development [86, 87, 89]. Furthermore, spindle assessment with the PolScope has been shown to facilitate the selection of embryos with high implantation potential for transfer [84]. It was demonstrated that a spindle can be detected in 75% of the oocytes, and its detection directly correlates with higher chances for achieving pregnancy. Furthermore, the number of spindle-positive oocytes decreases with increasing age of patients [88]. A recent study demonstrated that the size of the spindle can be measured by the PolScope, demonstrating that 90–120 μm^2 is the optimal size that predicts higher fertilization, blastocyst formation, and clinical pregnancy rates [90].

The use of the PolScope has also indicated that polar body position does not always accurately predict spindle location. In MII oocytes, the second meiotic spindle is expected to be adjacent to the first polar body (Figure 10.3b), making the first polar body a marker for appropriate orientation of the ICSI micropipette to avoid interference with chromosome alignment. However, observations by Silva et al. [86] and ourselves that the meiotic spindle is not always adjacent to the polar body (Figure 10.3c) have made use of the PolScope even more valuable. In the study of Konc et al. [88], of 320 tested oocytes, 66% exhibited a spindle that was positioned at a 45° angle from the PB. Furthermore, as mentioned earlier, in those oocytes that have not yet completed the formation of the first polar body, the PolScope can detect the presence of microtubules in the mid-body, suggesting that the second meiotic spindle has not yet been fully organized (Figure 10.3a), and ICSI should be postponed.

Appropriate ovarian stimulation protocols normally provide functional, fertilizable mature oocytes, while oocytes of poor quality may represent a disturbed hormonal balance. For example, exposure to high dosage of hMG has been shown to be associated with granularity of the perivitelline space [55]. Moreover, an extended exposure to high doses of this hormone may lead to the senescence of the mature oocyte before retrieval. As previously mentioned, oocyte maturation and ovulation are both stimulated by LH. However, studies have shown that the ovulatory response is less sensitive to this gonadotropin, requiring higher concentrations of the hormone [91]. Therefore, the relatively high concentration of LH in hMG effectively promotes oocyte maturation, but is insufficient to stimulate ovulation. Delayed administration of hCG in these patients entraps the mature oocytes in the follicle, leading to oocyte aging. One notable morphological marker in this case is the fragmentation of the first polar body [92]. The presence of aged oocytes can also explain the decreased quality of oocytes and lower fertilization rate in polycystic ovarian syndrome patients [93] who exhibit relatively high serum concentrations of LH throughout their menstrual cycle [94]. Nowadays, pure FSH preparations (recombinant FSH) are widely used for the stimulation of follicular growth and development. However, it has been demonstrated that introducing low concentrations of

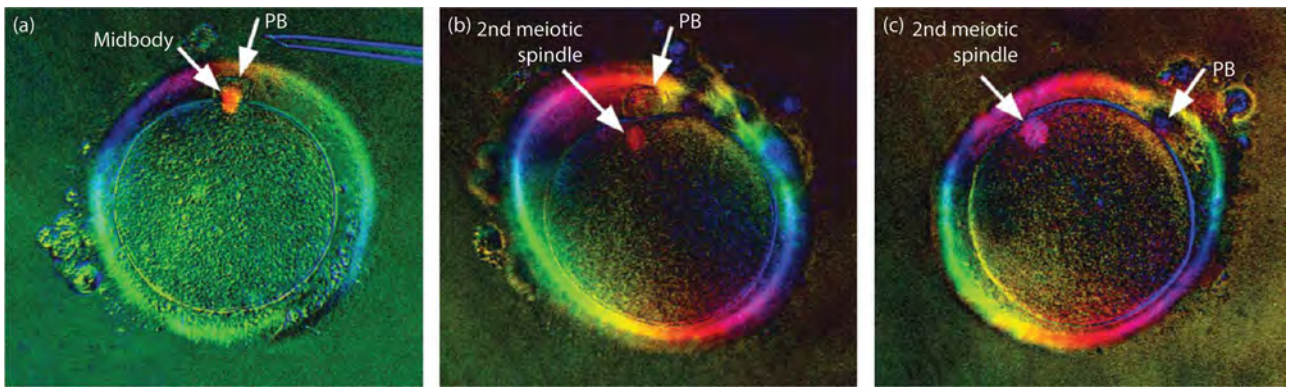

FIGURE 10.3 Microtubule images in metaphase II (MII) human oocytes. (a) Microtubules of the mid-body extending from the cytoplasm into the first polar body (PB). (b) Microtubules of the second meiotic spindle located adjacent to the PB. (c) Microtubules of the second meiotic spindle at a distal location from the PB.

LH (recombinant LH) in addition to FSH significantly improves IVF outcomes [95].

The accumulated experience worldwide indicates that the response to hormonal stimulation varies substantially among women and these individual variations are dependent not only on the stimulation protocol but also on the genetic background. It has been shown that the expression of different genes in granulosa cells, such as ADAMTS1 and HSPG2, is directly associated with oocyte quality and IVF outcome [96, 97]. These genes may serve as genetic markers for predicting ovulation response, facilitating the optimization of the stimulation regiment for each patient individually.

Epilogue

A baby girl is born with her ovaries containing about two million oocytes, all of which arrested at the prophase of the first meiotic division. This pool of oocytes remains dormant throughout childhood until the onset of puberty. In sexually mature females, at each cycle, one such "sleeping beauty" is kissed by the LH "prince" and awakened to continue its meiotic division. Once maturation has been completed, the oocyte is released from the ovarian follicle into the fallopian tube, a site at which it will eventually meet the spermatozoon and undergo fertilization. Hormonal stimulation protocols are designed to mimic the natural events that lead to the production of mature oocytes. In IVF patients, these oocytes are aspirated from the ovarian follicles prior to ovulation and allowed to meet the sperm cells in the Petri dish. A higher scale of assistance, designed to overcome the poor performance of spermatozoa, is offered by ICSI. The information regarding oocyte handling for this later modification of the classical IVF protocol has been summarized in this chapter.

APPENDIX

Laboratory protocol

The following protocol is used in our laboratory.

- Preliminary preparations for oocyte denudation
  1. Injecting dish: the droplets may be placed on the dish in any arrangement the laboratory prefers. Our laboratory recommends the following layout. Place nine droplets, 5 µL each, of MHM containing 10% serum, arranged in a 3 × 3 square within a shallow Falcon dish (type 1006). Place two additional droplets of 5 µL of 7%–10% PVP solution where the sperm will be placed. One of the PVP droplets will be elongated and flattened. Cover with oil. Place the dish on the heated area in the hood to warm up. In cases of extremely low sperm counts and/or motility, MHM as well as more than one PVP droplet can be used for sperm. In cases of very low sperm motility, add SpermMobile solution (GM501, Hamilton Thorne) into one or more of the MHM droplets, to a final dilution of 1:20, according to the manufacturer's protocol.
  2. Denuding dish: place a drop of 100 µL of hyaluronidase solution 80 IU/mL (Sage) and five 100 µL drops of MHM containing 10% serum in an embryo-tested 60-mm culture dish. Cover with oil and place on the heated area in the hood to warm up.
  3. Stripper tips: Prepare stripper tips with inner diameters of 275, 170, and 140 µM.
- Removal of the cumulus cells
  1. Place the cumulus–oocyte complexes into the drop of the hyaluronidase solution (up to five complexes at a time) and aspirate repeatedly through a Pasteur pipette for up to 40 seconds.
  2. Transfer the cumulus–oocyte complexes to a drop of MHM containing 10% serum and aspirate repeatedly through a 275 µM diameter stripper tip. Continue aspirating with 170 and 140 µM tip while passing the oocytes through the other four drops of the medium, until all coronal cells have been totally removed.
  3. Transfer the denuded oocytes to the MHM droplets in the injecting dish. It is recommended to place no more than one oocyte in each droplet.
- Microscopic evaluation
  1. Place the injecting dish containing the oocytes on the heated stage of an inverted microscope equipped with differential interference contrast.
  2. Evaluate oocyte morphology and meiotic status at 200× magnification.

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11

USE OF IN VITRO MATURATION OF OOCYTES IN A CLINICAL SETTING

What Is Its Role in ART?

Tuong M. Ho and Lan N. Vuong

Introduction

In vitro maturation (IVM) of oocytes is an assisted reproductive technology (ART) that involves collection of immature cumulus–oocyte complexes (COCs) at the prophase I stage, followed by maturation to metaphase II (MII) stage *in vitro* [1–4]. In contrast to *in vitro* fertilization (IVF), the typical IVM treatment cycle is characterized by minimal administration of follicle-stimulating hormone (FSH) or human menopausal gonadotropin (hMG) prior to oocyte retrieval, and no triggering of ovulation using human chorionic gonadotropin (hCG) [5, 6]. This chapter highlights the technical aspects of IVM and its current practice in the ART field, and includes a discussion about barriers to the use of IVM and approaches to overcome these obstacles.

History and development of human IVM

Human IVF and different ARTs have been developed over the last 40 years and have helped several millions of infertile couples to overcome childlessness. In fact, initial attempts to use IVM for human oocytes started early on to provide human oocytes for human IVF experiments because it was impossible to collect *in vivo* matured human oocytes at that time [1, 7, 8]. Edwards et al. achieved their landmark work in human IVF with human IVM oocytes [9]. Therefore, human IVF was actually developed based on the very early success of harvesting and IVM of human oocytes [9].

The first human live birth resulting from IVF was produced by natural cycle IVF, in which mature human oocytes were collected from pre-ovulatory follicles [10]. Subsequently, different ovarian stimulation protocols have been applied to increase the number of oocytes retrieved to improve the number embryos achieved and the chance of successful pregnancy per treated IVF cycle [11–14]. However, use of different ovarian stimulation protocols makes IVF more complicated and more expensive.

Despite its initial role in IVF research, there was almost no further development of IVM until the first baby conceived after IVM of immature oocytes derived from oocyte donors was reported by Cha and colleagues in 1991 [4]. Since then, the number of publications relating to IVM has increased, with the investigation of different protocols and a growing body of data reported (Table 11.1). Thus, IVM started to attract more interest from the ART community.

In 1994, Trounson et al. reported a case series describing babies born from immature oocytes collected from women with PCOS [15]. The trend to using a protocol for IVM in which different types of gonadotropins were administered before immature oocytes were recovered from small follicles started by the end of 1990s [16–18]. During the last two decades, IVM has been utilized for patients with PCOS to reduce the health risks associated with ovarian stimulation, for fertility preservation, or just as an alternative, more user-friendly approach to ART [19]. Recently, human IVM has been considered as a potential treatment for fertility preservation in women with cancer for whom ovarian stimulation is contraindicated or when the patient does not have enough time for ovarian stimulation due to the urgency of cancer treatment [20, 21]. The European Society of Human Reproduction and Embryology (ESHRE) Guideline on Female Fertility Preservation published in 2020 suggests IVM as a fertility preservation technique [22]. Over the last five years, a new biphasic IVM culture system has been developed to improve the efficacy of IVM. Recent data from this new IVM culture system, called capacitation (CAPA) IVM, shows promising results [23, 24].

In 2021, the Practice Committees of the American Society for Reproductive Medicine (ASRM), the Society of Reproductive Biologists and Technologists, and the Society for Assisted Reproductive Technology (SART) published a landmark document that presented an overview of published evidence supporting the conclusion that IVM should no longer be considered an experimental technique [3]. The potential for wider clinical application of IVM was suggested [3].

Advantages and disadvantages of IVM

IVM has several advantages over IVF. Firstly, IVM uses mild or no stimulation and therefore the risk of ovarian hyperstimulation syndrome (OHSS) in patients with polycystic ovaries (PCO) or PCOS is largely eliminated [2, 25, 26]. Another advantage is lower medication costs because the requirement for expensive gonadotropins is reduced [2, 27, 28]. Finally, IVM is more convenient for patients because of a much lower monitoring burden (frequent ultrasounds and blood tests are not required), reduced stress, and fewer patient observations [2, 27, 28]. Despite these advantages, IVM was not widely adopted by the profession because clinical outcomes after IVM were initially suboptimal, with live birth rates per cycle of <20% [29–31]. However, more recent studies in experienced centres have reported improved live birth rates of approximately 35%–40% [32–36]. Nevertheless, the chances of a live birth with IVM are currently still slightly lower than with IVF [35, 36].

Safety of IVM

One of the concerns regarding IVM is the health of babies after utilization of this procedure. Therefore, several studies have investigated the effects of IVM on embryos and children conceived using IVM. Embryos generated from IVM did not show an increase in imprinting [37]. In another study, IVM blastocysts showed similar

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TABLE 11.1 Milestones in IVM Development

Year	Development
1969	First experimental IVF with in vitro matured oocytes [9]
1991	First IVM baby resulting from immature oocytes derived from oocyte donors [4]
1994	First babies from IVM oocytes obtained from women with PCOS [15]
1999	First babies from IVM cycles with hCG triggering before oocyte retrieval [16]
2014	First live birth from IVM oocytes after oophorectomy in a patient with ovarian cancer [21]
2020	First report of birth after vitrification of IVM oocytes in a woman with breast cancer [20]
2020	First live births after biphasic IVM in women with PCOS [24]
2020	ESHRE Guideline on Female Fertility Preservation: IVM was regarded as a fertility preservation procedure [22]
2021	Practice Committees of the ASRM: IVM no longer considered an experimental technique; potential for wider clinical application of IVM suggested [3]

Abbreviations: ASRM, American Society of Reproductive Medicine; ESHRE, European Society of Human Reproduction and Embryology; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; PCOS, polycystic ovary syndrome.

rates of methylation and gene expression at germline differentially methylated regions (gDMRs) compared with IVF embryos, and expression of major epigenetic regulators was similar between the IVM and IVF groups [38]. Cellular morphology in IVM oocytes also does not appear to differ from oocytes matured *in vivo* according to transmission electron microscopy [39].

Initial data on the neonatal health and developmental outcome of children conceived using IVM showed no significant differences compared to children born after traditional IVF, with or without intracytoplasmic sperm injection (ICSI) [40–44]. However, the relatively small number of children conceived through IVM compared with IVF limits the accuracy of malformation and anomaly rates, and developmental outcomes cannot yet be adequately assessed.

Recently, a follow-up of babies born to women who participated in a randomized controlled trial comparing new IVM protocol with a pre-maturation step (CAPA IVM) and IVF showed that overall development up to 24 months of age was comparable in children born after CAPA IVM compared with IVF [45]. Another prospective cohort study also showed that the use of CAPA-IVM did not have any significant impact on childhood physical and mental development compared with children born as a result of natural conception, up to 15 months of age [46]. The updated data from the children follow-up studies assure the safety of the new CAPA-IVM technique.

Patient populations for application of IVM

Suggested indications for IVM include patients at risk of OHSS, those with limited time for ovarian stimulation, or where sustained elevations of estradiol are contraindicated [3]. Patient populations particularly suited to the use of IVM are described next.

PCO/PCOS

The fact that ovarian stimulation is not required means that IVM is especially suited to patients with PCOS. This group is at increased risk of exaggerated ovarian response, including OHSS, ovarian torsion, and thromboembolism associated with high estradiol levels [47–50]. In addition, because the reduced ability of immature oocytes retrieved from mid-antral follicles to resume meiosis and progress to the MII stage is a factor limiting the clinical efficiency of IVM [51], the higher antral follicle count (AFC) in women with PCOS [52, 53] makes them particularly suitable candidates for IVM. This is shown by higher IVM success rates in women with PCOS compared with normo-ovulatory women [54]. Recently, a large randomized controlled trial comparing IVM and IVF in women with a high AFC found that live birth after the first embryo transfer (ET) occurred in 96 women (35.2%) in the IVM group and 118 women (43.2%) in the IVF group (absolute risk difference −8.1%; 95% confidence interval [CI] −16.6%, 0.5%) [35]. Cumulative ongoing pregnancy rates at 12 months after randomization were 44.0% in the IVM group and 62.6% in the IVF group (absolute risk difference −18.7%; 95% CI −27.3%, −10.1%). OHSS did not occur in the IVM group, versus two cases in the IVF group. There were no statistically significant differences between the IVM and IVF groups with respect to the occurrence of pregnancy complications, obstetric and perinatal complications, preterm delivery, birth weight, and neonatal complications [35].

Normo-ovulatory patients

IVM can also be applied in normo-ovulatory women. Initially, clinical pregnancy rates in normo-ovulatory women were 4%–25% [17, 29, 31, 55], but these have improved to approximately 30% [56, 57]. For these women, reasons for choosing to undergo IVM are generally social, and may include financial considerations in countries where the cost of gonadotropins limits access to IVF, and the wish to avoid potential drug-related side effects and the psychological stress associated with IVF [49, 58]. IVM has also been cited as the ART of choice in countries where severe legal restrictions have reduced the success rate of standard IVF protocols [59].

AFC is an important consideration in the selection of suitable candidates for IVM among normo-ovulatory women [51, 58]. A minimum AFC of >5 has been suggested. In addition, it has been suggested that candidates for IVM should have a good prognosis in terms of achieving pregnancy using conventional ART, including the following patient characteristics: age ≤36 years; body mass index <30 kg/m^2; FSH levels <10 mIU/mL; estradiol levels <250 pmol/mL; and anti-Müllerian hormone levels >1.3 ng/mL [51, 58].

Fertility preservation

Given that IVM can be done within a relatively short timeframe without the need for ovarian stimulation, it is particularly useful for fertility preservation in patients with cancer who are unable to delay chemotherapy, or in women with breast cancer for whom exposure to elevated estradiol concentrations may accelerate their disease [60, 61]. While IVM is usually conducted during the follicular phase of the menstrual cycle, successful collection and maturation of oocytes has been demonstrated during the luteal phase in patients with cancer, thus making it a good option in those scheduled for imminent chemotherapy [60, 61].

Poor ovarian response

Some patients respond poorly to IVF, including those with low oestrogen levels, few or slow-growing follicles, or smaller follicles

(diameter <12 mm on day 15 of the treatment cycle) [62]. Poor responders to IVF do not appear to benefit from prolonged treatment or higher doses of gonadotropins, and fertilization and pregnancy outcomes may be negatively affected [62, 63]. Thus, alternative ARTs may be useful in this patient group. A case series reported positive outcomes after IVM in eight patients who were poor responders to IVF, with a clinical pregnancy rate of 37.5% [62].

Gonadotrophin-resistant ovary syndrome (GROS)

GROS is a rare endocrine syndrome characterized by hypergonadotropic anovulation (World Health Organization group 3) and consequent infertility. Women with GROS experience amenorrhea (primary or secondary) despite having an intact uterus and vagina, elevated levels of FSH and luteinizing hormone (LH), normal ovarian reserve, no concomitant autoimmune disease, and an inappropriate response to ovarian stimulation [64, 65]. The aetiology of GROS remains unclear. However, both genetics and immunology might play a role. Several mutations with loss of function and single nucleotide morphisms of the FSH receptor (FSHR) have been described to date [66–70]. The lack of response to ovarian stimulation in patients with GROS means that conventional IVF is not possible. Therefore, oocyte donation was previously considered the only option for ART in these patients. However, IVM is increasingly being recognized as a potential alternative [71–73], which would provide the opportunity for a woman with GROS to achieve fertilization of her own oocytes. Several live births after use of IVM in GROS have been reported [64, 72–76].

Rescue of oocytes that have failed to mature in stimulated cycles

Some patients undergoing IVF treatment yield high proportions of immature oocytes after conventional ovarian stimulation [77]. In these patients, IVM is a feasible rescue method for increasing embryo number [77]. A prospective study demonstrated the utility of rescue IVM, concluding that it should become routine practice in women with low functional ovarian reserve [78]. Rescue IVM produced an additional 1.5 embryos for transfer in women with low functional ovarian reserve and 1.6 in patients with normal functional ovarian reserve [78]. Pregnancy and delivery chances were also improved with rescue IVM in women with low functional ovarian reserve [78]. Different time dynamics were noted between women with low functional ovarian reserve and those with normal functional ovarian reserve.

Novel rescue IVM approaches are being developed, including the use of heterologous follicular fluid and supernatant of cumulus-granulosa cells in culture medium to mimic the intact follicular microenvironment [79]. While results appear promising, more data are required to understand the contribution of such approaches to improving clinical outcomes [77].

Unexplained primarily poor-quality embryos

Another reason for IVF failure can be the presence of follicular developmental abnormalities, which results in a lack of available embryos [80]. Management of this group of patients is particularly frustrating because the women appear to respond normally to ovarian stimulation, yet the reasons for very poor-quality embryos are largely unknown [80].

Positive outcomes with IVM have been reported in a study of patients with empty follicle syndrome (i.e. where no oocytes can be retrieved from mature ovarian follicles after controlled ovarian stimulation despite apparently normal follicular development and estradiol levels) [80]. Of seven patients who had failed to conceive after numerous IVF attempts, two pregnancies were achieved. The authors suggested that oocyte atresia or dysfunctional development in these two particular patients occurred in the more advanced stages of follicular development, and that IVM, with the final maturation occurring *in vitro*, had helped to overcome the processes involved, thus facilitating normal oocyte recovery [80].

Current IVM practices

Generally, IVM has been described as the technique whereby immature oocytes are collected from small follicles, usually <10 mm in diameter. Immature oocytes then are put in an IVM culture system, which allows the immature oocytes to develop and reach the MII stage. Mature oocytes are then inseminated with sperm to produce zygotes and embryos *in vitro*. The additional laboratory component of IVM includes the identification and collection of intact COCs and the IVM culture of intact COCs to obtain mature oocytes for IVF. The insemination technique and embryo culture procedure are similar to that of conventional IVF/ICSI [81].

Retrieved COCs are typically cultured in complex tissue culture-like medium with supplementation of a protein source and hormones for one to two days under atmospheric oxygen. Based on the use (or not) of FSH priming and hCG triggering, and the immature oocyte culture system, current IVM practices can be differentiated into four protocols: standard, hCG priming, biphasic (or CAPA), and rescue (Table 11.2, Figure 11.1) [19]. One of the benefits of a biphasic approach (CAPA IVM) is that most oocytes reach the MII stage (Figure 11.2).

Conventionally, IVM is performed without gonadotropin priming before immature oocyte retrieval. However, early evidence suggested that mild stimulation with FSH (FSH priming) improved both human oocyte yield and maturational competence [17, 18]. Conflicting data from small randomized controlled trials (RCTs) regarding the effectiveness of FSH priming on IVM success have been reported [82, 83]. Recently, a large RCT compared the efficacy of CAPA IVM and conventional IVF/ICSI in women with PCO treated with highly purified hMG 150 IU/day for two days [35]. The results showed that CAPA (biphasic) IVM was non-inferior to IVF in terms of live birth rate after the first ET [35]. Until now, there has been limited evidence regarding the optimal dosage of FSH/hMG used in IVM, but a common dosage is FSH 150 IU/day for two to six days in the follicular phase of the cycle.

The first successful application of hCG triggering before oocyte retrieval in IVM was reported in 1999 [16]. One early study reported that the pregnancy rate might be improved by priming with hCG prior to immature oocyte retrieval [84]. However, later reports provide conflicting results regarding the effectiveness of hCG priming in IVM [85, 86]. In the first decade of the new millennium, use of hCG triggering in IVM was adopted worldwide, mostly combined with FSH priming [87]. The most common approach was to prime with hCG 10,000 IU at 36 hours before the retrieval of immature oocytes. The largest report of IVM using hCG triggering, combined with FSH priming, included 921 women with PCOS [32]. The oocyte maturation rate was 71.2%, implantation rate was 21.5% with cleavage embryos, and the cumulative live birth rate over 12 months after an IVM cycle was 33.7% [32].

TABLE 11.2 Different *In Vitro* Maturation (IVM) Protocols in Clinical Practice

IVM Protocol	Features and Evidence
Standard (Figure 11.1)	• Conventional IVM protocol • With or without FSH priming before oocyte retrieval • No hCG trigger • After oocyte retrieval, only GV-stage COCs collected • All COCs matured to MII oocytes in one phase of IVM culture • Promising results [33]
hCG Priming (Figure 11.1)	• Modified IVM used in many ART centres worldwide • hCG trigger before oocyte pickup • With or without FSH priming before oocyte retrieval • Oocytes retrieved may be at different stages of maturation (MII, MI, or GVBD) • MII oocytes inseminated after a few hours, while MI and GVBD oocytes require IVM culture for 24–30 hours • Largest report of IVM using hCG triggering and FSH priming (n = 921 women with PCOS): oocyte maturation rate 71.2%, implantation rate 21.5% (cleavage embryos), cumulative live birth rate over 12 months after an IVM cycle 33.7% [32]
Biphasic (Figures 11.1 and 11.2)	• Also known as capacitation (CAPA) IVM • Includes a pre-IVM culture and the IVM culture • Pre-IVM medium contains C-type natriuretic peptide to block meiotic resumption and maintain oocytes at the GV stage for 24 hours, allowing GV oocytes to gain further cytoplasmic maturation and improved competence prior to IVM culture [88] • No hCG trigger • FSH priming can be given prior to oocyte retrieval • Only GV-stage COCs collected at oocyte retrieval • The first clinical reports of CAPA-IVM showed promising results [23, 24]. The CAPA-IVM system significantly improved oocyte maturation, embryo quality, and clinical pregnancy rates versus standard IVM in patients with PCOM. Larger data proved that CAPA-IVM was non-inferior to conventional IVF in terms of live birth rate after the first ET in women with high antral follicle count [35].
Rescue	• For poor quality, immature GV-stage oocytes collected after conventional ovarian stimulation, including full FSH stimulation and hCG triggering (these have no developmental potential and are usually discarded) • IVM performed to achieve mature oocytes (using any protocol) • The rationale and efficacy of rescue IVM is still questioned

Abbreviations: ART, assisted reproductive technology; COCs, cumulus–oocyte complexes; FSH, follicle-stimulating hormone; GV, germinal vesicle; GVBD, germinal vesicle breakdown; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; MI, metaphase I; MII, metaphase II; PCOM, polycystic ovarian morphology; PCOS, polycystic ovary syndrome.

Technical issues

Immature oocyte pickup

One of the most important steps that could be challenging for physicians when starting to perform IVM is the immature oocyte pickup (OPU) procedure. Differences between OPU procedures for IVM versus IVF are shown in Table 11.3.

Identify small cumulus–oocyte complex and additional culturing steps

The COCs that are harvested from small follicles are smaller in size and more compacted than those collected from stimulated IVF cycles. An additional filtration step can be applied to harvest very small COCs from follicle fluid. More culturing steps have to be prepared and applied in an IVM treatment cycle for immature oocytes. Embryologists have to obtain new skills in harvesting small COCs and workload is increased in ART laboratories in ART programs practicing IVM.

Fresh or frozen transfer

The traditional approach to ET after IVM was to transfer fresh embryos using hCG administration 36 hours before oocyte pickup (OPU), and endometrial preparation using estradiol and progesterone. This protocol has been used with reasonably good results [32]. However, some studies have reported comparatively low success rates with fresh ET in IVM [36, 89]. It is possible that different IVM methodologies and different endometrial preparation regimens might result in different outcomes. Recently, a randomized, controlled pilot study was designed to compare the effectiveness and safety of a freeze-only strategy and fresh ET in women with a high AFC undergoing IVM with a pre-maturation step (CAPA IVM, without hCG) [90]. The results suggested that use of a freeze-only strategy in patients with a high AFC undergoing IVM with a pre-maturation step could significantly increase the rate of ongoing pregnancy resulting in live birth compared with fresh ET. Other fertility outcomes and complication rates did not differ between the two groups [90], although larger studies with longer follow-up are needed to confirm the comparative safety of frozen versus fresh ET in IVM.

Clinical use of IVM

Role of IVM in modern ART

There is still room for further development of more efficient IVM protocols [91]. Recent knowledge about oocyte physiology and development can be translated into clinical practice to improve the efficacy of these protocols.

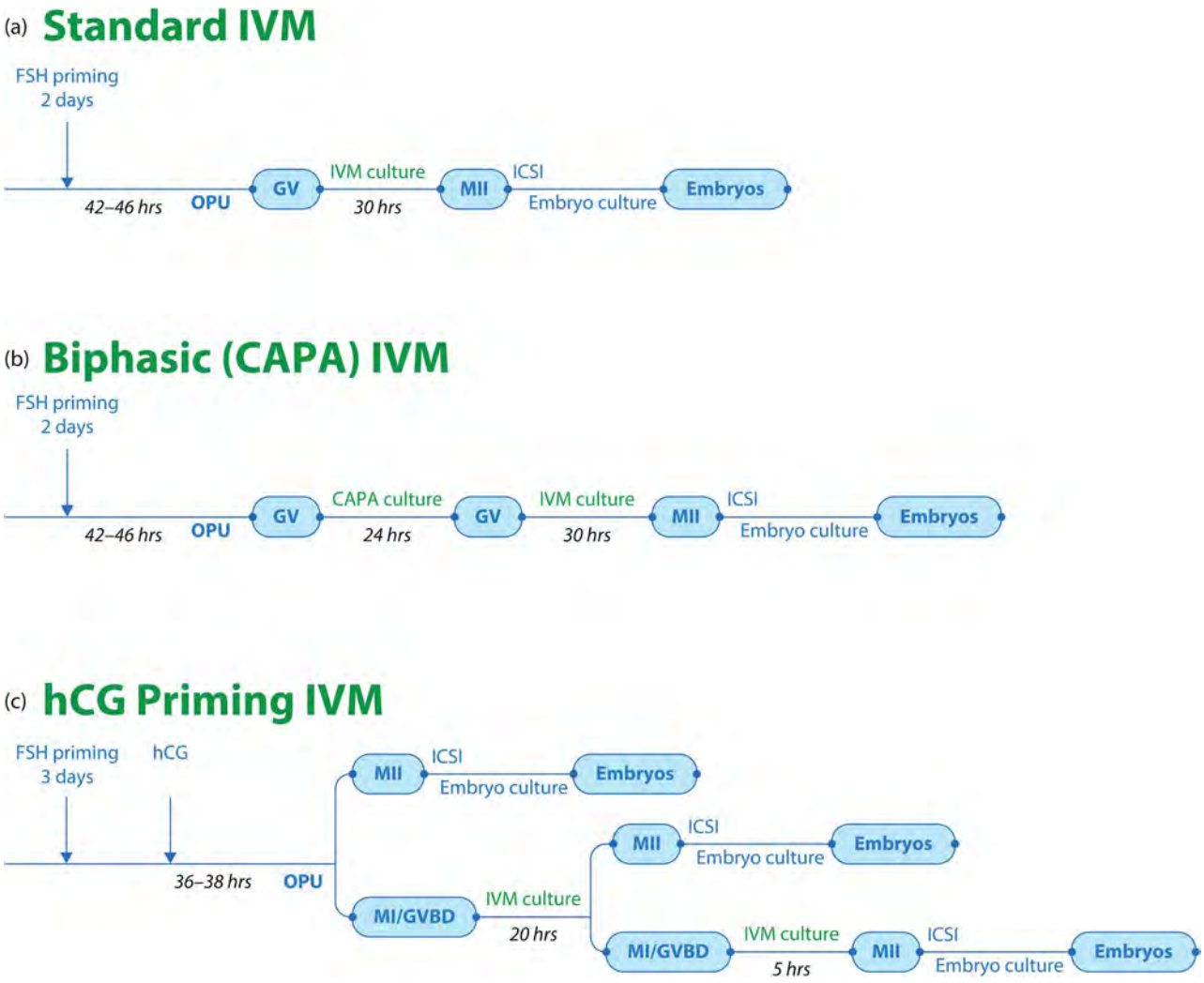

FIGURE 11.1 Current *in vitro* maturation (IVM) protocols, in which oocytes are harvested from small follicles (based on the authors' practice). (a) Standard protocol, without hCG before oocyte pickup (OPU) and one IVM culture step; (b) biphasic (CAPA) protocol, without hCG before OPU and biphasic IVM culture; and (c) hCG priming protocol, with hCG triggering before OPU and collected oocytes at different stages of maturity. *Abbreviations*: GV, germinal vesicle; ICSI, intracytoplasmic sperm injection; MII, metaphase II.

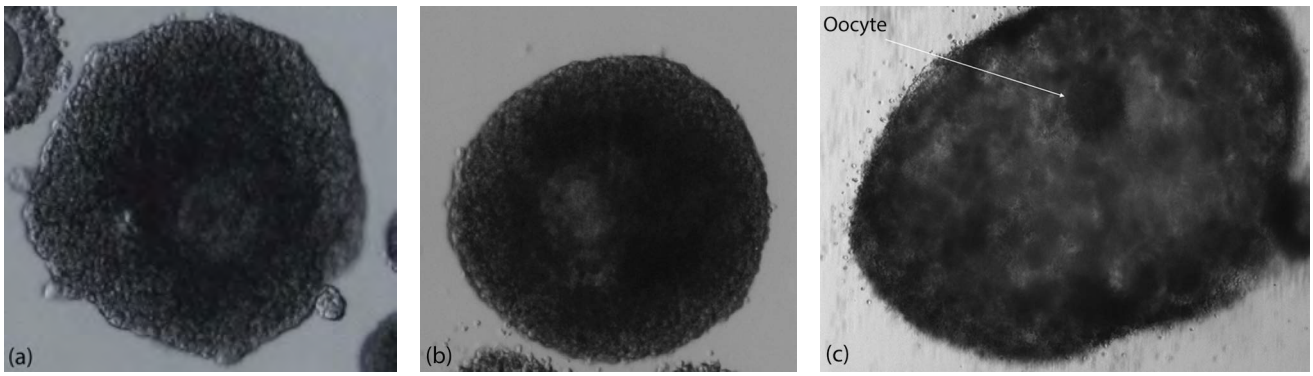

FIGURE 11.2 Cumulus–oocyte complex (COC) in the biphasic (CAPA) *in vitro* maturation (IVM) protocol. (a) COC after oocyte pickup (OPU); oocytes are at the germinal vesicle (GV) stage. (b) COC after CAPA culture; oocytes still at the GV stage. (c) COC after CAPA culture and IVM culture; oocytes reach the metaphase II (MII) stage.

TABLE 11.3 Differences between Oocyte Pickup Procedures for IVM versus IVF

Component	IVM	IVF
Timing	42 hours after FSH injection (standard or biphasic IVM) 36 hours after hCG injection (hCG IVM)	34–36 hours after hCG injection
Ovary characteristics	Ovary: smaller, not vascularized Follicle size: 2–10 mm Stromal tissue: thick, hard Mobile	Ovary: large, vascularized Follicle size: ≥14 mm Stromal tissue: soft, small Not mobile
Needle	19–21G Single/Double needle system: outer 17G; inner 19–21G Tubing length: 20–40 cm	17G Single/Double lumen Tubing length: ≥60 cm
Techniques	Aspiration and curetting No flushing	Aspiration ± Flushing
Medium	Heparin added (if necessary)	No heparin added
Duration	20–30 minutes per OPU	10 minutes per OPU

Abbreviations: FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; IVM, *in vitro* maturation; OPU, oocyte pickup.

IVM is no longer considered an experimental technique. The potential for wider clinical application of IVM was suggested by the Practice Committees of ASRM [3]. However, understanding which groups of patients will benefit from more widespread use of IVM remains a topic of debate. Groups of patients probably best suited for IVM include women at greater risk of adverse events during ovarian stimulation, including OHSS and other potential adverse reactions, and those with cancer who cannot wait for a full ovarian stimulation regime due to the urgency of initiating gonadotoxic cancer treatment or with hormonally sensitive cancer that is a contraindication for standard ovarian stimulation.

One approach is to consider IVM as an alternative ART treatment that is patient-friendlier and can reduce the treatment burden of standard IVF cycles with respect to medication cost, time, and stress relating to the stimulation and follow-up protocols. For young patients undergoing IVF who have a potentially high response to ovarian stimulation and a good prognosis with many extra, unused embryos (that may be cryopreserved or discarded), IVM could be a feasible alternative to IVF with more friendly treatment processes and a lower number of embryos generated, but a good cumulative live pregnancy rate.

Patients with good ovarian reserve but who have low response to ovarian stimulation could also be good candidates for IVM. In addition, IVM is the only option to help women with GROS to have children using their own oocytes [73].

There are several reasons why all major modern ART centres should have IVM facilities and protocols available. These include the likelihood of wider application of IVM in the future, the requirement to manage indications where IVM is the only option (e.g. fertility preservation, GROS), the increasing need for more patient-friendly ART treatment, and the fact that IVM protocols are improving thanks to advances in knowledge on human follicular and oocyte development.

Barriers to the application of IVM in clinical practice

As mentioned previously, worse outcomes (fewer embryos and lower pregnancy rates, especially cumulative pregnancy rate) with IVM versus IVF [36] have been the major barriers to more widespread implementation of IVM in clinical practice. In addition, there have been concerns about the health of infants born following IVM. High rates of chromosomal abnormalities have been reported in rescue IVM embryos [92, 93], although animal studies have shown a greater risk of imprinting defects in embryos from ovarian stimulation than from IVM [94]. Nevertheless, comparison of IVM with other ARTs (i.e. IVF, ICSI) and/or with spontaneous conception have found no differences in the incidence of congenital anomalies [40, 95, 96].

On a practical level, a lack of standardized protocols for IVM [3], leading to variable outcomes between centres depending on levels of expertise, might also negatively impact the uptake of IVM. Furthermore, IVM may be less attractive to some centres because of the associated increase in laboratory workload, particularly if the hCG-priming protocol is used.

While the costs associated with IVM are lower than those for IVF [2, 27, 28], IVF is covered by health insurance in many Western countries, whereas this may not be the case for IVM, meaning that out-of-pocket costs for patients might be higher. In contrast, for emerging economy countries that often have no reimbursement system for infertility therapies, IVM can be the most effective ART at the lowest out-of-pocket expense for the patient. In this sense, IVM could increase patient access to treatment in lower-income countries in Asia, South America, and Africa.

Finally, the availability of alternative strategies for reducing the risk of OHSS might also negatively impact the use of IVM. However, although other assisted reproductive medicine strategies, such as the use of gonadotropin-releasing hormone agonist trigger and freeze-only cycles, have reduced the rate of OHSS in high-risk women [97–99], cases of severe OHSS have still been reported with these approaches [100, 101]. IVM is the only ART that carries zero risk of OHSS, which is important in regions where critical care units are sparse.

Overcoming barriers to the clinical use of IVM

Strategies to overcome barriers to the use of IVM include specific training for clinicians to aspirate unexpanded COCs from small antral follicles and to adapt alternative therapeutic strategies for the most difficult cases. Specific training for embryologists is mandatory to handle these unexpanded COCs into prolonged pre-maturation "CAPA" systems. Additional research in the field is needed, especially on the health of babies born as a result of IVM. Another important factor is improved recognition of IVM as a women-friendly and efficient ART technique by fertility specialists. Finally, there is a need for increased allocation of funding for IVM research to further develop IVM into the first-line treatment for the aforementioned infertility indications.

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12

INTRACYTOPLASMIC SPERM INJECTION

Technical Aspects

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Background

The use of assisted reproductive technology (ART) to overcome infertility has increased steadily in the USA and worldwide. Based on 2019 estimates, approximately 448 ART clinics in the USA performed 171,206 ART procedures resulting in 77,998 live deliveries and 83,946 infants [1], contributing to 2.1% of all infants born in the USA, increasing from 2015's estimates of 1.5% [2]. Internationally, the number of non-donor aspirations increased more than 37% from 2010 to 2014 [3] and are projected to continue growing as ART becomes more widely available. Although today's trends indicate a wide acceptance of IVF, it was the development of ICSI that broadened the reaches of ART to serve patients with more severe cases of infertility.

Soon after the establishment of IVF, it became clear that as many as 40% of conventional IVF cycles were affected by fertilization failure or by an extremely low fertilization rate, even though spermatozoa were placed in close proximity to oocytes [4, 5]. This was particularly problematic in patients with diminished sperm motility and/or poor morphology (i.e. it presented a complex obstacle for spermatozoa to penetrate the zona pellucida (ZP), a thick glycoprotein layer surrounding the oocyte [4]. In such cases, gamete micromanipulation was thought to be the only way to overcome this problem. The different techniques developed in this regard focused on assisting the spermatozoon to penetrate the ZP by "softening" it enzymatically with trypsin or pronase [6], penetrating it chemically via localized exposure to acidified Tyrode's solution prior to sperm exposure, or by mechanically piercing the zone pellucida with a microneedle [7]. The placing of the spermatozoon beneath the ZP yielded consistent results, achieving a fertilization rate of ~20% [8]. However, these techniques were abandoned because of limiting factors such as the need for many functional spermatozoa with good progressive motility, and complications like polyspermy [7]. These initial efforts to assist sperm penetration soon became obsolete with the introduction of a microsurgical method for the insertion of spermatozoa directly into the oocyte.

Intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) involves the injection of a single spermatozoon directly into the cytoplasm of an oocyte. ICSI bypasses both the ZP barrier and sperm defects that compromise its ability to fertilize. The ability of ICSI to achieve higher fertilization and pregnancy rates regardless of sperm characteristics makes it the most powerful micromanipulation procedure yet for treating male factor infertility [9]. In fact, the therapeutic possibilities of ICSI range from cases in which, after sperm selection, the spermatozoon show poor progressive motility to its application in azoospermic men where spermatozoa are micro-surgically retrieved from the epididymis or the testis [10]. While the availability of ART remains variable among European countries given limitations on accessibility and funding, 35 of 43 countries permit treatments to single women and 23 to female couples [11]. ICSI has also made the consistent fertilization of cryopreserved oocytes possible [12], since cryopreservation can lead to physical damage such as premature exocytosis of cortical granules and ZP hardening that inhibit natural sperm penetration [13, 14]. When preimplantation genetic screening is to be performed on oocytes, the removal of the polar body requires the stripping of cumulus corona cells, thus supporting ICSI as the only insemination method to avoid polyspermy. When embryos need to be screened for genetic defects, the avoidance of contaminating spermatozoa on the ZP reduces the chance of unintended amplification by polymerase chain reaction.

While semen processing using a density gradient can remove viral particles from samples from men with HIV, ICSI is the preferred method of insemination by several groups for HIV-discordant couples because it virtually avoids the interaction of oocytes with semen [15–18], requiring fewer attempts to achieve a pregnancy with obviously reduced chances of viral exposure for the unaffected partner and to the conceptus. Reassuringly, no seroconversions have been reported following ART use for HIV-discordant couples [17]. Although it is still unclear whether COVID-19 viral particles are viable in semen [19], initial studies on sperm quality from men with a recent recovery from COVID-19 have shown that semen parameters were significantly lower than the baseline within the first two-month period after hospital discharge [19].

In this chapter, we describe the quintessential technical details involved in the proper execution of ICSI. We also present the clinical outcomes associated with ICSI and appraise its safety.

Ejaculate semen collection and processing

Semen samples are collected by masturbation after two to three days of abstinence and allowed to liquefy for at least 15 minutes at 37°C before analysis. When the semen has high viscosity, this can be reduced within three to five minutes by adding it to 2–3 mL of 4–(2 hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES)-buffered human tubal fluid (HTF) containing 200–300 IU of chymotrypsin (Sigma Chemical Co., St Louis, MO), which is capable of releasing spermatozoa [20]. Electroejaculation is applied in cases of spinal cord injury or psychogenic anejaculation [21].

Semen concentration and motility are assessed in a Makler® counting chamber (Sefi Medical Instruments, Haifa, Israel). Semen parameters are considered impaired when the sperm concentration is $<16 \times 10^6$/mL, the total motility is <42%, or normal morphology is exhibited by <4% of the spermatozoa [22]. Microscopic evaluation on morphology is usually made after

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spreading 5 µL of semen or sperm suspension on pre-stained slides (Testsimplets®; Boehringer, Munster, Germany); at least 100–200 spermatozoa are categorized.

For selection, spermatozoa are concentrated by centrifugation at 500 *g* for five minutes in HTF medium supplemented with 6% (v/v) human serum albumin (HSA; Vitrolife, Englewood, CO). Semen samples with $<5 \times 10^6$/mL spermatozoa or <20% total motile spermatozoa are centrifuged in HTF medium at 500–1800 *g* for five minutes. The resuspended pellet is layered on a discontinuous gradient (ISolate®, Irvine Scientific, Irvine, CA) on two layers (90% and 45%) and centrifuged at 300 *g* for 10 minutes. A single gradient (90%) is used when samples have a sperm density $<5 \times 10^6$/mL spermatozoa and <20% total motile spermatozoa. The densest portion containing the highest concentration of motile spermatozoa of approximately 800 µL is then processed by adding at least 5 mL of HTF medium and centrifuged at 600–800 *g* for 5–10 minutes to remove the silica gel particles. The concentration of the assessed sperm suspension is adjusted to $1–1.5 \times 10^6$/mL by the addition of HTF medium, and subsequently incubated at 37°C in a gas atmosphere of 5% CO_2 until utilization for ICSI.

Surgical retrieval of spermatozoa

In cases of irreparable obstructive azoospermia, a condition often caused by congenital bilateral absence of the vas deferens and associated with a cystic fibrosis gene mutation, spermatozoa are retrieved by percutaneous or microsurgical epididymal sperm aspiration [23–25]. Variable volumes of fluid (1–500 µL) are collected from the epididymal lumen by a glass micropipette or a metal needle. Since spermatozoa are highly concentrated, only microlitre quantities are needed. When the epididymal approach is not feasible, because of scarring [26], a specimen can be retrieved directly from the testicle by fine needle aspiration [27, 28].

In men with non-obstructive azoospermia (NOA), an open biopsy or, more specifically, a micro-TESE procedure is carried out. Once isolated, seminiferous tubules are mechanically minced in the OR to facilitate the release of spermatozoa [29]. Testicular sperm suspension is then brought to the laboratory and kept in culture for a few hours or overnight until utilization for ICSI.

If spermatozoa are not found, additional biopsies are taken. In these cases, all specimens are brought to the laboratory for further processing by exposure to collagenase type IV (1000 IU/mL) combined with 25 mg/mL of DNase I [26, 30, 31] for one hour, mixing the suspension every 10–15 minutes to enhance enzymatic digestion. Once large portions of undigested tissue, such as tubular walls and connective tissue, are removed, the digested suspension is centrifuged once at 500 *g* for five minutes or, if needed, at 1500–3000 *g* for five minutes. Pellets from supernatants and digested tissue are finally resuspended in medium with a final volume ranging from 20 to 200 µL. These samples are allocated into drops covered with oil for extended sperm search in an ICSI dish under an inverted microscope at 400× to assess for presence of germ cells.

Additional testing of the male gamete

Human spermatozoa undergo important modifications in the nuclear chromatin where sperm DNA is supercoiled as protection during transition within the male and female genital tracts. Shaping of the male gamete nucleus takes place in late spermiogenesis as its chromatin is undergoing a remarkable condensation that renders the sperm DNA transcriptionally inert and highly resistant to the environment. Following the morphological transformation of the nucleus in the seminiferous tubule, as spermatozoa transit through the epididymis, the chromatin is further stabilized through the establishment of disulphide bonds between the thiol-rich protamines [32]. However, DNA damage often remains inevitable in light of these protective measures and can provide insight into the fertilization potential of spermatozoa. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling (TUNEL) and Sperm Chromatin Structural Assay (SCSA; SCSA Diagnostics, Brookings, South Dakota, USA) assess DNA nick-end breaks that occur during mitotic development, but do not distinguish between the types of DNA damage. Similarly, the Comet assay, with its two different forms, the neutral and alkaline, also measures chromatin integrity, the former capable of distinguishing between the double- and single-stranded DNA breaks. On the account of DNA damage, our centre developed an algorithm to best allocate patient treatment plans. Specifically, patients with poor pregnancy outcomes after intrauterine insemination (IUI) were advised to undergo either of the two sperm chromatin fragmentation (SCF) tests. If SCF was within normal limits, patients were advised to proceed to standard *in vitro* insemination; however, patients with elevated SCF were advised to proceed directly to ICSI, and some extreme cases were directed to undergo testicular sperm retrievals [33]. Another test known as Cap-Score™ measures the percentage of spermatozoa undergoing capacitation, the physiological changes this cell must undergo to penetrate and fertilize an egg, by examining ganglioside monosialotetrahexosylganglioside (G_{M1}) localization patterns [34]. Using this assay, patients can minimize the time to achieve pregnancy by proceeding to the most effective ART treatment according to their Cap-Score™.

Several studies have suggested that suboptimal sperm morphology is often associated with aneuploidy, nuclear DNA damage, and, at times, impaired ICSI outcome [35, 36]. It has been postulated that infertile men have compromised DNA integrity as measured by these methods without a correlation with sperm concentration and/or morphology [37]. Conversely, by systematic observations carried out in our laboratory, we have identified an inverse correlation between DNA fragmentation and progressive motility [38]. Perhaps the reason why there is a lack of predictability between DNA integrity and pregnancy outcome with ICSI inseminations may be explained by the fact that only motile spermatozoa are utilized for injection.

The development of sperm sorting devices, such as a commercial microfluidic sperm sorting chip (Zymot Multi device; DxNow, Gaithersburg, MD), which selects spermatozoa based on the progression of their motility, have been shown to isolate sperm with higher genomic competence corroborated by their capacity to generate healthy pregnancies in comparison to sperm selected through standard processing procedures [39–41].

Sperm cryopreservation and thawing

The sperm suspension is supplemented with cryopreservation medium (Freezing Medium-Test Yolk Buffer with Glycerol, Irvine Scientific), and up to 600 µL aliquots of the final solution are placed in cryogenic vials (Nalgene Brand Products, Rochester, NY). The vials are exposed to liquid nitrogen vapor at –70°C for 15 minutes, and then plunged into liquid nitrogen at –196°C.

When needed for ICSI, vials are warmed to room temperature and HTF media is gently added to the thawed suspension and then centrifuged in medium to quickly remove the cryo-protectant. Epididymal spermatozoa and testicular tissue are cryopreserved in a similar manner to the ejaculated counterpart in an excess of cryo-protectant [42–44].

Collection and preparation of oocytes

Baseline bloodwork and pelvic ultrasound are performed on menstrual cycle day 2 for patients treated with gonadotropin-releasing hormone (GnRH) antagonist protocols [45]. Normal baseline parameters include follicle-stimulating hormone (FSH) <12 mIU/mL, oestradiol <75 pg/mL, progesterone <1 ng/mL, and anti-Müllerian hormone (AMH) >1.0 ng/mL. Pelvic ultrasound is performed to evaluate endometrial thickness and to assess the antral follicle count and identification of eventual ovarian cysts.

Controlled ovarian superovulation (COS), human chorionic gonadotropin (hCG) trigger, and oocyte retrieval are performed per standard protocols [45, 46] to maximize follicular response while minimizing the risk of ovarian hyperstimulation syndrome. In general, the hCG trigger is given when at least two lead follicles attain a mean diameter of 17 mm. Oocyte retrieval is performed under conscious sedation using transvaginal ultrasound guidance approximately 35–36 hours after hCG administration. Under the inverted microscope at 100×, the cumulus–corona cell complexes are scored as mature, slightly immature, completely immature, or slightly hypermature [47]. Thereafter, the oocytes are ideally incubated for about three and a half hours. Immediately prior to micromanipulation, the cumulus–corona cells are removed by exposure to HTF-HEPES-buffered medium containing 40 IU/mL of Cumulase® (Origio®, Måløv, Denmark). A timely cumulus removal [48, 49] is necessary to observe nucleus maturity and allow holding, visualization, and injection during ICSI. For final removal of the residual corona cells, the oocytes are repeatedly aspirated in and out of a 135–290 µm micropipette (EZ-Tip®, Research Instruments Ltd, Bickland Industrial Park, UK) mounted on a suction holder (STRIPPER®, ORIGIO, Måløv, Denmark). Each oocyte is then examined under the microscope for overall morphology and whether nuclear maturity, defined as metaphase II (MII) stage characterized by the absence of the germinal vesicle and extrusion of the first polar body, has been reached. ICSI is performed only in oocytes that have reached this level of maturity.

Gamete micromanipulation set-up

The holding pipette (HP-120-30; 120 µm outer diameter [OD]) and injecting pipette (IC-C1; 5–7 µm inner diameter [ID]) are both made from glass capillary tubes (Vitrolife AB, Göteborg, Sweden). Both pipettes are bent to an angle of approximately 30° at 1 mm from the tip to manipulate gametes while maintaining the tip of tools horizontally positioned in a plastic Petri dish (ICSI dish, model 351006, Falcon; Becton and Dickinson, Lincoln Park, NJ). Prior to injection, 1 µL of the sperm suspension is diluted with approximately 8 µL of a 7% polyvinylpyrrolidone (PVP) solution (90121, Irvine Scientific) and placed in the centre of the ICSI dish. The utilization of a viscous solution helps slow down spermatozoa and allows easier micromanipulation while preventing spermatozoa from sticking to the wall of the injection pipette. Drops 1–8 will contain a single oocyte each. When there are fewer than 100,000/mL spermatozoa in the sample, the sperm

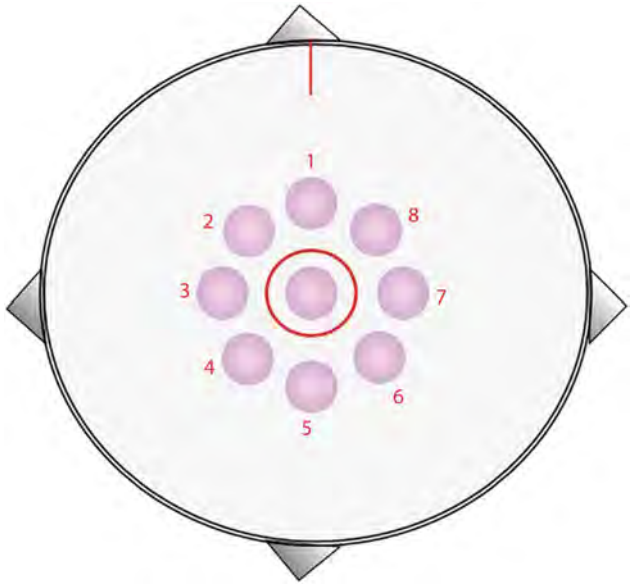

FIGURE 12.1 An intracytoplasmic sperm injection (ICSI) dish is made of 8 µL drops of ICSI medium plus one central drop overlaid with low-weight paraffin oil. The drops are labelled with a red pencil that is not embryo toxic. The central drop is marked with a circle while the surrounding drops are numbered 1–8 in a counter-clockwise fashion. The central drop is removed and replaced with polyvinylpyrrolidone and spermatozoa, while drops 1–8 will contain a single oocyte each. Specimens with very few spermatozoa are concentrated to a very small volume and placed in drop #8.

suspension is concentrated to approximately 5 µL and transferred directly in drop #8 (Figure 12.1) while individual oocytes are placed in the remaining drops of G-MOPS™ (Vitrolife) supplemented with HSA (90121, Irvine Scientific). These drops are covered with light-weight paraffin oil (OVOIL, Vitrolife). Following immobilization, an individual spermatozoon is aspirated at the three o'clock edge of the centre drop. For low concentration, a spermatozoon is retrieved from drop #8 and moved to the central drop containing viscous medium to remove debris, gain better aspiration control, and carry out sperm immobilization. The procedure is carried out on a custom designed heated stage (Eastech Laboratory, Centereach, NY) fitted on a Nikon TE2000U inverted microscope at 400× using Nikon Modulation contrast optics. The microscope is equipped with a customized micromanipulation set-up (NAI-20P, Narishige Co. Ltd, Tokyo, Japan) consisting of two motor-driven coarse control manipulators and two hydraulic micromanipulators. These custom manipulators have a modified low-position microscope mounting adaptor, a single power supply for the motor-drive coarse unit, and re-routed hydraulic Teflon tubing within the joystick. The micro-tools are controlled by two micro-injectors; one air control (IM-9B) tends to the holding pipette, and the other IM-6 is oil operated and fitted with a metal syringe to finely control pressure in the injection tool.

Selection, immobilization, and loading of the spermatozoon

Although ICSI does not require any specific spermatozoon pretreatment, gentle immobilization achieved through mechanical pressure is required to concurrently permeabilize the plasma

membrane and outer acrosomal membrane, which allows the release of the sperm cytosolic factor, phospholipase C zeta (PLCζ). This labile protein triggers a large influx of calcium ions from the endoplasmic reticulum into the cytoplasm, allowing the resumption of oocyte meiosis [50–52]. Qualitative and quantitative modifications of the plasma membrane occurring in the lipidic composition [53] and the absorption of specific proteins, such as beta-defensins and lipocalins, secreted by the epididymal epithelium result in changes of its electric charge and regulation of calcium ion influx of sperm cells [54]. The lack of all these changes is associated with a decreased ability of epididymal spermatozoa to bind and penetrate the oocyte in the case of standard *in vitro* insemination [53]. Owing to physiologic differences in the membrane characteristics of surgically retrieved spermatozoa, a more aggressive immobilization technique is necessary when using epididymal and/or testicular spermatozoa where the sperm tail is rolled over the bottom of the ICSI dish in a location posterior to the mid-piece [55]. This induces a permanent crimp in the flagellum rendering it kinked, looped, or convoluted (Figure 12.2). When aggressive immobilization was applied to surgically retrieved spermatozoa, the more extensive sperm tail disruption prior to oocyte injection appeared to improve the fertilization comparable to one achieved by ejaculated spermatozoa [55–57]. The findings were clarified in a later study where spermatozoa were mechanically immobilized and inserted into the perivitelline space of mouse oocytes [57] to allow ultrathin transmission electron microscopy (TEM) sections. These revealed consistent alterations of sperm plasma membrane including vesiculation, disruption of the acrosomal region, or even loss of the acrosomal content. All of the spermatozoa that were assessed had undergone some membrane disorganization of the head portion, in contrast to the majority of control intact sperm cells. This explains why the immobilization of sperm immediately prior to the ICSI procedure is fundamental for consistent fertilization [55, 57]. An explanation for the enhanced fertilization observed after aggressive immobilization may lie in the structural membrane differences between mature and immature spermatozoa. Immature gametes may require additional manipulation to promote membrane permeabilization, which enhances the post-injection events involved in sperm nuclear decondensation. For example, globozoospermia, a condition characterized by round-headed spermatozoa and partial or complete absence of the acrosome, impairs the sperm's ability to bind to the ZP. Although the sperm sample of globozoospermic men may retain normal parameters, they may not be able to fertilize oocytes even through ICSI, thus requiring the addition of an oocyte activating agent to achieve a successful pregnancy [58].

Motile spermatozoa are selected in viscous medium at 400× by observing its shape, light refraction, and motion pattern [59]. After the sperm suspension is loaded in a viscous medium, debris, other cells, bacteria, and immotile spermatozoa remain afloat at the PVP–paraffin oil interface. The viscous environment, by decelerating the spermatozoon, allows evaluation of its tri-dimensional motion pattern, permitting morphological assessment as well as favouring a finely controlled aspiration in the pipette tip. In cases where no motile spermatozoa are seen initially, the eighth drop may be replaced with the sample and supplemented with pentoxifylline to enhance sperm motility. Epididymal spermatozoa and testicular tissue are cryopreserved in the usual manner in an excess of cryo-protectant and spermatozoa are exposed to a motility enhancer (3 mmol/L pentoxifylline) to facilitate the identification of viable spermatozoa [60]. Once a motile spermatozoon is found, it is aspirated and moved into the PVP, where it is immobilized and loaded into the injection pipette for ICSI.

Oocyte injection

The oocyte is held in place by the suction applied to the holding pipette. Prior to piercing the oocyte, the injection pipette is blunted by tapping the tip against the edge of the holding pipette to minimize chances of slashing the oolemma. The inferior pole of the oocyte touching the bottom of the dish allows for better stabilization of the oocyte during the injection procedure. The injection pipette is lowered, and its level is adjusted to reach the focus in accordance with the outer right profile of the oolemma on the equatorial plane at three o'clock. The spermatozoon is then advanced into proximity of the bevelled opening of the injection pipette (Figure 12.3). The pipette is then pushed

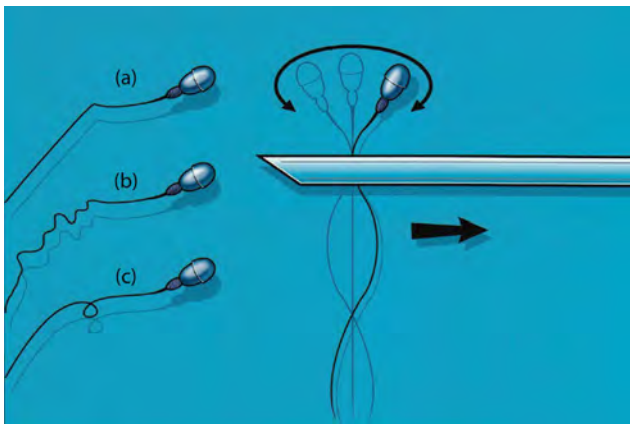

FIGURE 12.2 Aggressive immobilization of the spermatozoon for intracytoplasmic sperm injection. The correctly immobilized spermatozoon has its tail permanently kinked (a), convoluted (b), or looped (c).

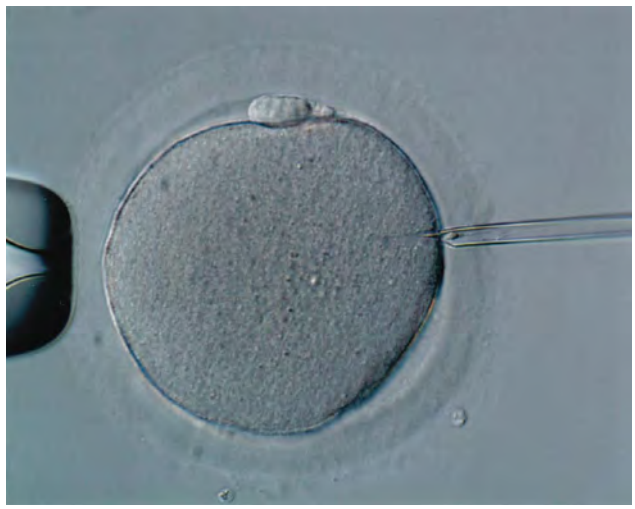

FIGURE 12.3 Intracytoplasmic sperm injection procedure. Prior to penetrating the oolemma, the spermatozoon is brought into proximity with the bevelled opening of the injection pipette.

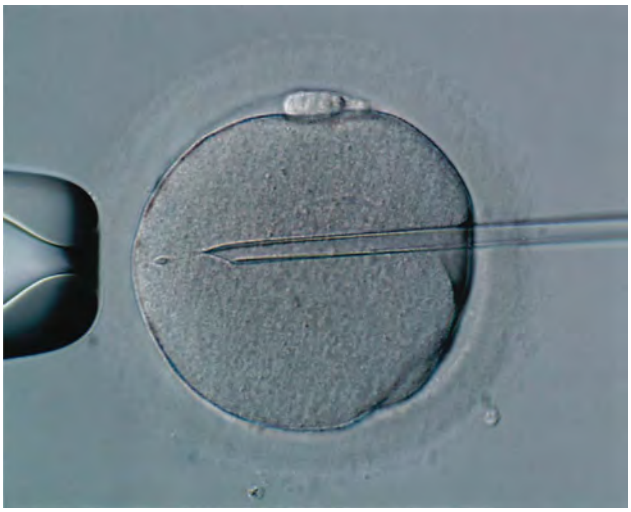

FIGURE 12.4 Intracytoplasmic sperm injection procedure. After the injection pipette has reached the approximate centre of the oocyte, a break in the oolemma is visible as a quivering of the convexities of the membrane above and below the site of penetration.

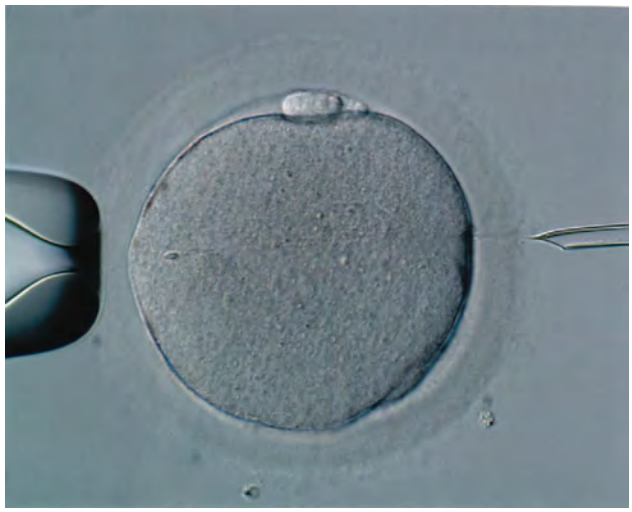

FIGURE 12.5 Intracytoplasmic sperm injection procedure. After the needle is withdrawn from the oocyte, the breach in the oolemma should be observed as a cone-shaped opening with its vertex towards the centre of the oocyte.

against the ZP, achieving its penetration through its inner surface to create an invagination of the oolemma. Once the pipette reaches the approximate centre of the oocyte, a breach in the membrane should occur. This is established by a sudden quivering of the convexities (above and below the oolemma invagination), along with a flow motion of the cytoplasmic organelles and the spermatozoon moving upwards into the pipette (Figure 12.4). The spermatozoon is then slowly ejected back into the ooplasm followed by the suction of the cytoplasmic organelle to exert an additional stimulus to trigger the oocyte to resume meiosis. To optimize the interaction with the ooplasm, the sperm cell should be ejected past the tip of the pipette to ensure an intimate position among the ooplasmic organelles that retain the sperm in place while withdrawing the pipette. While the injection pipette is withdrawn, eventual surplus medium is aspirated, resulting in the cytoplasmic organelles tightening around the sperm, and closing the breach generated during injection. Once the pipette is removed, the breach area is observed to make sure the point of entrance maintains a funnel shape with a sealed vertex pointing at the oocyte centre (Figure 12.5).

Evaluation of fertilization and embryo development

After injection, oocytes are loaded into specific culture dish (Embryoslide™, Vitrolife, Englewood, CO), covered in lightweight oil, and placed in an incubator with an integrated time-lapse system (Embryoscope™, Vitrolife, Englewood, CO) [61]. Cultured embryos can be monitored continuously and completely undisturbed while analysing the integrity of the cytoplasm in addition to the number and size of pronuclei. Photos are taken every 12–20 minutes by the device, and thereafter to depict blastomere cleavage, size, and timing in real time. In some cases, 72 hours after microinjection (the afternoon of day 3), those embryos with good morphology are transferred into the uterine cavity. This cleavage stage transfer approach is used particularly in male factor infertility. The number of embryos transferred depends on embryo availability, quality, and then obviously maternal age.

With the recent development of time-lapse imaging, most embryos are cultured to the blastocyst stage (day 5) to achieve higher implantation potential. The transfer of embryos that completed their full pre-implantation development more closely mirrors the events of natural conception, improving the likelihood that a healthy embryo will implant [62]. This allows for better selection of the best conceptus to maximize pregnancy rates following a single embryo transfer [62, 63].

A comprehensive medium specifically designed for time lapse culture (G-TL, Vitrolife) that support the embryo through its changing physiologic requirements allowing full implantation development [64, 65]. Blastocysts are evaluated according to well-established criteria in order to identify the ideal conceptus to subsequently transfer into the uterine cavity [66–68].

Extended sperm search

When no spermatozoa are identified at the initial semen analysis and even after high-speed centrifugation, an extensive search is carried out. An ICSI dish with the typical PVP solution placed in the central drop is loaded with the pelleted specimen supplemented with pentoxifylline to help augment sperm motility. Each drop is then browsed under 400× magnification and eventual spermatozoa identified are picked up and transferred to the PVP drop. The same procedure is performed for surgically retrieved specimens that have been freshly retrieved or recently thawed. Several dishes may have to be made and thoroughly searched for TESE patients until enough spermatozoa are found for injection.

In TESE specimens, sperm may be extremely scarce, requiring, in some cases, an extended sperm search lasting hours to complete depending on the number of oocytes awaiting injection [69]. As expected, the length of time required in acquiring spermatozoa affects clinical outcomes. In general, about 60% of testicular biopsies executed on non-obstructive azoospermic (NOA) men yield injectable spermatozoa (Figure 12.6). At our

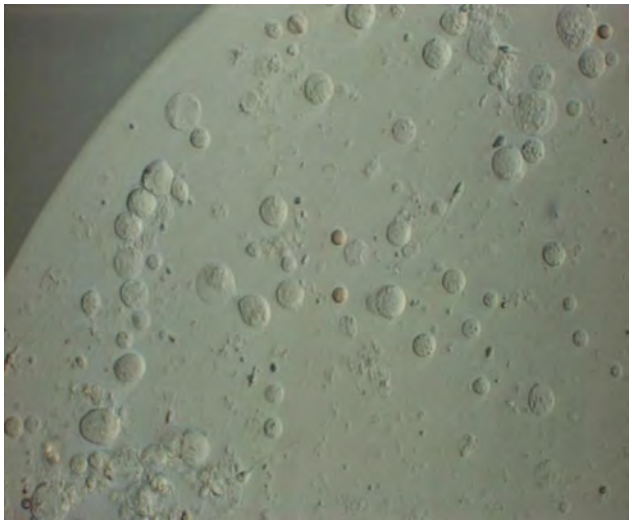

FIGURE 12.6 An example of a testicular sample for an extensive sperm search that has yielded spermatozoa for pickup and injection.

centre, when the time required for extended search time were allotted in groups, requiring from 30 minutes to one hour, one to two hours, two to three hours, and more than three hours, the fertilization rates were 46.5%, 44.9%, 35.5%, and 28.0%, respectively ($P < 0.01$); with the overall clinical pregnancy rates of 41.0%, 34.0%, 31.7%, and 24.2%, respectively ($P < 0.01$); and the overall live birth rates of 35.1%, 26.1%, 21.6%, and 18.4%, respectively ($P < 0.01$) (Figure 12.7). In spite of the fact there was a negative effect on the outcome by increasing search time, the extended search is still important and valuable, as it represents the only opportunity for a man with NOA to bear his own biological child. In fact, even in searches lasting more than three hours, achieving pregnancy is still possible as long as a viable spermatozoon is identified [69].

Clinical results with ICSI

In a cross-sectional survey of ART procedures performed in 76 countries in 2014, the International Committee for Monitoring Assisted Reproductive Technologies reported that 67.4% (619,811 of 919,732) of all cycles utilized ICSI. However, there was considerable variation in ICSI rates, ranging from 53.2% in Asia to 85.5% in Latin America [3]. In examining the incidence of ICSI in the United States, ICSI use increased from 36.4% in 1996 to 76.2% in 2012 and has remained a strong prevalence with 76.6% in 2019 [1, 70]. At our centre, there has been a steady and progressive utilization of ICSI starting at 32.2% in 1993, rising to 48.8% in 1995, reaching 73.6% by 2002, and accounting for more than 90% in 2018 [71, 72].

At our centre, between 1993 and 2021, ICSI has been used in 47,332 cycles compared to 13,737 standard *in vitro* insemination (sIVI) cycles with conventional insemination. ICSI has yielded comparable reproductive outcomes in comparison to conventional sIVI but is also capable of consistently overcoming unforeseen sperm cell dysfunction. The overall fertilization rates after ICSI and sIVI were 73.9% (299,388/404,860) and 60.7% (76,969/126,813), respectively. However, with standard *in vitro* insemination, the fertilization rate is calculated over the total number of oocytes retrieved, so once adopted this denominator for ICSI, the fertilization rate is comparable between the two insemination methods (59.8% ICSI vs. 60.7% IVF). Clinical pregnancy rate, as defined by the presence of a fetal heartbeat on ultrasound, was 39.3% (14,156/36,043) for ICSI compared to 40.2% (4584/11,402) for IVF. Thus far, 21,420 children have been born by the two ART procedures, of which 14,726 were conceived with ICSI. We have performed 47,332 ICSI cycles. Of these, a proportion of 92.2% ($n = 43,643$) of all ICSI cycles were performed using ejaculated spermatozoa, and the remainder involved specimens that were surgically retrieved from the epididymis or testis at our centre. In cycles utilizing ejaculated spermatozoa, a total of 369,662 MII oocytes were injected, resulting in a survival rate of 97.2%. Of those that survived, 80.9% of oocytes fertilized normally, with only one pronucleus and three pronuclei in 2.0% and

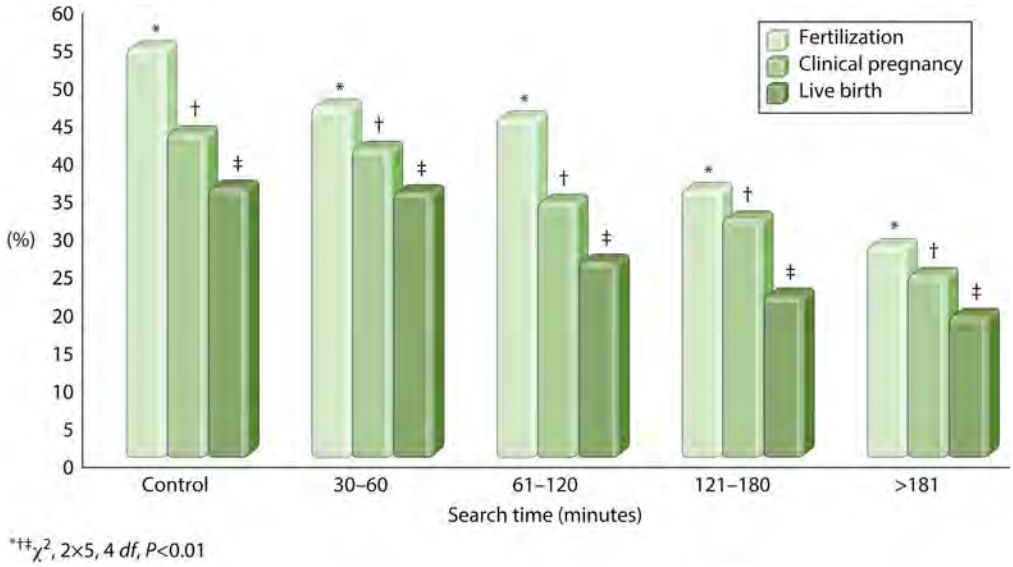

FIGURE 12.7 Pregnancy outcomes according to the length of time spent searching for testicular spermatozoa.

TABLE 12.1 Intracytoplasmic Sperm Injection Outcomes in Men with Severe Oligospermia (<1 × 10⁶/mL of Spermatozoa)

Parameter	Value
Cycles	3577
Mean concentration (10^6 per mL ± SD)	0.17 ± 0.25
Mean motility (% ± SD)	20.5 ± 38.8
Mean morphology (% ± SD)	0.14 ± 0.52
Fertilization (%)	10,621/17,769 (59.8%)
Clinical pregnancy (%)	1256 (42.8%)

2.9% of oocytes, respectively. No fertilization was noted in 11.1% of oocytes.

Our centre also treats severely oligozoospermic men with a concentration of <1 × 10^6/mL of spermatozoa. The outcomes of ICSI cycles in these men are highlighted in Table 12.1. If the initial semen specimen examination showed no spermatozoa, high-speed centrifugation was used.

Table 12.2 summarizes the fertilization and clinical pregnancy rates in ICSI cycles using ejaculated, epididymal, and testicular spermatozoa. When examining the three different sperm sources encompassing all maternal ages, the ejaculated cohort displayed the highest fertilization rates despite encompassing older women ($P < 0.001$). Epididymal spermatozoa achieved a somewhat lower fertilization rate but reported the highest clinical pregnancies as defined by the presence of at least one fetal heartbeat. Cycles using testicular spermatozoa had the lowest fertilization rates overall, despite including the youngest women ($P < 0.001$). The pregnancy rates for patients using testicular spermatozoa were somewhat lower compared to the other sources. It must be noted that this analysis is purely descriptive, because the surgically retrieved spermatozoa address different clinical indications.

A total of 1295 cycles were performed with epididymal spermatozoa and 1996 cycles were performed with testicular samples. When the fertilization and pregnancy characteristics were analysed according to whether or not the sample was cryopreserved, we observed that after cryopreservation, epididymal samples had lower motility parameters ($P < 0.0001$) and pregnancy outcomes ($P < 0.0001$), though without affecting fertilization rate. When testicular samples were used for ICSI, zygote formation and the ability of the resulting embryo to implant was unaffected (Table 12.3).

The characteristics for 51,457 ICSI cycles including all semen sources are described in Table 12.4.

Of the 21,082 patients presenting with positive βhCG (52.6%), 3244 were biochemical (15.4%) and 869 were blighted ova (4.1%). Among 15,811 patients in whom a viable fetal heartbeat was observed, 1768 had a miscarriage or were therapeutically aborted. The clinical pregnancy rate was 39.4% per embryo transfer (15,811/40,099). A total of 16,843 neonates were born from 14,043 deliveries, including 8376 baby girls and 8467 boys, with an overall frequency of multiple deliveries of 22.8% (3202/14,043): 2989 twins (21.3%), 209 triplets (1.5%), and four quadruplets (0.03%).

Safety of ICSI

At present, 1.5%–6% of children born in developed countries are conceived via ART, where two-thirds of the aspiration cycles included ICSI [3, 73–75]. It is well established that assisted reproduction is associated with adverse perinatal outcomes, including increased risks of preterm delivery, low birth weight, and neonatal mortality [76, 77]. In recent years, there has been considerable

TABLE 12.2 Intracytoplasmic Sperm Injection Outcomes Using Ejaculated, Epididymal, and Testicular Spermatozoa

Parameter	Ejaculated	Epididymal	Testicular
Maternal age (years)	38.4 ± 5	34.2 ± 5	32.8 ± 6
Cycles	43,643	1351	2028
Fertilization rate (%)	238,877/316,691 (75.4)	9317/13,219 (70.5)	10,420/21,242 (49.0)
Clinical pregnancy (%)	18,786 (43.0)	661 (48.9)	807 (39.8)

TABLE 12.3 Semen Parameters and Intracytoplasmic Sperm Injection Outcomes According to Retrieval Sites and Specimen Condition

	Spermatozoa			
	Epididymal		Testicular	
Outcome	Fresh	Frozen/Thawed	Fresh	Frozen/Thawed
Cycles	401	932	1301	800
Density (10^6/mL ± SD)	37.1 ± 56	19.2 ± 27	0.44 ± 6	0.22 ± 0.9
Motility (% ± SD)	19.1 ± 19[a]	4.2 ± 10[a]	2.4 ± 9[b]	0.8 ± 5[b]
Fertilization (%)	3058/4269 (71.6)	6114/8673 (70.5)	7171/14585 (49.2)	3790/7652 (49.5)
Clinical pregnancies (%)	223 (60.8)[c]	363 (46.2)[c]	493 (44.7)	239 (39.6)

Notes:

[a] Student's t-test, two independent samples, effect of epididymal cryopreservation on sperm motility, $P < 0.0001$.

[b] Student's t-test, two independent samples, effect of testicular cryopreservation on sperm motility, $P < 0.0001$.

[c] χ^2, 2 · 2, 1 df, effect of epididymal cryopreservation on clinical pregnancy rate, $P < 0.0001$.

TABLE 12.4 Evolution of Intracytoplasmic Sperm Injection (ICSI) Pregnancies in 51,457 Cycles

No. of		Positive Outcomes	
ICSI cycles	51,457		
Embryo replacements	40,099		
Positive human chorionic gonadotropin	21,082	Pregnancy	52.6% (21,082/40,099)
Biochemical pregnancies	3244		
Blighted ova	869		
Ectopic pregnancies	238		
Positive fetal heartbeats	15,811	Clinical pregnancy	39.4% (15,811/40,099)
Miscarriages/therapeutic abortions	1609		
Deliveries	14,043	Delivery rate	35.0% (14,043/40,099)

work investigating health outcomes in IVF and ICSI children beyond the neonatal period [78, 79]. Follow-ups of children following ART use are highly recommended and needed, however, still onerous [78–80].

ICSI's safety has often been criticized because the fertilizing spermatozoon neither binds to the ZP nor fuses with the oolemma [81]. Bypassing these physiologic steps together with the arbitrary selection of the spermatozoon has been a reason for concern [81–84]. In addition, few studies have been conducted on the effect of male gamete quality on the development of ICSI-conceived children [79]. Thus far, ICSI offspring reaching adolescence and beyond have provided sufficient information to allay these qualms. A study comparing the incidence of congenital abnormalities in ART-conceived and naturally conceived children found no significant difference between the groups [85]. Another series investigating the cognitive development of children born after sIVI or ICSI at ages 3, 5, and 11 found no detrimental effects of their conception on their cognitive abilities when compared with naturally conceived children [86]. In one study evaluating the neurodevelopmental outcomes of two-year-old children born after ART and natural conceptions, the authors found that most two-year-old ICSI children were healthy and were developing normally, as measured by the Bayley Scales of Infant Development [87]. Similarly, no significant difference was observed among the development of two-year-old children when comparing *in vivo* (ovarian stimulation or intrauterine insemination) and *in vitro* (IVF or ICSI) treatments [87]. In a different follow-up of nine-year-old children, it was found that ICSI children and their naturally conceived counterparts had similar behavioural outcomes and IQ scores [88]. Our own centre's evaluation of three-year-old ICSI-conceived children born from fathers with spermatogenic failure displayed no significant delay in developmental skills compared to those ICSI-born children from normozoospermic men [79].

The specific concerns regarding ICSI, whether real or theoretical [89–92], involve the insemination method, the use of spermatozoa with genetic or structural defects, and the possible introduction of foreign genes. Several epidemiological studies of assisted reproduction children report a near twofold increase in the risk of infant malformations [93], a recurrent reduction in birth weight [94], certain rare syndromes related to imprinting errors [95–97], and even a higher frequency of some cancers [98]. However, current evidence does not prove that there is an increased risk of imprinting disorders and even less so childhood cancers in ICSI children [99, 100]. Epigenetic imbalances have been similarly linked to the exposure of the embryos to long-term culture [101]. To date, Beckwith–Wiedemann, Angelman, Prader-Willi, and Silver-Russell syndromes have been associated with ART procedures [97, 102] and have been found to be equally distributed among the *in vitro* conception methods. At present, there is no evidence that the ICSI insemination itself is responsible for any increase in epigenetic disorders, findings that have been confirmed in animal studies [103].

Considering the thorough investigation into the health and development of children born from ICSI, focus has shifted towards the reproductive health of these individuals. Initially, a study conducted in Belgium found that men born from ICSI were three times more likely to have sperm concentrations below the WHO 2010 reference [104] value of 15 million/mL and four times more likely to have total sperm motility below the WHO reference value of 40% [105]. However, a more recent study offered reassuring data regarding the reproductive capabilities of men conceived through ICSI [106]. Researchers found no significant differences among spermatozoa concentrations from spontaneously conceived men and participants conceived through sIVI or ICSI. Evaluation of serum reproductive hormones from both groups also suggested normal testosterone production, and no correlation was observed between the semen parameters of participants conceived from sIVI or ICSI and their fathers, suggesting those men conceived from ART have comparable reproductive health to their naturally conceived peers [106].

Conclusions

ICSI has established itself as the most reliable technique to overcome fertilization failure via male factor infertility. By pinpointing the beginning of fertilization, it has helped us to better understand important aspects of early gamete interaction. The observed high performance of aggressively immobilized spermatozoa suggests a more efficient destabilization and consequent permeabilization of the sperm membrane, which is responsible for a prompter release of the oocyte-activating factor [107]. These profound physiologic changes induced on the sperm membrane by the action of the injection needle seem to be critically important for immature, surgically retrieved spermatozoa, as confirmed in mammalian studies [55, 108]. It has been demonstrated that the positive outcome of ICSI is largely independent of the basic sperm parameters such as concentration, motility, and morphology. This is particularly evident with cryptozoospermia or when no spermatozoa are present in the ejaculate [59]. It is in these azoospermic men that the surgical isolation of spermatozoa together with ICSI is able to yield fertilization and support embryo development. The possibility of bypassing the steps of testicular and epididymal sperm maturation, acrosome reaction, binding to the ZP, and fusion with the oolemma now permits infertility due to various forms of male factor to be addressed successfully. In fact, in cases of men diagnosed as NOA, as long as a viable spermatozoon is isolated, there is a chance of generating a conceptus. However, we should be cautious about the utilization of ICSI in relation to the acquired evidence that sub-fertile men have a higher frequency of genetic abnormalities that may be passed on through their gametes [109]. Therefore, the earlier concern focused on ICSI insemination itself has shifted to the screening of the sub-fertile man who may transmit his genetic features to the offspring, specifically boys [110, 111]. A large,

worldwide experience suggested that men with extreme male factor conditions caused by a clear genetic make-up such as Klinefelter's syndrome or Yq micro-deletions can be successfully treated by ICSI and still generate healthy offspring [72, 112]. The potential effects of ART on child development should always be kept in mind and the monitoring of child health can be accomplished by a parent-administered questionnaire that provides a cost- and time-effective approach to measuring the child's physical and psychological well-being. In recent years, many studies have provided information on the health of children born after ART, and therefore, current evidence shows that the outcomes of singletons born at term following ART are reassuring [113]. The increased awareness of the risks related to multiple gestations has supported measures aimed at obtaining singleton births, with obvious benefits for the long-term welfare of the offspring.

Despite its success in treating male factor infertility, ICSI does not necessarily achieve the same standard when treating non-male factor infertility. A meta-analysis evaluating clinical outcomes from ICSI in couples with non-male factor showed no advantage to using the procedure to treat non-male factor infertility versus using standard IVF [114]. Similarly, fertilization rates per oocyte retrieved are observed to be lower in ICSI cycles than the standard IVF procedures for these couples as well [115]. In light of the worldwide popularity of ICSI, it is imperative to apply the technique thoughtfully to uphold its fundamental role in infertility treatment [116].

In the evaluation of the infertile male, we still rely on the basic semen analysis measures that can indicate a compromised germ cell maturation process that is altered during spermatogenesis. However, it is still unclear whether semen analysis data provide information on an individual spermatozoon's real potential to generate offspring, rather than reflecting the current status of spermatogenesis. That said, the importance of utilizing additional tests to evaluate spermatogenic characteristics is paramount in providing more insight into the fertilization capacity of sperm [33, 39, 52]. Spermatozoa are not just a vehicle that delivers the male genomic contribution to the oocyte. Upon fertilization, the spermatozoon provides a complete, highly structured, and epigenetically marked genome that, together with a defined complement of RNAs and proteins, plays a distinct role in early embryonic development. Often the origin of male infertility has been associated with specific gene imbalances; although, in many cases, the cause remains idiopathic. Future research will be focused on exploring the effects of genetic variants such as single-nucleotide polymorphisms, copy number variants, differential genome packaging, differential methylation, proteomic changes, and diverse sperm RNAs in order to enlighten the conundrum represented by what we define as male infertility.

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13

HUMAN EMBRYO BIOPSY PROCEDURES

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Introduction

In 1968, Gardner and Edwards reported the possibility of biopsying rabbit embryos with the purpose of sex selection [1]. In this study, rabbit embryos were placed under a dissecting microscope and, using two pipettes and a pair of scissors, some trophectoderm cells were removed from the embryo. The excised cells were then fixed and stained to visualize the Barr Body (i.e. female embryos). Biopsied blastocysts were then transferred to a receiving female rabbit and, once the fetuses were surgically birthed, the sex was confirmed. The first attempt at embryo manipulation on mouse embryos dates back to 1989 [2]. Embryos on day 3 of development were flushed from the female oviduct and one of the eight blastomeres removed mechanically with a pipette. The blastomere was then used for biochemical studies distinguishing between female (XX) and male (XY) embryos by comparing the activity of an X-linked gene with a reference autosomal gene. The first attempt in humans was performed in 1989 when Handyside and colleagues biopsied *in vitro* fertilized embryos at the 6- to 10-cell stage by removing a blastomere through a hole in the zona pellucida (ZP) using a drilling pipette [3]. Due to previous developments in DNA amplification technologies (i.e. PCR), they were able to perform molecular studies where female embryos could be discriminated from male embryos by amplifying target regions on the X chromosome. In 1990, the first pregnancy from a biopsied embryo was reported [4].

Since its first application in the late 1960s, embryo biopsy methodologies have changed radically, not only in the technical approach and the instruments employed for collecting the cellular specimen but also in the developmental stage at which the collection takes place. These changes reflect the continuous improvements that the field of assisted reproduction has undergone over the last decades, including micromanipulation devices and tools, incubation and culture systems, along with the increasing knowledge of embryo physiology generated through clinical experience and research studies. In this chapter, we will discuss the developments in embryo biopsy methods, providing a foundation on the advantages and disadvantages for each approach, and building up a consequential timeline that delineates how preimplantation genetic testing (PGT) has developed into a robust diagnostic tool for the investigation of embryos' monogenic and chromosomal inheritance.

Embryo biopsy applications

Embryo biopsy is employed in combination with PGT. PGT is mainly employed in cases where the couple in treatment is found or suspected to be at higher risk of having an offspring affected by genetic or chromosomal abnormalities. The analysis of the biopsied cells allows the detection of genetic abnormalities and the subsequent deselection from transfer of the associated embryo.

PGT-A for aneuploidies

An abnormal number of chromosomes (i.e. aneuploidy) is frequently detected in human embryos. This occurrence mainly originates from meiotic errors in the oocyte and therefore affects every cell of the ensuing embryo. The frequency of meiotic errors has been found to be related to women's age and follows a U-shaped curve [5]. Moreover, aneuploidy is the most common genetic abnormality detected in miscarried products of conception [6]. For this reason, PGT-A is mainly employed in patients of advanced maternal age, recurrent pregnancy loss, and repeated implantation failure.

PGT-M for monogenic disorders

Monogenic disorders are caused by a defect in the nucleotide sequence of specific genes. The defective sequence encodes for an altered protein that is unable to carry out its biological function, resulting in abnormal tissue, organ, or systemic physiology, depending on the gene involved (e.g. cystic fibrosis). These defects usually run in the family and are inherited from the parents. Their manifestation mainly depends on their specific type of inheritance (i.e. recessive, dominant, X-linked). However, some monogenic conditions can arise *de novo*, with spontaneous mutations occurring in either the germinal tissue of the parent or in the developing embryo.

PGT-SR for chromosomal structural rearrangements

Chromosome structural rearrangements include inversions and translocations. The first involves the detachment, 180° rotation, and reattachment of a genetic fragment. This event can be harmless if the breaking points are outside of a coding region; however, it can lead to aberrant levels of gene products or dysfunctional genetic regulation if it involves active regions of the chromosome. Translocations involve the detachment of a section of the chromosome and its migration and reattachment to another chromosome. The most typical of these events are Robertsonian translocations where acrocentric chromosomes (i.e. 13, 14, 15, 21, and 22) fuse together. Healthy carriers of a balanced Robertsonian translocation bear almost (if not all) genes without pathological implications. However, their gametes can inherit either normal, balanced, or unbalanced karyotypes. For this reason, they are at higher risk of conceiving a fetus with an abnormal karyotype (and subsequent higher risk of miscarriage).

PGT-HLA for HLA haplotyping

In some countries, couples may access PGT for HLA-typing (i.e. human leukocyte antigen, HLA or major histocompatibility complex, MHC). PGT-HLA allows the selection of an embryo based on its inheritance of specific genes regulating the immune system. This type of embryo selection can be employed to generate a child whose organs are compatible with a sibling affected by a congenital or acquired disorder for whom a hematopoietic stem cell transplantation (HSCT) is needed.

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Types of embryo biopsy

Embryo biopsy is the procedure that allows the collection of embryonic cellular material and its DNA to subsequently use as substrate for genetic analysis. The information gathered from the biopsy specimen is used to infer the chromosomal and genetic composition of the whole embryo. Embryo biopsy can be performed at different developmental stages of the embryo or the gametes (Figure 13.1). These include polar body (PB) (hence on the mature oocyte prior to injection on day 0 and/or at the zygote stage on day 1 post insemination), cleavage (on day 3), and blastocyst stage biopsy (on day 5, 6, or 7). According to the latest report from the ESHRE PGT Consortium, which reflects clinical data collected between 2016 and 2017, the embryo developmental stage at which the biopsy is carried out varies according to the downstream PGT analysis [7]. Cleavage stage biopsy is employed in over 75% of PGT-M and 65% of PGT-SR cases, whilst the remaining involve blastocyst biopsy. On the other hand, blastocyst biopsy was carried out in over 85% of PGT-A cases and over 90% of instances where PGT-A was performed in combination with PGT-M or PGT-SR. Each approach has its advantages and limitations mainly revolving on (i) quantity of DNA material harvested (which impacts the robustness and reproducibility of the genetic analysis), (ii) representativeness of the biopsy specimen of the whole embryo's genetic status, (iii) impact of the procedure on embryo viability, and (iv) operational efficiency of the procedure (e.g. percentage of detected healthy embryos over number of biopsy procedures carried out).

Despite the developmental stage at which the procedure is carried out, this consists of three steps: (i) opening of the ZP, (ii) removal/collection of the specimen (either PB(s), blastomeres, or trophectoderm cells), and (iii) tubing of the specimen (or cell fixation on a glass support in case of fluorescence in situ hybridization or FISH analysis).

Zona pellucida opening

ZP opening can be carried out using three different approaches: mechanical, chemical, or through laser [8]. The mechanical method was the first employed and it involved the breaking of the ZP using a sharp microneedle operated through the micromanipulator [9]. This application is nowadays very rare due to access to more advanced techniques (i.e. laser). The chemical approach entails a topical dissolution of the ZP using Tyrode's acid [10]. This was achieved by loading the chemical agent into a microneedle and its subsequent release near the ZP area where access was required for biopsy. At its introduction, this technique was widely used during cleavage stage biopsy, as the acid was very effective at dissolving the ZP. However, the possible harmfulness of the acid to the embryo has led many IVF laboratories to move away from this approach, in favour of laser. The laser approach is currently the most used method for opening the ZP. It involves the use of a medical grade laser beam to quickly and accurately create a hole with a user-defined diameter in the ZP [11]. Several randomized trials reported no differences among the three different approaches for zona breaching [12]. However, the laser-assisted method is currently the most used due to its standardization, reproducibility, and time effectiveness. Nevertheless, irrespective of the method used, ZP opening can have itself a negative effect on embryo development. One study demonstrated that ZP drilling can compromise the subsequent hatching process and lead to the development of blastocysts with smaller diameter and thicker ZP [13].

Polar body biopsy

PB biopsy was developed as a pre-implantation diagnostic approach alternative to cleavage stage biopsy. Genetic defects are the main cause of spontaneous abortions during the first trimester of pregnancy, with chromosomal abnormalities responsible for about 65% to 70% of them [14]. Most of the aneuploidies found in embryos and fetuses are of maternal origin [15, 16]. This phenomenon is due to a progressive damage of the meiotic apparatus in the oocyte that is developmentally suspended in dictyotene (prophase of meiosis I). When ovulation occurs and maturation (i.e. meiosis) is resumed, the impairments accrued by the meiotic molecular machinery can cause abnormal chromosomal segregation, resulting in aneuploidy [17]. The degree of damage to this apparatus is dependent on female age, and its consequences are confirmed by the drastic increase in aneuploidy rate in patients of advanced maternal age. Being the by-product of female meiosis, polar bodies reflect the content of the oocyte. Therefore, their analysis has the advantage of predicting the maternal contribution to the embryonic genotype, without interfering with the embryo itself. Furthermore, PB biopsy is the only pre-implantation diagnostic alternative that can be offered to couples living in countries where embryo biopsy (post-fertilization) is not allowed.

PB biopsy can be performed using two strategies: (i) simultaneous and (ii) sequential biopsy. In the simultaneous PB biopsies, the two polar bodies (PB1 and PB2) are collected at the same time between six and nine hours after insemination [18, 19]. On the other hand, the sequential approach consists in the biopsy of the two polar bodies at different time points: PB1 is removed one hour after oocyte retrieval and PB2 after fertilization assessment (16–18 hours post-insemination). The opening of the ZP is performed either with a laser or mechanically. In case of sequential biopsy, a second opening in the ZP may be necessary, but it should be avoided whenever possible as it may have a negative effect on blastocyst hatching. After biopsy, the oocytes or zygotes can be cryopreserved or transferred back to the culture dish for

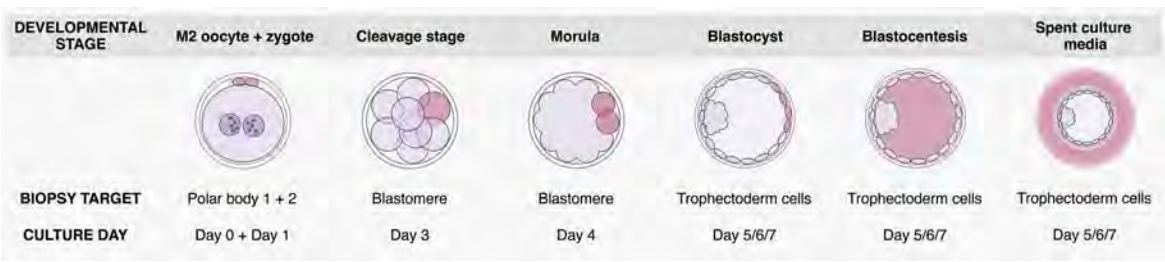

FIGURE 13.1 Developmental stages at which biopsy and embryonic DNA collection can be performed. Cellular (i.e. polar bodies, blastomere, and trophectoderm cells) and extracellular (i.e. blastocoel and spent culture media) targets are shown in pink.

further *in vitro* development. Beside the advantages mentioned previously, genetic investigations based on PB biopsy are limited to the maternal genome, whereas the paternal genomic contribution and the potential mitotic errors occurring in the first stages of embryonic development are completely neglected.

Furthermore, PB biopsy is very time-consuming and not cost-effective, as biopsies need to be performed at inconvenient hours, and two samples per embryo must be collected and analysed regardless of their further development. In a reference study highlighting the limitations of this type of biopsy, the diagnostic accuracy was also questioned. The concordance between karyotype results from PB biopsy and blastocyst biopsy was as low as 60% [20]. This lack of concordance between methodologies may be due to the more comprehensive analysis provided by the blastocyst biopsy approach, which considers not only the maternal contribution but also paternal meiotic errors and mitotic segregation errors occurring during embryonic development. These intrinsic biological and technical limitations make PB biopsy of limited value in the diagnostic field. In fact, due to this evidence, PB biopsy applications are infrequent and limited to specific situations.

Cleavage-stage biopsy

Cleavage embryo biopsy or blastomere biopsy is performed on day 3 post-insemination, when the embryo displays between 6 and 14 cells, before it reaches the compaction stage. The genetic constitution of the whole embryo is inferred from the genetic content of the cell biopsied. In certain circumstances, two blastomeres have been collected to either improve genetic testing accuracy (i.e. by doubling the amount of starting DNA) and/or to minimize the risk of no diagnosis in case the first biopsied cell had lysed. Nevertheless, one cell biopsy is strongly recommended, as the removal of two cells involves a depletion of about 25% of the embryonic mass, with negative effects on the clinical outcome [21]. The timing of cleavage biopsy can correspond with the beginning of the blastomeres compaction process, which starts at around 8 cells and completes around 32 cells, prior to cavitation. This phenomenon makes detachment and isolation of individual blastomeres difficult. For this reason, embryos are commonly exposed to Ca^{2+}/Mg^{2+}-free media for 5–10 minutes prior to biopsy. The absence of these salts in the culture solution results in the loosening of cell adhesion, allowing for easier manipulation. However, embryo exposure to this environment should be minimized to avoid major impact on cytoskeleton stability and intracellular communication processes [22]. During cleavage-stage biopsy procedures, the blastomere can be removed mainly by using two approaches: aspiration and extrusion. Both of these methods require the zona to be opened through, as mentioned earlier, Tyrode's acid, mechanical piercing, or, more commonly, using laser pulse. The aspiration method involves making contact between the pipette and the target blastomere through the ZP opening, followed by gentle aspiration of the cell until a good hold is established. After that, the blastomere is carefully pulled out of the ZP. This is the most common method for cleavage-stage biopsy. The extrusion method entails the application of mechanical pressure with a blunt pipette against the ZP, without direct contact with the cell, aiming to dislodge the target blastomere from the remaining embryo through the ZP opening.

After blastomere removal, cell nucleus should be visualized prior to tubing. Biopsied blastomere can then be fixed on a microscope glass support for FISH or tubed for DNA amplification.

There is growing evidence that even at this very early stage of development, a portion of embryonic cells have lost totipotency and are already partially committed into a specific cell lineage [23, 24]. Moreover, the spatial organization and points of contact between cells may be crucial for correct embryonic development. Compaction reversal and blastomere removal may therefore impact the ability of the embryo to follow specific architectural organization and hinder the formation of tissues required for further development and implantation.

Clear evidence about detrimental effects of cleavage-stage biopsy on embryo reproductive competence was provided by several studies. Scott et al. compared the implantation rate of top-quality sibling embryos, where one of them was submitted to cleavage-stage biopsy and the other was used as a control [25]. Day 3 blastomere biopsy was significantly associated with reduced implantation rate, with only 30% of biopsied embryos resulting in post-transfer sustained implantation, compared to 50% of the unbiopsied embryos.

The usefulness and efficacy of PGT based on blastomere biopsy are also affected by issues associated with the analysis of single cell DNA, impacting both technical (e.g. low amount of DNA results in higher chance of allele drop out (ADO), preferential amplification (PA), chimerical DNA molecules formation, and amplification failure), and biological aspects (single cell results in reduced representativeness of embryo's genetic constitution, e.g. mosaicism). Indeed, mosaicism (i.e. the coexistence of two or more karyotypes within the same embryo) has been detected at higher rates at the cleavage stage, compared to other stages of preimplantation development [26]. In conclusion, despite the vast experience in its use worldwide, cleavage-stage biopsy appears to be gradually replaced by safer and more robust approaches like trophectoderm biopsy, especially in cases where multicellular specimens provide higher diagnostic robustness (i.e. PGT-A).

Morula biopsy

The human embryo reaches the morula stage between day 3 and day 5 post-insemination, when approximately 16–32 blastomeres are highly compacted. Similar to the procedure used for cleavage-stage biopsy, the embryo must be exposed to Ca^{2+}/Mg^{2-} free medium to loosen intercellular junctions, thus allowing cell removal. Compared to cleavage-stage biopsy, morula-stage biopsy allows the procurement of more than one cell, thus improving overall diagnostic robustness and reliability. Nonetheless, this approach is rarely used due to the necessity of thoroughly reversing the compaction process, with possible downstream effects on embryo physiology and developmental ability.

Blastocyst biopsy

Following the morula stage, the cavitation process takes place, resulting in the formation of the blastocoel, a fluid filled cavity surrounded by trophectoderm cells (TE). As the blastocyst expands and the blastocoel increases in volume, the inner cell mass (ICM) becomes visible, protruding towards the centre of the cavity. ICM and TE are the first two distinguishable cell lineages of the developing embryo. While TE cells differentiate into extraembryonic tissues (e.g. placenta, chorion), ICM cells form the "embryo proper" from which eventually derive every cell, tissue, and organ of the ensuing fetus. In 2004, the TE biopsy approach was first described clinically by de Boer et al. [27], while the first live births following the procedure were reported in 2005 [28, 29]. Due to the more advanced developmental phase of the embryo, blastocyst-stage biopsy allows the collection of multiple cells (i.e. between 5 and 10) without interfering with the ICM (Box 13.1). Blastocyst biopsy is usually performed between

BOX 13.1 TECHNICAL INFORMATION ON TROPHECTODERM BIOPSY

LABORATORY REQUISITES

- Laboratory experienced and proficient in extended embryo culture
- Adequate equipment including:
  - Incubators in accordance with the workload
  - Micromanipulation station equipped with laser system

Equipment

- Laminar airflow cabinet
- Inverted microscope with micromanipulation system and heated stage
- Medical grade Laser (usually integrated in a x40 objective)
- Cooling rack
- Thermostat/incubator
- Incubator for embryo culture
- Vitrification system
- –20°C freezer

N.B. All devices should be calibrated, and their performance routinely validated.

Materials

- Capillaries for standard manipulation (i.e. 140 µm for biopsy specimen, 240 µm for vitrification, 300 µm for blastocyst transfer)
- Micromanipulation dishes (e.g. ICSI dishes)
- Sterile PCR tubes (i.e. 0.2 mL)
- Pre-warmed culture oil (i.e. 37°C)
- Holding micropipette (conventionally at 35° angle)
- Biopsy micropipette (conventionally at 35° angle, 10–15 µm diameter for PB biopsy; 30–35 µm diameter for cleavage-stage biopsy; 25–30 µm diameter for blastocyst biopsy)

Reagents

- Pre-warmed HEPES or MOPS buffered medium
- Biopsy sample washing solution
- Biopsy sample loading solution
- PVP for micropipette priming (optional)
- Surface decontaminant effective on DNA and DNase

Biopsy procedure

Pre-set-up

- Decontaminate surfaces, instruments, and work areas with appropriate, non-embryo toxic solutions (e.g. Oosafe)
- Wear protective clothing to minimize chance of sample DNA contamination (e.g. face mask, powder-free gloves)
- Embryologist must work in accordance with validated protocols
- Ensure all the material required is available, sterile, and within the expiration date
- Ensure correct traceability through labelling of dishes and tubes

Set-up

- Plate out dishes using pre-warmed (37°C) HEPES or MOPS-buffered media
- Overlay with pre-warmed (37°C) culture oil
- Place in thermostat (non-gassed) to equilibrate
- Set up micromanipulator with holding and biopsy pipettes
- Calibrate laser

Procedure

- When TE cells are herniating, transfer the embryo to biopsy dish avoiding blastocyst collapse.
- Orientate the embryo to have the ICM close to the side of the holding pipette and the herniating cells on the side of the biopsy pipette.

(Continued)

- Make minor adjustments to the embryos orientation to have the point of herniation on the same plane as the holding pipette.
- Make contact with the herniating cells gently aspirating them inside the biopsy pipette.
- Through combined action of suction and traction, acquire 5–10 cells within the pipette and expose cell junctions outside of the ZP.
- Apply a couple of laser beams to the cell junctions while applying traction on the aspirated cells until detached. Alternatively, apply "flicking" method by releasing the blastocyst from the holding pipette and move its lower edge against the upper edge of the biopsy pipette. Maintaining both pipettes in focus, swiftly move the biopsy pipette up, excising the target cells using the friction between the pipettes.

Tubing

- Prepare biopsy wash dishes immediately before use at room temperature, aliquoting 20 µL drops.
- Transfer the biopsied cells to the first drop and rinse them through serial wash drops.
- Transfer the specimen into the sterile, prelabelled PCR tube in 1 µL volume.
- To minimize DNA degradation, the PCR tube containing the specimen should be kept in a cooling rack until transfer to a –20°C freezer.

CONTINUOUS PERFORMANCE MONITORING

In order to achieve consistent outcomes, TE biopsy should be performed only by trained operators [56]. To avoid service disruption and work overload, more than one qualified operator should be available in the clinic. Operators' performance should be periodically monitored to ensure consistency of service. Main parameters to be tracked include:

- Rate of conclusive (aneuploid/euploid) and inconclusive diagnosis (amplification failure/non-concurrent result)
- Embryo degeneration rate after biopsy and embryo survival rate after warming
- Pregnancy rate and live birth rate per transfer

day 5 and day 7 post-insemination, depending on the embryo's development rate, expansion stage, and presence/identifiability of the ICM. There are three main protocols in the literature that describe how to perform a TE biopsy (Figure 13.2). The first method was published by McArthur and colleagues in 2005, then Capalbo et al. published two other methods in 2014 and 2016 [29–31].

The major difference among these protocols is when ZP opening is performed.

The protocol described by McArthur et al. entails a laser-assisted ZP opening at the cleavage stage, followed by extended culture up to the blastocyst stage [29]. ZP opening on days 3 to 4 post-insemination facilitates TE cells herniation, which, under pressure from the enlarging blastocoel, extrude from the ZP, making their biopsy easier. This approach is commonly used and shows both advantages and limitations. The main benefit of this method involves an easier ZP opening procedure, as the space between blastomeres and ZP is larger and therefore the laser (or acid) is less likely to affect the embryo. However, the procedure exposes the embryo to (i) suboptimal culture conditions for longer, as it needs to be removed from the incubator twice (i.e. for ZP opening and biopsy); and (ii) the risk of ICM herniation through the ZP opening, which makes the biopsy procedure more challenging.

The method presented by Capalbo et al. in 2014 avoids manipulation of the embryo on day 3 [30]. On the other hand, it requires simultaneous ZP opening and TE cells excision on the day of full blastocyst expansion. Blastocyst hatching is indeed left to occur spontaneously. This approach allows the embryologist to select the portion of TE to biopsy, rather than having to target the cells extruded through the ZP opening. Once the ICM is clearly visible, the blastocyst is anchored to the holding pipette orienting the ICM between 7 and 11 o'clock. This approach avoids direct anchoring of the ICM by the holding pipette, while keeping it as far as possible from the biopsy area.

The second protocol described by Capalbo et al. in 2016 suggests a ZP opening using a laser early on the day of blastocyst formation, followed by further incubation [31]. This approach promotes and expedites TE herniation in the following hours. Following TE cells extrusion cells, the herniating cells are collected using a combination of gentle suction from the biopsy pipette and laser beam pulses directed at cell junctions to separate the target cells from the rest of the embryo. Similar to McArthur's strategy, this approach requires the embryo to be manipulated over two sessions; however, ICM herniation is avoided. Moreover, compared to the first method described by Capalbo, ZP opening may speed up logistics as cell herniation is facilitated compared to spontaneous hatching.

In the study discussed earlier, Scott et al. also assessed the impact of blastocyst stage biopsy on embryo's reproductive potential [25]. Differently from embryos biopsied at the cleavage stage, embryos that underwent TE biopsy had comparable implantation rates to non-biopsied sibling embryos. The reason for the higher performance of blastocyst stage compared to cleavage stage biopsy are mainly three: (i) TE is a non-embryonic tissue and the risk of interfering with cells committed to ICM differentiation is avoided; (ii) the biopsy is performed after embryonic genome activation, hence the embryo has higher reproductive potential compared to an embryo at an earlier developmental stage; and (iii) a smaller proportion of embryonic mass is removed (5–10 cells out of around 100). Furthermore, blastocyst stage biopsy ensures a more accurate and robust diagnosis. Specifically, all validation studies to date reported a 98%–100% correct prediction of meiotic errors through comprehensive chromosomal

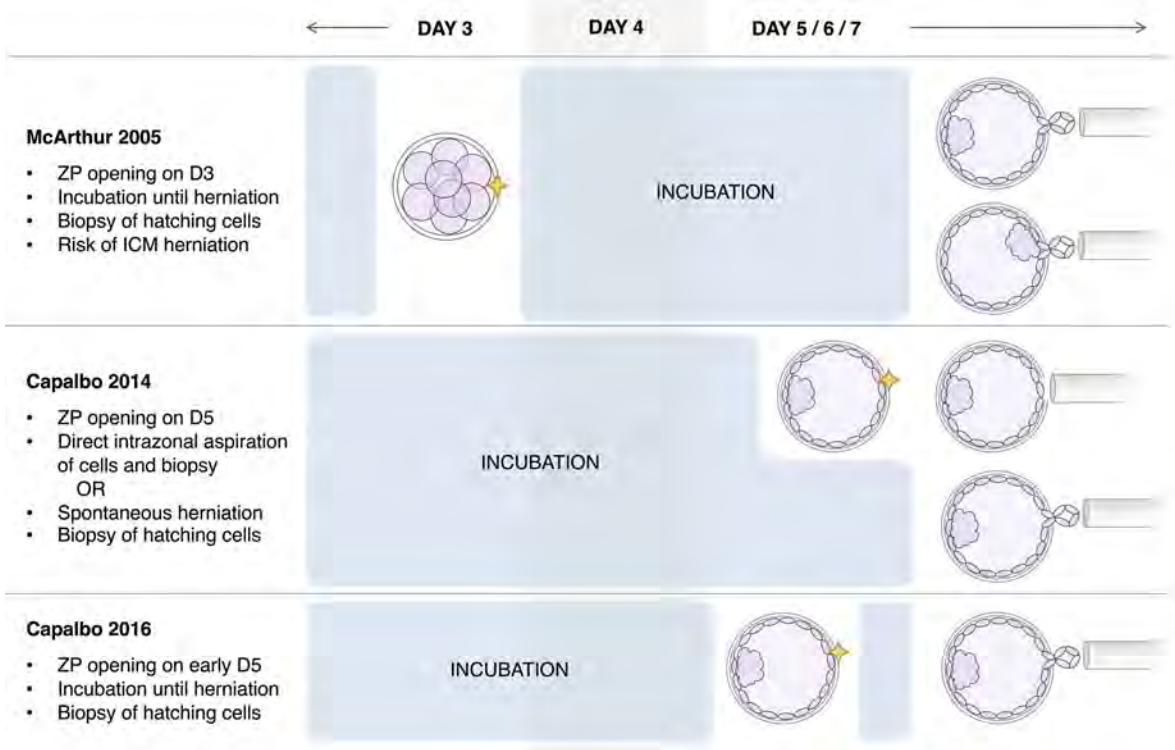

FIGURE 13.2 Three different strategies for zona opening and trophectoderm biopsy. McArthur 2004 involves ZP opening on day 3 of culture and subsequent biopsy of herniating cells at the blastocyst stage (day 5/6/7). Limitations of this approach include potential herniation of ICM cells and double exposure of the embryo to suboptimal conditions (i.e. outside of the incubator). Capalbo 2014 involves ZP opening on day 5 and immediate biopsy of TE cells by contacting the cells within the intra-zonal space. This method avoids both double exposure of the embryo and ICM herniation; however, it may be more technically challenging for less experienced operators. Capalbo 2016 involves ZP opening on day 5 followed by short incubation to allow herniation of TE cells. This method requires double exposure of the embryo to suboptimal conditions; however, it avoids ICM herniation.

screening (CGT), a significant improvement from previous cytogenetic approaches (i.e. FISH) [32, 33]. The collection of multicellular biopsies, combined with sensitive next-generation sequencing technologies also allow the detection of intermediate chromosome copy numbers, possibly associated with mosaicism. However, technical accuracy in detecting mosaicism and its impact on embryo's health and reproductive competence are still at the centre of current scientific debate [34].

Non-invasive biopsy

Although there is no evidence of detrimental effects on the embryos when the biopsy procedure is carried out by experienced professionals, the learning curve for biopsy is steep. As more IVF laboratories look to implement PGT procedures, they face expensive and time-consuming training for their laboratory personnel. For this reason, several groups have investigated and developed non-invasive strategies for collection of embryo-derived DNA which could be employed as alternatives to embryo biopsy. To date, two non-invasive methods have been proposed, both relying on the collection of cell-free embryonic DNA: blastocoel fluid (BF) aspiration (i.e. blastocentesis) and spent culture media.

Blastocentesis

Blastocentesis consists in the aspiration of the BF from the embryonic cavity using a minimally invasive procedure employing an ICSI needle inserted through the TE wall [35–37]. During the aspiration of the fluid, which is enriched of fragmented DNA of embryonic origin, aspiration of cellular material should be avoided as excluded, free-floating cellular fragments may not be representative of the rest of the embryo. Once the sample has been obtained, it is then tubed for any genetic-molecular analysis. However, blastocoel-derived DNA has been shown to be present in low quantity and poor quality [38]. Accordingly, a number of studies have reported varying efficiencies in DNA amplification, ranging between 34.8% and 87.5% [37, 39–42]. Furthermore, significant differences affect concordance rates at single chromosome level between blastocentesis and conventional biopsy procedures [35, 40, 43–45].

Despite its easier application compared to TE biopsy, this technique requires further development and validation before clinical application can be considered.

Spent culture media

Embryonic DNA can also be found in the culture media where the embryo is being cultured in (i.e. spent culture media, SCM). Different studies have reported the presence of embryonic DNA in the SCM as early as day 2 or 3 of development [46]. This approach is completely non-invasive as it is based on the collection of cell-free DNA accumulated in the culture media and it does not involve embryo manipulation [47, 48]. Although DNA quantity and integrity appear to be superior in SCM compared

to the BF, overall parameters are inferior to TE biopsy specimens [49]. Nonetheless, cell-free DNA availability is dependent on both length of embryo culture prior to SCM collection and external DNA contamination [50]. It has been shown that culture media change on day 4 of culture, followed by SCM collection on day 5/6/7 can increase DNA yield and reduce maternal DNA carry-over from either cumulus cells or in cell-free state [51]. However, although improvements in DNA amplification rate (i.e. >95%) have been obtained by several groups [51–53], the overall diagnostic efficiency of this approach significantly varies across settings and remains unsatisfactory compared to TE biopsy approach even in the best scenarios (e.g. ~80% [50, 51, 54, 55]). Nevertheless, the extremely attractive prospect of obtaining a genetic diagnosis for an embryo without having to biopsy any of its cells surely warrants further endeavours aimed at improving the diagnostic output of this strategy.

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14

ANALYSIS OF FERTILIZATION

Thomas Ebner

Introduction

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) cycles have shown that women have only a finite number of gametes out of a pool of collected oocytes that are viable for generating a term pregnancy. This demonstrates the need for simple methods of pre-implantation embryo assessment in the prediction of pregnancy rates. In this respect, intensive research has been done at the zygote stage on day 1 of pre-implantation development.

Independently of the mode of fertilization, a sperm-borne enzyme called phospholipase C-ζ enters the oocyte and activates it via the inositol-3-phosphate pathway. In detail, this molecule binds to the corresponding receptor at the endoplasmic reticulum where it causes Ca^{2+} release in the form of oscillations. This Ca^{2+} response drives the extrusion of the second polar body and the formation of both pronuclei.

While conventional IVF more or less mimics natural fertilization, ICSI is a rather invasive procedure circumventing some of the major steps in the process of oocyte activation and fertilization. Consequently, the ICSI schedule differs slightly from the IVF one [1]. This delay is attributed to the time needed for the sperm to pass through the oocyte outer complex, particularly the cumulus and corona cells, along with the zona pellucida. Fusion of the spermatozoon with the oolemma and incorporation into the oocyte plasma, on the other hand, seem to occur very rapidly [2] via an orchestrated interaction between Izumo-1 (sperm) and Juno (egg) receptor proteins [3]. In ICSI, fertilization usually has to be assessed approximately two hours earlier (e.g. 16–18 hours post-insemination) than in IVF (18–20 hours post-insemination) in order to find identical developmental stages [4].

Timing of fertilization events

Either active propulsion (conventional IVF) or direct deposition (ICSI) ensures presence of a spermatozoon in the cytoplasm. There is evidence from time-lapse imaging studies indicating that regular fertilization follows a definite course of events, though the timing of these events may vary between eggs [5, 6]. Time-lapse technique further allows for the annotation of time specific morphological changes during oocyte activation, fertilization, and further embryo development which is referred to as morphokinetics.

With respect to this, a time-lapse user group proposed guidelines on the nomenclature of human embryo development, including the dynamic fertilization process [7]. Per the definition, the time at which insemination occurs (IVF or ICSI) is called t0. Consequently, tPB2 marks the time at which the second polar body is extruded and tPN marks the time at which the fertilization status is confirmed. For proper analysis of the time period in which the two pronuclei are visible (VP), their appearance (tPNa) and fading (tPNf) should be documented. It is important to note the time of time-lapse pronuclear assessment (tZ) since the pronuclear pattern is a dynamic event and its morphology can change between tPNa and tPNf [8].

Approximately 90% of the oocytes showed circular waves of granulation within the cytoplasm [5] after ICSI. During this granulation phase, the head of the spermatozoon decondensed. Subsequently, the second polar body was extruded. A characteristic fertilization cone, probably reflecting an interaction between the male chromatin and the oocyte's cortex, was not always observed [6]. The next steps would involve the central formation of the male pronucleus and the peripheral formation of the female counterpart. The latter was then drawn towards the male pronucleus until the two abutted. Data from the literature suggest [9] that during this process the male pronucleus rotates onto the female one, in which the chromatin condensates on the side facing the centre of the egg, in order to also align its chromatin towards the spindle forming between both pronuclei. Both pronuclei then increase in size, and their nucleoli move around and arrange themselves near the common junction [5].

Within both nuclei, nucleoli form at sites on the DNA known as the "nucleolar organizing regions" located on the chromosomes where the ribosomal genes are situated [10]. This means that the nucleoli are the active sites of rRNA synthesis. During the course of development, nucleoli tend to fuse due to an increase in protein synthesis [5, 11]. It should be emphasized once again that IVF zygotes reach the final stage of nucleolar organization at a later time than ICSI zygotes.

The size and distribution patterns of the nucleoli may serve as prognostic parameters of the events of fertilization, the completion of meiosis, and the cell cycle, leading to the first mitotic division, the normality of the chromatin complement in the two nuclei, and the formation with chromosome attachment of the mitotic spindle [10].

In particular, asynchrony in formation and polarization of nucleoli (Figure 14.1a) may severely impair further development of the pre-implantation embryo [12–16]. Consequently, good-quality embryos can arise from oocytes that had more uniform timing from injection to pronuclear abuttal [5].

Pronuclear grading

According to the aforementioned agreement [5], pronuclear pattern assessment using time-lapse imaging should be done immediately before tPBf. However, embryologists are faced with several pronuclear patterns at the time of fertilization assessment. Based on original data from Wright et al. [17], Scott and Smith [13] were the first to attribute zygote morphology with a certain prognostic value for subsequent implantation. In particular, the alignment of nucleoli at the junction of the two pronuclei was found to be a selection criterion for embryo transfer. Since this zygote score did not exclusively rely on the pronuclear pattern but also comprised multiple other parameters, including the appearance

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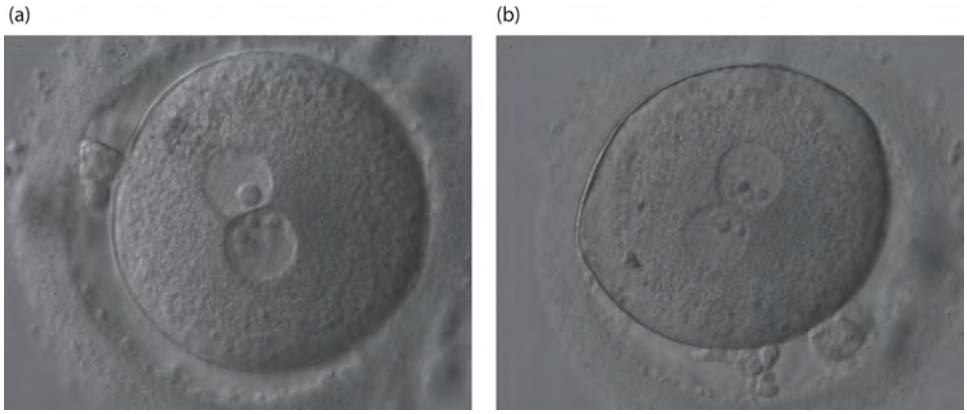

FIGURE 14.1 (a) Bad-prognosis zygote with an asymmetric pronuclear pattern corresponding to pattern 4 [14] or Z3 [16]. (b) Zygote showing optimal pronuclear pattern 0B [14] or Z1 [16] and a clear halo.

of cytoplasm and timing of nuclear membrane breakdown, the actual impact of pronuclear morphology on further outcomes remained unclear.

Thus, Tesarik and Greco [14] were the pioneers in predicting pre-implantation development by focusing exclusively on the number and distribution of nucleoli (nucleolar precursor bodies [NPBs]) in each pronucleus. They considered inter-pronuclear synchrony, evaluated 12–20 hours post-IVF/ICSI, as being more important than the actual NPB polarity at the site of pronuclear apposition since they presumed that polarization of nucleoli is not evident from the beginning of pronuclei formation, but rather appears progressively with time [11]. According to Tesarik and Greco [14], the optimal synchronized pattern 0 yields 37.3% good-quality embryos compared to all other patterns (27.8%). In addition, the frequency of developmental arrest of pattern 0 zygotes was only 8.5% as compared with 25.6% in the other patterns.

Since all these previous reports were of retrospective character, particular importance must be assigned to a prospective multicentre study of Montag and Van der Ven [4]. These authors highlighted that cycles with transfer of at least one embryo derived from pattern 0B (Figure 14.1b), but not pattern 0A, resulted in significantly higher rates of pregnancy (37.9%) and implantation (20.5%) than nonpattern 0B cycles (26.4% and 15.7%). Similar results have been published by others [15] who found significantly increased pregnancy rates (44.8% vs. 30.2%) if embryos derived from zygotes with pattern 0 were transferred. Obviously, NPB polarization at the area of pronuclear contact outdoes pronuclear symmetry.

Scott et al. [16, 18] further refined their score by also creating a single observation zygote score. This so-called Z-score was comparable with the score introduced by Tesarik and Greco [14], since patterns Z1 and Z2 resemble patterns 0B and 0A. Several other authors successfully used the zygote scores of Scott et al. [13, 15] and Tesarik and Greco [14] for prognostic purposes [4, 15, 19–22]. Though the grading systems differ slightly in some of these papers, the conclusion is a common one. Zygotes showing pronuclei with approximately the same number and alignment of NPBs in the furrow between the nuclei had the best prognosis in terms of subsequent implantation.

It is noteworthy that Salumets et al. [23] failed to show any correlation between zygote score and pregnancy rate. This is of particular interest because this group only analysed single-embryo transfers and, consequently, the actual implantation potential could be accurately estimated. Though two different scores were applied [13, 14], no correlation to treatment outcome could be demonstrated. This discrepancy in literature results may be explained by the use of different culture media and stimulation protocols and differences in timing of fertilization assessments (e.g. the inclusion of early cleavage in the Scott and Smith [13] scoring system).

An increased incidence of subsequent blastocyst formation in zygotes with optimal patterns of the pronuclei [16, 21, 24] seems to be consistent with the reported increase in terms of pregnancy rate. Theoretically, a lower blastocyst formation rate in abnormal zygotes could be related to their chromosomal status since there is information from the literature that several pronuclear patterns seem to be associated with aneuploidy [25–28].

In detail, Kahraman and colleagues [27] found a 52.2% rate of chromosomal abnormality in biopsied embryos derived from suspicious zygotes (showing an asymmetric distribution of NPBs), which was significantly lower than the observed 37.6% in the normal control zygotes. Others [28] also confirmed that the position of pronuclei within the cytoplasm, the size and distribution of nucleoli, and the orientation of polar bodies with respect to pronuclei were highly predictive of the presence of chromosomal abnormalities in the corresponding embryos. In this study [28], zygotes with abutted pronuclei, large-sized nucleoli, and polar bodies with small angles subtended by pronuclei and polar bodies were the configurations associated with the highest rates of euploidy. Using the Z-score, it could be shown [26] that Z1 patterns had a significantly higher rate of euploidy (71%) as compared to Z3 (35%) and Z4 (36%) patterns. The same also holds true for the score of Tesarik and Greco [14], since pattern 0 was associated with a minimal rate of aneuploidy (26%), whereas patterns with poor prognosis showed higher rates of up to 83% [25].

It is important to note that not all studies published to date suggest complete reliance on zygote morphology [23, 29]. One problem is that overall up to 14 different zygote scoring systems have been published so far. On the basis of those papers that made their way into a recent meta-analysis [30], it can be concluded that there is a lack of conclusive data on the clinical efficacy of zygote scoring.

Further evidence on the limited potential of pronuclear scoring comes from time-lapse imaging since none of the tested scoring systems [18, 31] were shown to predict the live birth outcome [8]. On the other hand, tPNf occurred significantly later in embryos

resulting in live birth and was never observed earlier than at 20 hours and 45 minutes [8].

A definite difference between IVF and ICSI cycles with regard to the frequency of good patterns (pattern 0 according to Tesarik and Greco [14]) was reported [4]. In particular, superior pronuclear patterns were observed in ICSI cycles. This phenomenon may be due to the aforementioned accelerated course of development in ICSI [1, 32]. Zygotes showing this most advanced stage of nuclear polarization seem to reach that stage earlier after ICSI than after conventional IVF [4].

However, the study did not evaluate the position of the pronuclei relative to the presumed polar axis. This arrangement has been reported to relate to embryo quality [33, 34]. Edwards and Beard (35) suggested that the oocyte may establish this polarity by either ooplasmic or pronuclear rotation towards the second polar body. Such a resetting of a new axis after fertilization is governed by cytoplasmic contraction waves organized by the sperm centrosome [35]. Embryos unable to achieve optimal pronuclear orientation, possibly due to shorter cytoplasmic waves [5], may exhibit poor morphology (e.g. uneven cleavage or fragmentation) [33].

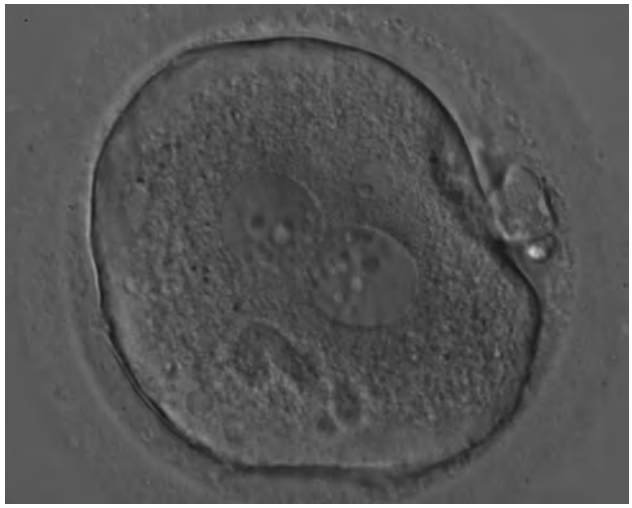

FIGURE 14.2 Zygote showing two pronuclei with an additional smaller nucleus (2 o'clock position) possibly containing chromosomal material.

Abnormal pronuclear formation and patterns

Single-pronucleate (1PNs) zygotes can be obtained following IVF and ICSI at frequencies ranging from 2% to 5% (36). They were reported to show a trend towards higher frequency in ICSI [36].

Karyotyping indicated that following IVF more than half of 1PN embryos are in fact diploid, but these studies [37, 38] did not differentiate between diploidy produced by fusion of both pronuclei or fertilization by parthenogenetic activation. However, in further studies it could be demonstrated that when embryos were diploid, approximately half of them were fertilized [37, 38]. Two mechanisms could be responsible for this observation: asynchronous appearance/fading [39] or fusion of both pronuclei [40]. If there is no other choice, such IVF embryos could be considered for transfer, particularly if the single pronucleus is larger than regular size. With respect to this, a recent time-lapse study [41] identified a cut-off value for the single pronuclear area (713 μm^2) and diameter (31 μm^2), which allowed to distinguish between those 1PN-zygotes that made it to blastocyst stage and those which did not (AUC 0.662 and 0.661, respectively). For the prediction of blastocyst quality AUC was even higher, e.g. 0.848 and 0.827 [41]. Of note, IVF 1PN zygotes performed better as compared to the ICSI counterpart [41].

Recent data [42] on PGT-A cycles challenge the dogma that 1PN zygotes derived from ICSI should rather not be transferred [43, 44] since close to 70% of such abnormally fertilized oocytes turned out to be diploid and some of them did result in live births.

The presence of 3PN zygotes after IVF is the most common fertilization anomaly in humans. This is mostly caused by dispermy (3PN, two polar bodies), and the majority of the corresponding embryos will cleave but stop development at later stages [36]. In ICSI, some 4% [36] of zygotes show digynic triploidy, meaning that a single sperm is present in the egg but the second polar body was not extruded (non-disjunction). In this case, the chromosomes of the three pronuclei are organized in a single bipolar spindle at syngamy, indicating that only one centrosome deriving from one sperm is active. Time-lapse imaging has shown that close to 75% of tripronuclear zygotes directly cleave into three cells [45], a phenomenon which is known as trichotomous mitosis (6).

Within 3PN zygotes, a special case is the presence of 2PNs with a third additional small nucleus which Capalbo et al. [42] referred to as 2.1PN zygotes (Figure 14.2). Since the same authors have shown that the presence of a smaller nucleus can be associated with chromosomal loss or gain, the decision to select such embryos for transfer has to be carefully weighted. Due to the sometimes-small size of these additional nuclei, there is of course a high risk of missing them during routine fertilization checks, especially when using objectives of lower magnification. Again time-lapse imaging would facilitate the scoring process and would increase the change not to miss 2.1PN zygotes.

Peripheral positioning of pronuclei

Regardless of the pronuclear pattern that the oocyte reflects, it is generally accepted that both pronuclei should be located in the centre of the female gamete. Cytoplasmic inclusions, such as dense granularity, large refractile bodies, and/or vacuoles, may displace both pronuclei. However, this scenario can also happen in zygotes with normal homogeneous ooplasm. Any deviation from the presumed optimal central arrangement (e.g. peripheral apposition of both pronuclei) (Figure 14.3) is most likely associated with reduced developmental capacity [33]. Considering the fact that the first cleavage plane runs through the contact zone of both pronuclei, it is a frequent phenomenon that the corresponding embryo will show uneven cleavage. This scenario is more frequent in conventional IVF than in ICSI (3.3% vs. 11.8%), probably due to varying sites of sperm entrance in IVF [34] (e.g. near-spindle penetration of the zona, which in turn could force eccentric formation of pronuclei [9]).

Non-juxtaposition of pronuclei

Another problem occasionally arising during fertilization is a failure in alignment of both pronuclei (Figure 14.4), which is caused by an intrinsic defect of the cytoskeleton, or the parental centrosome may cause a complete failure in alignment [13]. While it is quite uncommon in assisted reproduction technologies (approximately 1%–2%), it is rather detrimental since the

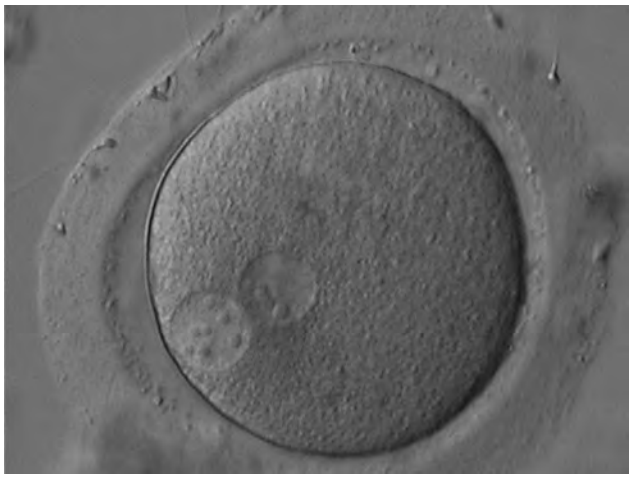

FIGURE 14.3 *In vitro* fertilization zygote showing peripheral apposition of both pronuclei.

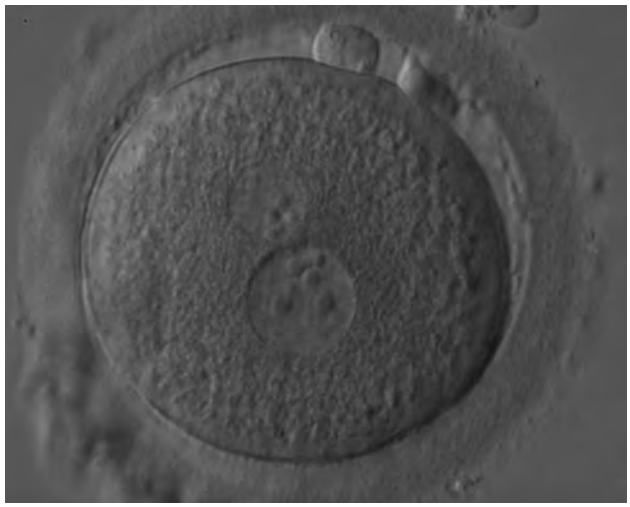

FIGURE 14.5 Zygote with uneven pronuclear size.

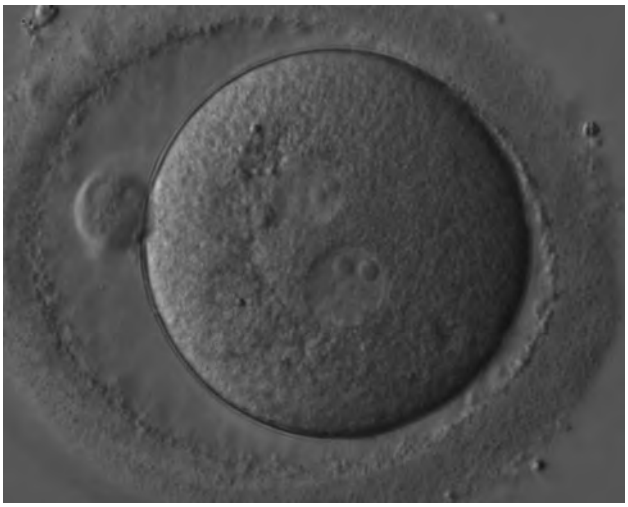

FIGURE 14.4 Zygote with failure in alignment of both pronuclei.

vast majority of zygotes with unaligned pronuclei fail to cleave or show developmental arrest at early stages [14] if not resulting in chromosomal aberrations at all [28]. Morphokinetic deviations from the considered normal development includes a higher rate of trichotomous mitosis and an increased interval between tPNf and t2 [39].

Uneven size of pronuclei

Though the female pronucleus usually is smaller than its male counterpart [5], more extensive differences in size (>4 µm) may be observed *in vitro* (Figure 14.5). This divergence most likely is the result of problems arising during male pronucleus formation, since *in vitro*-matured oocytes from ICSI with labelled spermatozoa showed the proximity of the fluorescent sperm mid-piece remnant to the smaller pronucleus [46]. Uneven pronuclear size severely affects the viability of the corresponding embryos since more than 87% were found to be aneuploid, mostly mosaics [47, 48]. This fact probably led them to arrest at a significantly higher rate than zygotes with pronuclear diameters showing no excessive differences. In addition, a higher incidence of day-2 multinucleation was observed [47].

Undocumented zygotes

Interestingly, 1% of all zygotes do not show pronuclei at all [36]. Manor et al. [48] demonstrated that 57% of such undocumented zygotes are normal diploid. If two polar bodies were present on day 1, corresponding embryos may be considered for transfer in case insufficient bipronucleated embryos are available. The most probable reason for this failure in detection is an abnormal developmental speed and/or inaccurate timing of fertilization control. It has also been reported that pronuclei may be hidden to extensive cytoplasmic granularity [36].

Cytoplasmic halo

Immediately prior to pronuclear growth, a microtubule-mediated withdrawal of mitochondria and other cytoplasmic components contracts from the cortex towards the centre of the oocyte, leaving a clear halo around the cortex [5]. Since the presence of a halo effect (Figure 14.1b) within the ooplasm may be recognized in 65%–85% of all zygotes [21, 23, 49], it is less applicable for scoring purposes than the pronuclear pattern. Nevertheless, this particular morphism was found to be correlated with better embryo quality [18, 23], increased blastocyst formation on day 5 [50], and a higher pregnancy rate [49].

The physiological role of mitochondrial redistribution in zygotes is still unknown, but it has been speculated that clustering of mitochondria to perinuclear regions may be involved in cell cycle regulation [51–53] (e.g. by means of calcium mobilization and ATP liberation [54–56]). In addition, location of mitochondria next to the pronuclei would allow immature mitochondria, as seen in zygotes [57], to complete maturation, presuming that some input from the nucleus is needed [53].

There is a certain disagreement between most of the studies dealing with cytoplasmic appearance at zygote stage. Some did not distinguish between several types of haloes, thus pooling symmetrical and polar haloes [13, 23], whereas others presuppose that symmetrical [49] or extreme haloes [50] are abnormal.

In view of this lack of uniformity, our working group [21] set up a prospective trial to investigate the actual influence of certain subtypes of haloes on the pre-implantation development of IVF and ICSI embryos. In this paper, haloes were measured accurately in order to see if a light or extreme halo effect would have any impact on subsequent developmental stages. Based on our findings, it was concluded that any halo effect, irrespective of its grade and dimension, is of positive predictive power in terms of blastocyst quality and, consequently, clinical pregnancy rate [21]. Neither the method used for insemination (IVF or ICSI) nor the presence of areas of dense cytoplasmic granulation or larger vacuoles affected the zygote in terms of halo performance. Furthermore, it was demonstrated that the pronuclear pattern and halo formation are two distinct parameters [21]. In contrast to the pronuclear pattern, no association between halo formation and genetic status of the fertilized egg has been observed [58].

The only available time-lapse study on the presence of the halo effect [59] reports higher rates of irregular cleavages, particularly rapid cleavage, cell fusion, and asymmetrical division in halo-negative zygotes. Additionally, the prolonged presence of the halo was associated with lower ongoing pregnancy rates.

Conclusion

During evaluation of zygote morphology, it has to be considered that both halo and pronuclear formation follow a fixed schedule. Since direct ooplasmic placement of a viable spermatozoon is performed in ICSI, thus bypassing most steps of fertilization (including acrosome reaction and zona binding), the further course of development will be somewhat accelerated as compared to conventional IVF. It is of interest that more physiological sperm selected on the basis of its potential to bind to hyaluronic acid did not influence the pronuclear score [60].

Pronuclear morphology and halo characteristics turned out to be unstable independent factors within the dynamic process of fertilization. The degree and morphology of the halo per se have no influence on further outcome. However, the presence of such a halo had positive predictive power. Consequently, halo formation in combination with optimal pronuclear patterns (e.g. those with alignment of fused nucleoli) will characterize a subgroup of oocytes showing a developmental advantage compared to zygotes lacking these positive predictors.

This is in line with recent findings indicating that during syngamy those zygotes with an accelerated breakdown of the pronuclear membranes at 22–25 hours post-insemination or post-injection implanted significantly more frequently than those with delayed dissolution [61]. This is not to forget the reported positive correlation between the occurrence of the first mitotic division and the rates of implantation and clinical pregnancy [62–64].

Recently, promising strategies have been published combining the morphological information of zygote stage with other developmental stages [65–69]. In detail, sequential assessment of cultured human embryos allowed for accurate prognosis in terms of good-quality blastocyst development [67, 68]. Others [65] found a relatively high outcome predictability after IVF using a combined score for zygote und embryo morphology and growth rate. Finally, day-3 embryo transfer with combined evaluation at the pronuclear and cleavage stage compared favourably with day-5 blastocyst transfer [66].

This suggests that zygote stage, although being an important developmental phase, should not be used solitarily as a prognostic parameter, but rather morphological information from day 1 should be pooled with that of earlier and/or later stages in order to maximize benefit and minimize the numbers of embryos transferred. The contribution of deep learning and artificial intelligence techniques to automatic detection and particularly scoring of pronuclei is not yet assessable but first publications are available [70].

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15

CULTURE SYSTEMS FOR THE HUMAN EMBRYO

David K. Gardner

Introduction

Embryo culture is often mistaken for a relatively simple procedure, when in reality it is a complex task, requiring a high level of training for embryologists, together with proactive quality control and quality assurance programs to ensure the optimum performance of the laboratory and equipment. Further, a sufficient number of suitable workstations and incubation chambers are required to maintain a stable environment for embryo development *in vitro*. Evidently, embryo culture is far more involved than simply using the appropriate culture media formulations. Consequently, in order to optimize embryo development *in vitro* and maintain viability to ensure the delivery of a healthy baby, it is essential to consider embryo culture as a system in its entirety. The embryo culture system consists of the media, macromolecules, gas phase, type of medium overlay, the culture vessel, the incubation chamber, ambient air quality and even the embryologists themselves. The concept of an embryo culture system exemplifies the interactions that exist not only between the embryo and its physical surroundings, but between all parameters within the laboratory (Figure 15.1) [1]. Only by taking such a holistic approach can one optimize embryo development *in vitro* and maintain success rates.

Working *in vitro*, literally "in glass," means that stressors are present in the culture system, which are not present within the lumen of the female reproductive tract. Stressors identified in the embryology laboratory which can have a negative impact on gametes and embryos include: transient temperature shifts as gametes and embryos are manipulated, increases in medium osmolarity if the medium is not covered with an oil overlay, changes to the levels of carbon dioxide and hence changes in pH when embryos are taken in and out of an incubator, potential physical stress should pipetting be too vigorous, atmospheric oxygen (even transient exposures are detrimental and cumulative), and the accumulation of ammonium from amino acids [2]. Of practical significance, the earlier stages of development, particularly prior to compaction, are the most susceptible to such stressors, and this is represented schematically in Figure 15.2. Furthermore, stressors have the capacity to interact with each other and create negative synergies which has significant adverse consequences for embryo development and viability [2, 3].

Finally, it is important to appreciate that it is not feasible to make a good embryo from poor quality gametes (current investigations on oocyte rejuvenation through mitochondrial transfer not withstanding). Rather, the role of the laboratory is to maintain the inherent viability of the oocyte and sperm from which the embryo is derived. Hence, the success of IVF is dependent on the quality of the ovarian stimulation determined by the physician, the preparation/development of a receptive endometrium, as well as on patient factors including the impact of their lifestyle choices (especially diet), hence emphasizing the need for a broader perspective of patient management as well as laboratory management. Consequently, in order to ensure consistent successful outcomes, it is paramount that appropriate communication pathways exist between physicians and scientists to ensure all variables are considered and discussed, and that action plans are in place, so that changes can be rapidly implemented in response to any concerns.

The human embryo in culture

Serendipitously for the development of human IVF, the human embryo exhibits a considerable degree of plasticity, enabling it to develop under a wide variety of culture conditions. Indeed, it appears that the human preimplantation embryo is the most resilient of all mammalian species studied to date with regards to its ability to tolerate a range of culture environments. However, this should be perceived as a reflection of the ability of the human embryo to adapt to its surroundings and not our ability to culture it. Undoubtedly, having to adapt to sub-optimal collection and/or culture conditions comes at the cost of impaired viability and potentially compromised pregnancy outcomes [2, 4, 5]. Therefore, it is important to focus on the generation of healthy embryos, as it is clear that embryo development in culture, even to the blastocyst stage per se, does not necessarily equate to the development of a viable embryo [1, 6, 7]. Implantation rate (fetal heart rate) is a key parameter utilized to evaluate the performance of the IVF laboratory in this regard, as it provides relatively quick information on cycle performance. However, the definition of viability is best defined as the ability of the embryo to implant successfully and give rise to a normal healthy term baby. Hence, only live birth rates and cumulative pregnancy rate per retrieval reflect the true efficacy of an IVF laboratory.

Today, clinics are not only faced with a multitude of embryo culture media to choose from and what gas phases to employ, but also with the decision of whether to transfer at the cleavage or the blastocyst stage. Data accumulated over the past 25 years indicate an increase in pregnancy and implantation rates and reduced pregnancy loss following blastocyst culture [8, 9]. This, combined with a move to perform preimplantation genetic screening through trophectoderm biopsy and next generation sequencing, and embryo vitrification at the blastocyst stage [10], means it is important that laboratories are able to support extended culture. It is therefore the aim of this chapter to discuss the types of media and culture systems currently available to support viable blastocyst development, and to describe how such systems can be successfully implemented in a clinical setting.

Significance of single embryo transfer for the laboratory

It is evident that with the development of enhanced culture systems and better methods for embryo selection (see Chapter 16 by Sakkas and Gardner) [7] and cryopreservation (Chapters 20

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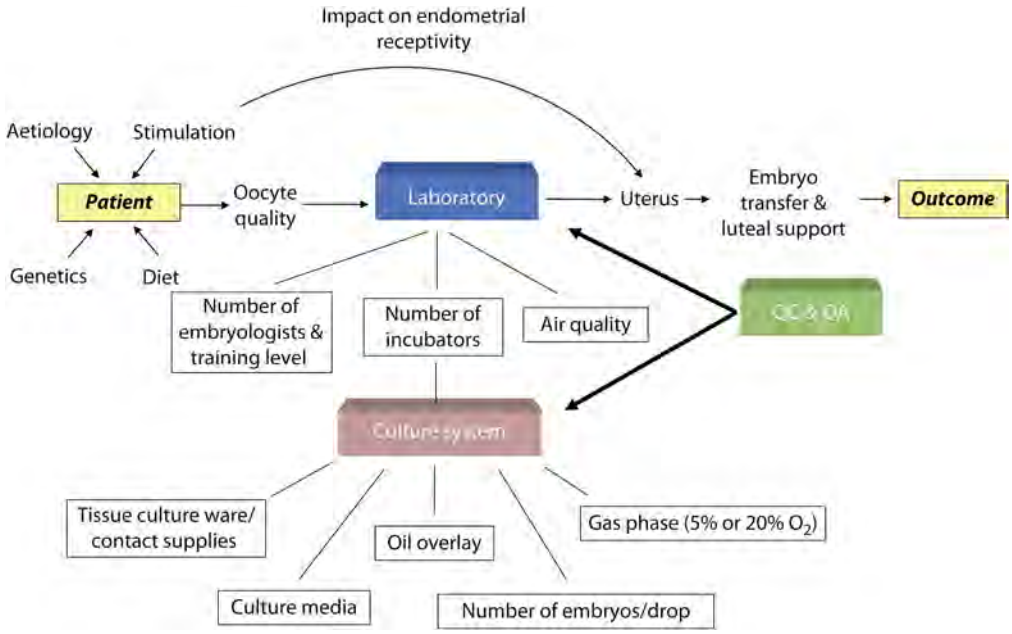

FIGURE 15.1 Holistic analysis of the human IVF laboratory and transfer outcome. This figure serves to illustrate the complex and interdependent nature of human IVF treatment. For example, the stimulation regimen used not only impacts on oocyte quality (and hence embryo physiology and viability [245]), but can also affect subsequent endometrial receptivity [186, 246–248]. Furthermore, the health and dietary status of the patient can have a profound effect on the subsequent developmental capacity of the oocyte and embryo [249, 250]. The dietary status of patients attending IVF is typically not considered as a compounding variable but growing data would indicate otherwise. In the schematic, the laboratory has been broken down into its core components, only one of which is the culture system. The culture system has in turn been broken down to its components, only one of which is the culture media. Therefore, it would appear rather simplistic to assume that by changing only one part of the culture system (i.e. culture media), that one is going to mimic the results of a given laboratory or clinic. One of the biggest impacts on the success of a laboratory and culture system is the level of quality control and quality assurance in place. For example, one should never assume that anything coming into the laboratory that has not been pre-tested with a relevant bioassay (e.g. mouse embryo assay), is safe merely because a previous lot has performed satisfactorily. Only a small percentage of the contact supplies and tissue culture ware used in IVF comes suitably tested. Therefore, it is essential to assume that everything entering the IVF laboratory without a suitable pre-test is embryo toxic until proven otherwise. In our program the 1-cell mouse embryo assay (MEA) is employed to pre-screen every lot of tissue culture ware that enters the program, i.e. plastics that are approved for tissue culture. Around 25% of all such material fails the 1-cell MEA (in a simple medium lacking protein after the first 24 hours) [213]. Therefore, if one does not perform QC to this level, one in four of all contact supplies used clinically will be embryo toxic. In reality many programs cannot allocate the resources required for this level of QC and when embryo quality is compromised in the laboratory it is the media that are held responsible, when in fact the labware are more often the culprit. (Modified from [1], with permission.)

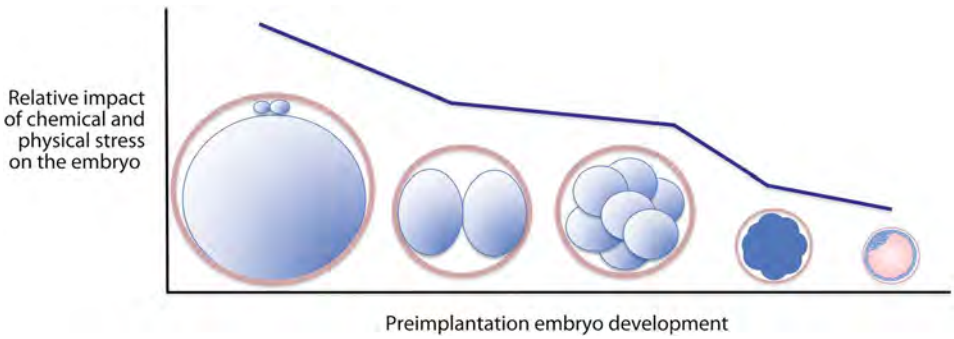

FIGURE 15.2 Relative impact of chemical and physical stress on preimplantation embryo (from oocyte to blastocyst stage), representing the stage-specific differences in the embryo's response to stress. The fertilized oocyte being more sensitive than the cleavage stage embryo, which in turn is more susceptible to stress than an embryo post-compaction. Of all stages the blastocyst is least perturbed by such factors. (From [3], with permission.)

to 22), that single embryo transfer (SET) is the standard of care for our patients [11], thereby alleviating one of the greatest problems associated with IVF, that being multiple births [12]. One of the impacts of uptake of SET is the increased reliance the laboratory has on a successful cryopreservation program. Therefore, an important consideration in assessing the efficacy of any culture system is its ability to produce high quality embryos that can survive cryopreservation as this has a significant impact on cumulative pregnancy rates per retrieval. Of significance, the culture conditions themselves have a profound effect on cryopreservation outcome, particularly the inclusion of hyaluronan in the media, discussed later in this chapter.

Dynamics of embryo and maternal physiology

Before attempting to culture any cell type, be it embryonic or somatic, it is important to consider the physiology of the cell in order to establish its nutrient requirements. The mammalian embryo represents an intriguing scenario in that it undergoes significant changes in its physiology, molecular regulation and metabolism during the preimplantation period. The preimplantation human embryo is a highly dynamic entity which changes its needs as development proceeds. Indeed, it goes from being one of the most quiescent tissues in the body (the pronucleate oocyte), to being amongst the most metabolically active (the blastocyst) within just four days [4, 13, 14]. Interestingly, the pronucleate oocyte, like the MII oocyte from which it was derived, exhibits relatively low levels of oxygen consumption and prefers the carboxylic acid pyruvate as its primary energy source [13, 15]. Lactate can be utilized as sole substrate only from the 2-cell stage, while glucose is only consumed and utilized in relatively small amounts by the cleavage stage embryo [16]. The balance of mitochondrial and cytoplasmic metabolism is critical at these early stages of development to maintain adequate levels of ATP production [17]. However, despite the low levels of biosynthetic activity at these early stages of development there is an increasing awareness of a significant amount of remodelling of the nucleus. For example, there are major changes in methylation and acetylation levels, with many of the processes involved still to be elucidated [18–20]. Nevertheless, what is critical is that many key developmental events, such as activation of the egg and regulation of methylation and acetylation, are regulated by proteins whose activity is dependent on metabolic activity and appropriate regulation [21–24]. Therefore, maintenance of metabolic homeostasis at these early stages is paramount for the maintenance of viability (Figure 15.2).

As development proceeds and energy demands increase with cell multiplication, transcription following activation of the embryonic genome, subsequent increases in protein synthesis and the formation of the blastocoel, there is a concomitant increase in energy requirement and in glucose utilization. By the blastocyst stage, the embryo exhibits high oxygen utilization and an ability to readily utilize glucose, along with other energy sources. However, in spite of high levels of oxygen consumption, the blastocyst produces significant amounts of lactate, a phenomenon known as aerobic glycolysis, also referred to as the Warburg effect after Otto Warburg who first characterized this idiosyncratic metabolism in cancers [25]. As an aside, Warburg proposed that the mitochondria in cancer cells were dysfunctional, hence the significant production of lactate. However, it transpires that the mitochondria of cancer cells can be very active, and the lactic acid produced, which creates an acidic microenvironment, is actually a means to facilitate tissue invasion, or in the case of the blastocyst to promote successful implantation [26, 27]. The mechanisms underpinning the metabolic shift during preimplantation development have been reviewed and remain an ongoing area of research [13, 14].

TABLE 15.1 Differences in Embryo Physiology Pre- and Post-Compaction

Pre-Compaction	Post-Compaction
Low biosynthetic activity	High biosynthetic activity
Low QO_2	High QO_2
Pyruvate preferred nutrient	Glucose preferred nutrient
Requirement for specific amino acids including alanine, aspartate, glutamate, glycine, proline, serine and taurine	Requirement for a more comprehensive group of amino acids
Maternal genome	Embryonic genome
Individual cells	Transporting epithelium
One cell type	Two distinct cell types: ICM & Trophectoderm

Table 15.1 highlights some of the differences between the pre- and post-compacted embryo. In many ways the physiology of the cells of the embryo prior to compaction, hence before the formation of a transporting epithelium, can be likened to unicellular organisms [28]. This in part explains why those amino acids present at high levels in the oviduct and classified as "non-essential" for tissue culture purposes, are beneficial to the cleavage stage embryo, as they ensure several key cell functions, as described later.

Within the human female reproductive tract, the nutrients available mirror the changing nutrient preferences of the embryo. At the time when the human embryo resides in the oviduct, the fluid within is characterized by relatively high concentrations of pyruvate (0.32 mM) and lactate (10.5 mM), and a relatively low concentration of glucose (0.5 mM) [29]. In contrast, uterine fluid is characterized by relatively low levels of pyruvate (0.1 mM) and lactate (5.87 mM), and a higher concentration of glucose (3.15 mM), consistent with the changes in embryo energy production. These nutrient gradients do not only provide appropriate stage specific energy substrates, but also provide stage specific signals important not just for metabolism but also for the control of molecular signalling [14].

Susceptibility of the preimplantation embryo to stress

Although the early embryo is highly adaptive to its environment, it does so at the cost of normal cellular processes and checkpoints that may be essential for viability. Therefore, as a result many embryos can appear to be morphologically normal while at a cellular level are actually highly perturbed and unlikely to be viable [5, 28]. It is clear from animal models, where invasive assessments allow additional insight, that disruptions to molecular pathways including stress response pathways frequently occur in the absence of any changes to embryo morphology. Furthermore, frequently these perturbations are permissive of implantation but affect subsequent fetal growth [30–32]. Consequently, a key focus

of the embryology laboratory should be to ensure its gamete collection and culture system are able to maintain normal cellular physiology in order to ensure the health of the embryo.

Cleavage stages versus post-compaction embryo and stress

As a result of its more "primitive" physiology, the pre-compaction stage embryo is highly susceptible to stress compared to the post-compaction stage embryo. A stress applied *in vitro* at the 2PN to the 8-cell stage can have harmful effects on normal cellular physiology and viability of the subsequent blastocyst and fetus (Figure 15.2) [28, 33–35]. At these early stages of development prior to activation of the embryonic genome, the embryo possesses only limited capacity at a molecular level to respond to a stress. In somatic cells, when a cell finds itself in a hostile environment it can activate a cascade of molecular signalling pathways to engage systems to maintain normal development. However, the pre-compaction stage embryo has a limited capacity for gene transcription [36] and, therefore, the human embryo prior to the 8-cell stage is highly vulnerable to any perturbed environment. Further, at these early stages of embryo development prior to compaction there is limited capacity to maintain normal cellular functions such as regulation of intracellular pH (pHi) [37, 38], alleviation of oxidative stress and ionic homeostasis [5, 28]. Therefore, a stress applied prior to compaction can result in major disruptions to subsequent viability. In contrast, the application of the same stress post-compaction (i.e. post the formation of a transporting epithelium) and post-embryonic genome activation typically has limited negative impact on subsequent developmental competence [28, 34, 35]. Additionally, stress can be masked at the level of morphological assessment and may only become evident downstream of the stress itself. For example, the detrimental effects of a stress applied at the early stage of development during handling and culture of the oocyte and 2PN may not be evident until the blastocyst stage. Effects can be at a sub-cellular level with the embryo having reduced metabolic capacity, high levels of apoptosis, and altered molecular profile, which ultimately result in a reduction in pregnancy rates [33–35]. Therefore, the conditions employed for gamete collection and culture of the human cleavage stage embryo directly affect the ability of the embryo to implant and form a viable pregnancy, independent of morphological assessments within the laboratory. The inability of morphology alone to distinguish viable and non-viable embryos highlights a major limitation in the field and reaffirms the need for the development of more diagnostic procedures to quantitate normal development [7, 39] (see Chapter 16 by Sakkas and Gardner).

Composition of culture media

There are several extensive treatises on the composition of embryo culture media [40–50], and it is beyond the scope of this chapter to discuss in detail the role of individual medium components. However, two key components, amino acids and macromolecules, will be considered briefly due to their significant impact on cycle outcome. Understanding their effects on embryo physiology will greatly assist clinics to make a more informed decision regarding their choice of culture media.

Amino acids

It is certainly the case that the human embryo can grow in the absence of amino acids. The real question is how well do they develop in their absence and how healthy are the resultant embryos? There are several reasons for the inclusion of amino acids in embryo culture media. Oviduct and uterine fluids contain significant levels of free amino acids [51–56], while both oocytes and embryos possess specific transport systems for amino acids [57] to maintain an endogenous pool [58]. Amino acids are readily taken up and metabolized by the embryo [59, 60]. Table 15.2 lists the roles amino acids can fulfil during the pre- and peri-implantation period of mammalian embryo development.

TABLE 15.2 Functions of Amino Acids during Preimplantation Mammalian Embryo Development

Role	Reference
Biosynthetic precursors	[238]
Energy source	[239]
Regulators of energy metabolism	[4, 17]
Osmolytes	[154]
Buffers of pHi	[37]
Antioxidants	[240]
Chelators	[241]
Signalling	[242, 243]
Regulation of differentiation	[74, 244]

Oviduct and uterine fluids are characterized by high concentrations of the amino acids alanine, aspartate, glutamate, glycine, proline serine and taurine [51–56]. With the exception of taurine, the amino acids at high concentrations in oviduct fluid bear a striking homology to those amino acids present in Eagle's non-essential amino acids [61]. Studies on the embryos of several mammalian species, such as mouse [62–65], hamster [66, 67], sheep [68, 69], cow [70, 71], and human [72, 73], have all demonstrated that the inclusion of amino acids in the culture medium enhances embryo development to the blastocyst stage.

More significantly, it has been demonstrated that the preimplantation mouse and cow embryo exhibits a switch in amino acid requirements as development proceeds. Up to 8-cell stage non-essential amino acids and glutamine increase cleavage rates, [71, 74, 75], i.e. those amino acids present at the highest levels in oviduct fluid stimulate the cleavage stage embryo. However, after compaction, non-essential amino acids and glutamine increase blastocoel formation and hatching, while the essential amino acids stimulate cleavage rates and increase development of the inner cell mass in the blastocyst [33, 74]. Importantly, amino acids have been reported to increase viability of cultured embryos from several species after transfer to recipients [40, 69, 74] along with increasing embryo development in culture. In the mouse, equivalent implantation rates to *in vivo* developed blastocysts have been achieved when pronucleate embryos were cultured with non-essential amino acids to the 8-cell stage followed by culture with all 20 amino acids from the 8-cell stage to the blastocyst [49, 74].

Of note, those amino acids classified as non-essential act as strong intracellular buffers of pH due to their zwitterionic nature [66], and are able to chelate toxins. As discussed, prior to compaction the blastomeres of the mammalian embryo appear to behave like unicellular organisms and therefore use exogenous amino acids to regulate their homeostasis. In contrast, post compaction and the generation of a transporting epithelium, the embryo is able to regulate its internal environment and is not as dependent on the non-essential amino acids to regulate intracellular function [37]. However, the terms non-essential and essential have

little meaning in terms of embryo development and differentiation, rather they reflect the requirements of certain somatic cells *in vitro* [61], and consequently their use is rather restrictive when it comes to the embryo's changing requirements. Indeed, a case can be made for the inclusion of specific essential amino acids such as methionine and cysteine during cleavage stage development, the former for its role in methylation pathways and the latter for its antioxidant capabilities (see section "Antioxidants" later in the chapter) [76]. Clearly, the process of culture media optimization remains ongoing.

Of great practical and clinical relevance is that even a transient exposure (~ five minutes) of mouse pronucleate oocytes to medium lacking amino acids impairs subsequent developmental potential [77]. During this five-minute period in a simple medium the pronucleate oocyte loses its entire endogenous pool of amino acids, which takes several hours of transport to replenish after returning the embryo to medium with amino acids. This has direct implications for the collection of oocytes, and more importantly the manipulation of denuded oocytes during ICSI, where the inclusion of amino acids in the holding medium will decrease or prevent intracellular stress (see more on this later in this chapter). Hence, media lacking amino acids should not be used for any oocyte or embryo handling or culture. Consistent with this recommendation, the work of Ho et al. [78] on gene expression in mouse embryos cultured in the presence of amino acids was comparable to that of embryos developed *in vivo*. In contrast, mouse embryos cultured in the absence of amino acids, i.e. in a medium based on a simple salt solution, exhibited aberrant gene expression and altered imprinting of the H19 gene [79].

Macromolecules

Most culture media for the human embryo contain serum albumin as the protein source. Historically, serum was employed worldwide, however the use of serum is no longer condoned due its extensive documented detrimental effects on embryos [69, 80–83].

Although serum albumin is a relatively pure fraction of blood, it is still contaminated with fatty acids and other small molecules. The latter has been shown to include an embryotrophic factor, citrate, which stimulates cleavage and growth in rabbit morulae and blastocysts [84]. Not only are there significant differences between sources of serum albumin [85, 86], but also between batches from the same source [85, 87, 88]. Therefore, when using serum albumin or any albumin preparation, it is essential that each batch is screened by the manufacturer for its ability to adequately support mouse embryo development and human sperm survival prior to clinical use. Furthermore, new concerns with regards to the use of human serum albumin have been raised since it has been revealed that serum albumin, added as the protein supplement, is the source of detectable levels of Di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate, as well as polybrominated diphenyl ethers in human embryo culture media [89, 90]. In addition to these compounds, serum albumin preparations also contain variable levels of contaminants that include, carbohydrates, amino acids, transition metals, and growth factors. These contaminants will be modifying the compositions of the base media in a way that is essentially variable between lots and uncontrolled [91]. Such data infer that the use of serum albumin in clinical IVF warrants renewed consideration.

To this end, recombinant human albumin is available, which eliminates the problems inherent in using blood derived products and could lead to the standardization of media formulations. Recombinant human albumin has now been shown to be as effective as blood derived albumin in supporting fertilization [92] and embryo development, and its efficacy has been proven in a prospective randomized trial [93]. Significantly, embryos cultured in the presence of recombinant albumin exhibit an increased tolerance to cryopreservation [94]. Historically its clinical use has been restricted by price, however, with costs of such recombinant product falling, it is appropriate to re-evaluate its clinical use.

A further macromolecule present in the female reproductive tract is hyaluronan, which in the human and mouse uterus increases at the time of implantation [95, 96]. Hyaluronan is a high molecular mass polysaccharide that can be obtained endotoxin- and prion-free from a yeast fermentation procedure. Not only can hyaluronan improve mouse and bovine embryo culture systems [97, 98], but its use for embryo transfer results in a significant increase in embryo implantation [97, 99, 100]. In the largest prospective trial to date, which enrolled 1282 cycles of IVF, it was determined that the use of hyaluronan enriched medium was associated with significant increases in clinical pregnancy rates and implantation rates, both for day 3 and day 5 embryo transfers. The beneficial effect was most evident in women who were >35 years of age, in women who had only poor-quality embryos available for transfer, and in women who had previous implantation failures [100, 101]. A recent Cochrane report on 21 studies reported improved pregnancy and take-home baby rates when hyaluronan is included in the transfer medium [102], and a further study by Adeniyi and colleagues, analysing more than 3000 transfers, also concluded that the presence of high levels of hyaluronan in the transfer medium improved live birth events [103].

Of note, another highly beneficial effect of the inclusion of hyaluronan in the culture medium is on the cryo-survival of cultured embryos, data being obtained from a number of species including the human, mouse, sheep and cow [94, 99, 104–106]. As IVF programs are moving to transfer fewer embryos, there is an increasing need to be able to cryopreserve supernumerary embryos. The ability of a culture system to increase an embryo's ability to survive cryopreservation, thereby increasing cumulative pregnancy outcomes, is an important factor in deciding which culture system to use in the laboratory.

A cautionary tale of working in vitro

Even though the formulations of embryo culture media have improved significantly over the years, and for the most part have become more physiological in their basis, there is nothing physiological about a static drop of medium in a polystyrene culture dish or well. Therefore, one has to be careful about *in vitro* artefacts created by a static environment. A good example of this is the production of ammonium by both embryo metabolism of amino acids [107] and by the spontaneous breakdown of amino acids in the culture medium once incubated at 37°C (Figure 15.3a) [62]. Amino acids are increasingly used by human embryos as development proceeds [108], and by the blastocyst stage it produces around 25 pmol/embryo/h of ammonium [107] which can accumulate in the surrounding medium. Furthermore, the spontaneous breakdown of amino acids at 37°C contributes to the overall ammonium present in the medium. Ammonium build up in culture medium not only has negative effects on embryo development and differentiation in culture [62, 68, 109], but can affect subsequent fetal growth rates and normality at a concentration of around 300μM [33, 110]. Furthermore, it has been shown that ammonium affects embryo metabolism, pHi regulation and gene expression in both the mouse and human [111–113], and that

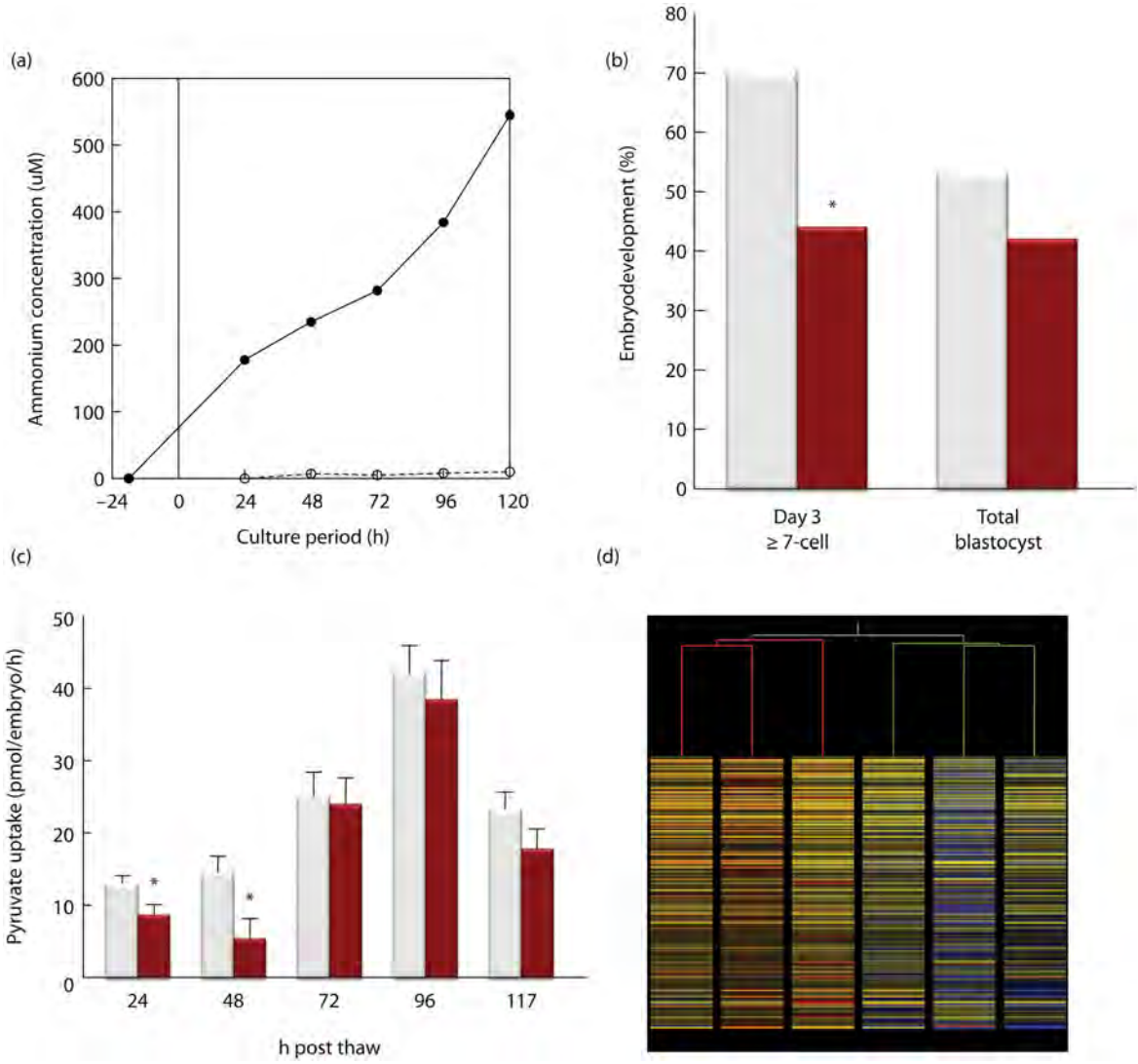

FIGURE 15.3 (a) Production of ammonium into the culture medium (lacking embryos) by the spontaneous breakdown of amino acids in culture media. Solid circles, KSOM^{AA}; Open circles, G1/G2. The media were placed in the incubator at 4 pm the day before culture for equilibration purposes. The line at time zero represents when embryos would be placed into culture (although these measurements were taken in the absence of embryos). Medium KSOM^{AA} contains 1 mM glutamine and therefore releases significant levels of ammonium into the culture medium. Media G1/G2 do not contain glutamine, but rather the stable dipeptide form, alanyl-glutamine, and therefore these media do not release significant levels of ammonium. At a concentration of just 75 µmol/l ammonium can induce a 24-hour developmental delay in mouse fetal development by day 15 and induces the neural tube defect exencephaly in 20% of all fetuses [33]. (b) Ammonium significantly reduces the development of the cleavage stage human embryo. Pronucleate oocytes were exposed to an increasing ammonium gradient as depicted in 3a. Control media (open bars), presence of ammonium (red bars); significantly different from no ammonium, $P < 0.05$. (From [112].) (c) Ammonium significantly compromises human embryo metabolism. Pyruvate uptake was significantly reduced by ammonium at 24 and 48 hours of culture. Control media (open bars), presence of ammonium (red bars); significantly different from no ammonium, $P < 0.05$. (From [112].) (d) Ammonium significantly impairs human blastocyst gene expression. Heat map representation with hierarchical clustering of altered genes in human blastocysts following ammonium exposure and separation of control (green lines) and three ammonium (red lines) samples into distinct branches. Gene expression is related to colour, with red representing the highest levels of gene up-regulation and blue down-regulation. (From [112].)

perturbations induced by ammonium are further compromised by its interaction with atmospheric oxygen (discussed in more detail later) [114].

There is growing data on the appearance of ammonium in the culture medium over time [62, 109, 115, 116] and its toxicity to embryos, including the human [109, 112, 117]. Of relevance, an analysis of the impact of culture media composition on the live birth rates and subsequent development of the children conceived has been reported by Dumoulin and colleagues [118, 119]. In their studies the effects of two commercial media were analysed in a day 2 transfer program, and it was determined that difference existed in embryo growth kinetics, and subsequent birth weight, which persisted through the first two years of life. Of relevance to this discussion is that one of the two media used contained free

glutamine, and hence in this group the embryos (which exhibited the growth delay) were exposed to a level of ammonium documented to adversely affect human embryo development and physiology (Figure 15.3b–d).

As amino acids are such important regulators of embryo development it is essential to alleviate this *in vitro*-created problem. An immediate answer is to renew the culture medium every 48 hours, thereby controlling ammonium concentration within non-toxic limits. A second, and complimentary, solution is to replace the most labile amino acid, glutamine, with a dipeptide form such as alanyl-glutamine. This dipeptide has the advantage of not breaking down at 37°C. Therefore, media containing this stable form of glutamine produce significantly lower levels of ammonium. Further, the overall levels of amino acids can be titrated down to an effective concentration.

Monoculture or sequential media: One size fits all or a tailored approach?

It was established in the 1960s that it was feasible to culture the 1-cell mouse embryo to the blastocyst stage in medium lacking amino acids. In the intervening decades, it has become apparent that amino acids have a significant role to play during embryo development (discussed earlier), and that medium ideally should be renewed/replenished at least every 48 hours to ensure minimal accumulation of embryo-toxic ammonium. Subsequently, all culture systems have become, by default, dynamic [50]. From a practical point of view, therefore, the amount of work and embryo manipulations required are the same whether one is working with sequential media or a monophasic system (i.e. one medium formulation for the entire preimplantation period).

However, the two approaches to embryo culture do have some fundamental differences. Specifically, monoculture is based on the principle of letting the embryo choose what it wants during development. In contrast, sequential media were developed to accommodate the dynamics of embryo nutrition and to mirror the environment of the female reproductive tract in which the embryos is exposed to a gradient of nutrients as it passes along the oviduct into the uterus [29, 55, 56]. The significance of these nutrient gradients to the embryo in culture warrants further research as existing data on the mouse indicates that such gradients *in vitro* do impact embryo viability following transfer. For example, when the mouse zygote is cultured to the 8-cell stage and then transferred, embryo viability is highest after exposure of the embryo to a high lactate concentration (>20 mM D/L lactate), while when the embryo is cultured post-compaction to the blastocyst stage, viability is highest after exposure to lower levels of lactate (<5 mM D/L lactate) [120]. These data, support the hypothesis that the physiology of the developing conceptus is regulated by the relative concentrations of nutrients available at specific stages of development [121].

With the advent of time-lapse microscopy, we have seen the emergence of media designed specifically for the purpose of uninterrupted culture, with the aim of minimizing the build-up of ammonium [122]. These media have been shown to be effective, but further work is warranted in this area, such as the development of sequential media in situ, upon which existing media drops are not necessarily renewed but supplemented with a second formulation to give rise to a modified culture environment during for the post-compaction stages. Further, should embryo culture be extended beyond day 5, it is strongly recommended to renew the culture medium given the high metabolic rate of the blastocyst and therefore its capacity to greatly reduce the levels of available nutrients within the surrounding culture medium.

How far behind embryo development in vivo is development in vitro?

Historically, embryos cultured *in vitro* lag behind their *in vivo* developed counterparts [123, 124]. However, with the development of sequential media based on the premise of meeting the changing requirements of the embryo and minimizing trauma coupled with use of reduced oxygen concentrations in the gas phase, *in vivo* rates of embryo development can now be attained *in vitro* in the mouse [4, 49, 125]. The one proviso is that each laboratory must have sufficient quality systems in place to ensure the optimum operation of a given culture system. Such advances in culture systems represent a significant development for the laboratory, for now there exists a means of producing blastocysts at the same time and with the same cell number and allocation to the inner cell mass as embryos developed in the female tract [1, 49]. Using culture media in a highly controlled environment, as detailed throughout this book, it is possible to attain high rates of human embryo development to the blastocyst stage. Using an oocyte donor model to evaluate the efficacy of culture approaches, where the age of the oocytes is typically under 30 years, it is possible not only to obtain a blastocyst formation rate of 65%, but the resultant viability (as determined by fetal heart beat following transfer) is >65% (Table 15.3). As such, oocyte donors represent as close to a human "gold standard" as one can have in an infertility clinic. With this in mind, ensuring one can attain blastocyst development of greater than 50% and implantation rates of over 50% when using donated oocytes is a good potential starting point for introducing blastocyst culture clinically, or if oocyte donation is not offered, then patients under 35 years of age could be used when analysing laboratory KPIs and transfer outcomes.

Culture systems

Several key components of the culture system are reviewed here, all of which need to be optimized as all directly impact upon media performance.

Incubation chamber

Whatever incubation chamber is chosen, a key to successful embryo culture is to minimize perturbations in the atmosphere around the embryo. The two key perturbations to avoid are pH and temperature changes. This means that ideally the environment in which the embryo is placed is not disturbed during the culture period. Practically this is difficult to achieve in a busy

TABLE 15.3 Viability of Human Embryos Conceived *In Vitro* Using an Oocyte Donor Model

Mean blastocyst development (%)	65.1
Mean number of blastocysts transferred	2.05
Mean age of recipient	40.3
Fetal Heart (per blastocyst transferred) (%)	68.0
Clinical Pregnancy Rate (per retrieval) (%)	85.2
Twins (%)	59.9

Note: All pronucleate oocytes were cultured for 48h in medium G1 at 5% O_2, 6% CO_2, and 89% N_2. On day 3 of development embryos were washed and transferred into medium G2 under same gaseous environment. Embryos were cultured in groups of 4 in 50 µl drops of medium under Ovoil (Vitrolife AB, Sweden) in 60 mm Falcon Primaria dishes. All embryos were transferred on day 5 of development. n = 950 patients [50].

clinical laboratory. The use of an individual incubation chamber, such as a modular incubator chamber or glass desiccator (such as that used to grow Louise Brown), which can be purged with the appropriate gas mix, can alleviate such concerns [77]. Using such incubator chambers, each patient's embryos can be completely isolated within an incubator, the gas phase and for the most part temperature, being unaffected when the incubator door is opened. We like to consider such chambers as "a womb with a view." However, a downside of this approach is that only three modular chambers can be placed in one incubator, thereby necessitating the acquisition of sufficient incubators. An alternative to the use of modular chambers is the use of inner doors within an incubator to significantly reduce fluctuations in the gaseous environment upon opening the incubator door. Several incubator manufacturers make incubators with inner doors. A more recent move has been the production of incubators with a greatly reduced working volume, such that rather than two double stacks of conventional incubators (giving four working chambers), one can now have three rows of smaller incubators, stacked three high, giving a total of nine chambers. This approach allows the successful allocation of one chamber to just one or two patients, thereby stabilizing the culture environment.

Incubators with infrared (IR) as opposed to thermocouple (TC) CO_2 sensors are quicker at regulating the internal environment of the chamber and are less sensitive to environmental factors and subsequently are better able to maintain a constant CO_2 level in the incubator. Therefore, incubators equipped with IR sensors will provide a more stable environment for embryo development. With regards to temperature changes, incubators with an air jacket are less susceptible to large temperature fluctuations as those with a water jacket. Again, the use of inner doors will aid in minimizing environmental fluctuations within the chamber.

Alternatives to "classic" tissue culture incubators are mini benchtop incubators which allow for direct heat transfer between the chamber and culture vessel. Such chambers also allow for a direct flow of pre-mixed gas and therefore minimize changes in pH. More recently such chambers have seen the inclusion of time-lapse capability, facilitating the constant monitoring of embryos without the need to remove them from their culture environment. Consequently, this approach has been shown to have inherent advantages for embryo development, by minimizing handling and variations in temperature and pH [3, 126].

What is evident is that it is imperative to have sufficient numbers of incubator chambers to match the caseload. This is especially true when performing extended culture. It is important to consider the number of times an incubator will be opened in a day and to keep this to a minimum. Further, it is advisable to have separate incubators for media equilibration and for embryo culture, thereby minimizing the amount of access to incubators containing embryos.

pH and carbon dioxide

When discussing pH it is noteworthy that the pHi of the embryo is around 7.2 [38, 127, 128], whereas the pH of the media routinely range from 7.25 to 7.4. Specific media components, such as lactic and amino acids directly affect and buffer pHi respectively. Of the two isomers of lactate, D- and L-, only the L form is biologically active. However, both the D- and L-forms decrease pHi of the embryo [38]. Therefore, it is advisable to use only the L-isomer of lactate and not a medium containing both the D and L forms. While high concentrations of lactate in the culture medium can drive pHi down [38], amino acids increase the intracellular buffering capacity and help maintain the pHi at around 7.2 [37]. As the embryo has to maintain pHi against a gradient when incubated at pH 7.4, it would seem prudent to culture embryos at lower pH. However, the pH of a CO_2/bicarbonate buffered medium is not easy to quantitate. A pH electrode can be used, but one must be quick, and the same technician must take all readings to ensure consistency. A preferred and more accurate approach is to take samples of medium and measure the pH with a blood-gas analyser. A final method necessitates the presence of phenol red in the culture medium and the use of Sorensons's phosphate buffer standards. This method allows visual inspection of a medium's pH with a tube in the incubator and is accurate to 0.2 pH units [43, 45].

When using bicarbonate buffered media, the concentration of CO_2 has a direct impact on medium pH [43]. Although most media work over a wide range of pH (7.2 to 7.4), it is preferable to ensure that pH does not go over 7.4. Therefore, it is advisable to use a CO_2 concentration of between 6% and 7% to yield a medium pH of around 7.3. The amount of CO_2 in the incubation chamber can be calibrated with a Fyrite, although such an approach is only accurate to ± 1%. A more suitable method is to use a hand-held IR metering system which can be calibrated and are accurate to around 0.2%.

When using a CO_2/bicarbonate buffered medium it is essential to minimize the amount of time the culture dish is out of a CO_2 environment to prevent increases in pH. To facilitate this modified paediatric isolettes designed to maintain temperature, humidity and CO_2 concentration can be used. However, should it not be feasible to use an isolette, then the media used can be buffered with either 20–23 mM HEPES [129] or MOPS [130] together with 5–2 mM bicarbonate [131]. Such buffering systems have been shown to work effectively on their own and also in combination [132], and do not require a CO_2 environment to maintain pH. Further, an oil overlay reduces the speed of CO_2 loss and the associated increase in pH.

Oxygen

Atmospheric oxygen is ~20% and very few tissues in the body ever see such high levels. The concentration of oxygen in the lumen of the rabbit oviduct is reported to be 2%–6% [133, 134] whereas the oxygen concentration in the oviduct of hamster, rabbit, and rhesus monkey is ~8% [135]. Interestingly, the oxygen concentration in the uterus is lower than in the oviduct, ranging from 5% in the hamster and rabbit to 1.5% in the rhesus monkey [135].

Importantly, it has been demonstrated that optimum embryo development of all non-human mammalian species occurs at an oxygen concentration below 10% [85, 136, 137]. The fact that human embryos can grow at atmospheric oxygen concentration (~20%), and give rise to viable pregnancies, has led to some confusion regarding the optimal concentration for embryo culture. Consequently, the validity of having to use a reduced oxygen concentration for human embryo culture is continually challenged. The continued use of 20% oxygen in a human IVF culture system is a good example of something that has been used for over four decades and does give pregnancies; however, the question remains, does 20% oxygen adversely affect the physiology of the developing embryo before implantation?

It was initially established in the mouse model that 20% oxygen impacts embryo development as early as the first cleavage [138]. Of great interest, it was determined that 20% oxygen is detrimental to embryo development at all stages, but with the

greatest detrimental effects imparted at the cleavage stages [138]. These findings have now been evaluated clinically, and it has been determined that 20% oxygen reduces developmental rates and delays completion of the third cell cycle [139], indicating a heightened sensitivity to oxidative stress during the cleavage stages. Furthermore, it has been established in animal models that embryos cultured to the blastocyst stage in the presence of 20% oxygen have altered gene expression and perturbed proteome compared to embryos developed *in vivo* [28, 140–142]. In contrast, culture in 5% oxygen had significantly less effect on both embryonic gene expression and proteome. Similarly, 20% oxygen has been shown to adversely affect embryonic metabolism [5]. Recent data has revealed that not only does 20% oxygen compromise the utilization of both carbohydrates and amino acids throughout the preimplantation period [113], but that atmospheric oxygen also impairs the ability of the embryo to regulate against an ammonium stress [114]. Therefore, not only does oxygen induce its own trauma on the embryo, but it also increases the embryo's susceptibility to other stressors present in the culture system or laboratory [3]. Furthermore, atmospheric oxygen has recently been linked to changes to the embryonic epigenome (reviewed by [2, 3, 143]).

In support of the utilization of physiological levels of oxygen in human embryo culture, clinical data including a randomized controlled trial, support this move to more physiological conditions with lower oxygen concentrations increasing both implantation and live birth rates [144–147]. A more recent analysis on the effects of oxygen concentration on cumulative pregnancy rates has determined that 5% oxygen is associated with significantly improved outcomes compared to 20% oxygen [148].

However, in spite of the animal and clinical data describing the detrimental effects of atmospheric oxygen, it has been reported in an on-line survey, in which 265 clinics from 54 different countries participated, that <25% of IVF human embryo culture is performed exclusively under physiological (~5%) oxygen [149]. Although this survey represents only a small fraction of the world's IVF clinics, what is notable from an extensive literature review is a clear geographic difference with regard to the use of 5% oxygen. Hopefully, since the publication of the Christianson paper, fewer clinics now used atmospheric oxygen. I presented a case for the clinical introduction of physiological oxygen in human IVF over 30 years ago [43]. In the intervening decades the rationale for the discounting of atmospheric oxygen has become compelling. Consequently, the time has come to confine the use of 20% oxygen to the annals of human IVF and to ensure physiological levels of oxygen are used by all human IVF clinics [143]. It is unethical to do otherwise.

Osmolality

Ions in any medium make the largest single contribution to osmotic pressure [48]. Mouse [150] and hamster [151] embryos will develop in a wide range of osmolalities (200 to 350 mOsmols). Although conventional embryo culture media has an osmolality of between 275 and 295 mOsmols, enhanced development of mouse embryos appears to occur at reduced osmolalities [152, 153]. However, such studies were performed using simple embryo culture media, i.e. balanced salt solutions, in the absence of amino acids. It is now evident that the inclusion of osmolytes, such as betaine, or specific amino acids, such as glycine, in the culture medium can reduce any osmotic stress [5, 66, 154–157], thereby allowing apparently normal embryo development to occur over a wider range of osmotic pressures and ion concentrations. In spite of this, paying attention to the medium osmolality is an important variable to include in the quality assurance of the IVF laboratory, and it is essential to monitor the culture system to ensure that the osmolality of the medium used does not exceed ~300 mOsmols. To ensure the osmolality of any given culture medium, it is important to use an oil overlay and to cover the drops of medium as quickly as possible. Whenever possible, prepare only one dish at a time and use cold medium to minimize evaporation and consequent increase in osmolality. This is especially important when working in a laminar flow hood, as moving air across the dish as it is being prepared will further increase media evaporation. For incubators run without humidity, as is the case for some time-lapse incubation systems, osmolality increases have been reported [158], but the effect is dependent upon several variables including the types and volume of oil used for the medium overlay, drop size, and culture dish design [159].

Incubation volumes and embryo density

Within the lumen of the female reproductive tract the developing embryo is exposed to microlitre volumes of fluid [160]. In contrast, the embryo grown *in vitro* is subject to relatively large volumes of medium of up to 1 ml [161]. Consequently, any autocrine factor(s) produced by the developing embryo will be diluted and may therefore become ineffectual. It has been demonstrated in the mouse that blastocyst formation and cell number increase when embryos are grown in groups (up to 10) or reduced volumes (around 20 µl) [162–165]. Similar results have been obtained with sheep [68] and cow embryos [166, 167], and more recently positive effects of culturing embryos in groups has been reported for the human [168], although other clinical studies failed to observe any effects [169]. Consequently, it does appear that the preimplantation mammalian embryo produces a factor(s) capable of stimulating development of both itself and surrounding embryos (Figure 15.4) [170]. Interestingly, it has been reported that during individual culture atmospheric oxygen impairs mouse embryo development, with delays during early cleavage culminating in a resultant decrease in both inner cell mass and trophectoderm development in the blastocyst [171, 172]. When 5% oxygen was used for individual embryo cultures, development was significantly improved. The negative impacts of individual culture were further improved when embryos were culture in microwells (such as those for time-lapse incubation), as opposed to drops of medium under oil [173]. While high pregnancy rates are obtained following single embryo cultures in time-lapse systems run with 5% oxygen, future studies may still confirm the best design of microwells for human embryo development [174, 175].

What day should embryo transfer be performed?

In the early days of human IVF embryos were transferred between days 1 and 3 at either the pronucleate or cleavage stages. The reason for this stems primarily from the inability of past culture systems to support the development of viable blastocysts at acceptable rates. However, with the advent of physiological culture media [6] it became feasible to perform day 5 blastocyst transfers as a matter of routine in an IVF clinic [176, 177]. This now facilitates an answer to the question: On which day of embryo development should embryos be transferred? Before answering this question, the potential advantages and disadvantages of blastocyst culture and transfer are considered.

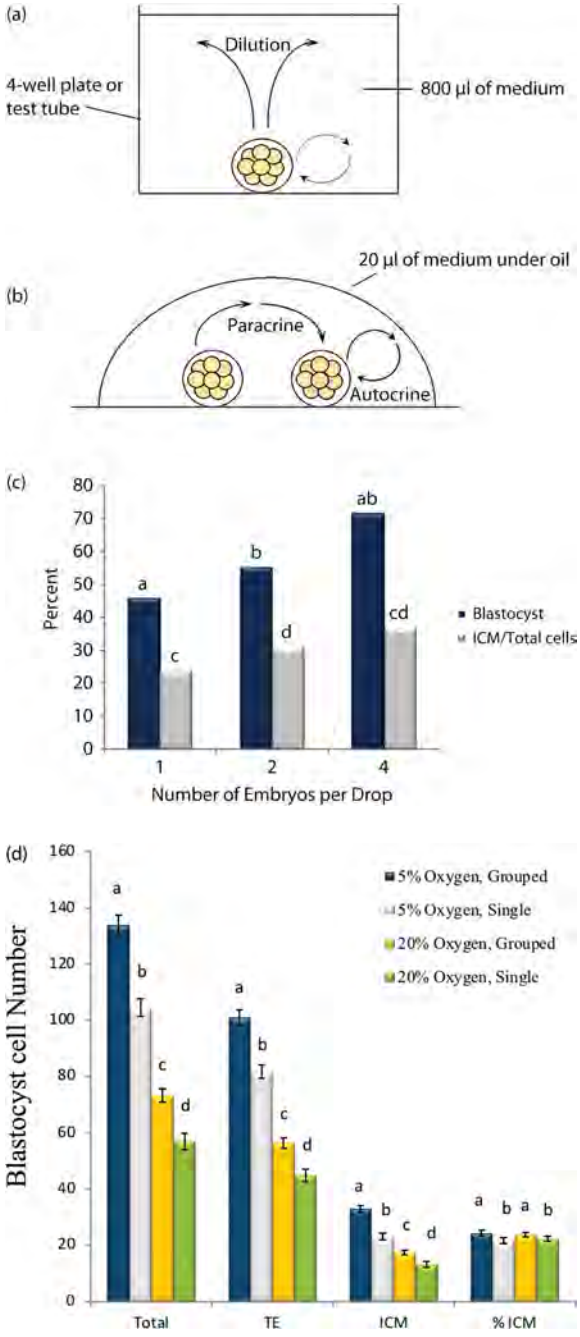

FIGURE 15.4 Effect of incubation volume and embryo grouping on embryo development and differentiation. (a) A single embryo cultured in a four-well plate or test tube, any factor produced by the embryo will become ineffectual as a result of dilution. (b) Culture of embryos in reduced volumes and/or groups increases the effective concentration of embryo-derived factors, facilitating their action in either a paracrine or autocrine manner. (c) Effect of embryo grouping on bovine blastocyst development and differentiation. Bovine embryos were cultured either individually or in groups of two or four in 50 µl drops of medium. Like pairs are significantly different ($P < 0.05$). (Data from [167].) (d) Cell numbers on day 5 of mouse embryo culture in 5% or 20% oxygen, in groups of 10 or individually. Different letters represent significant differences between treatments; Total, TE, ICM < 0.001, %ICM < 0.05. (Data from [171].)

Blastocyst transfer: Advantages and disadvantages

The potential advantages of blastocyst culture and transfer have been well documented [178–181]. Advantages include:

1. Synchronizing embryonic stage with the female tract. This is important as the levels of nutrients within the fallopian tube and uterus do differ, and therefore the premature transfer of the cleavage stage embryo to the uterus could result in metabolic stress [4]. Asynchronous transfer of the cleavage stage embryo to the uterus (as opposed to the oviduct), is documented to result in poor transfer outcomes and compromised fetal development in laboratory and domestic animals [182, 183]. Furthermore, the uterine environment during a stimulated cycle cannot be considered normal. Certainly, it is known from animal studies that the hyperstimulated female tract is a less than optimal environment for the developing embryo, resulting in impaired embryo and fetal development [184–186]. Therefore, it would seem prudent to shorten the length of time an embryo is exposed to such an environment before implantation.
2. When embryos are selected for transfer at the 2- to 8-cell stage the embryonic genome has only just begun to be transcribed [36, 187], and therefore it is not possible to identify from within a given cohort those embryos with the highest developmental potential. Only by culturing embryos past the maternal/embryonic genome transition and up to the blastocyst does it become realistic to identify those embryos with limited or no developmental potential. Assessment of embryos at either the pronucleate oocyte or cleavage stages is best considered an assessment of the oocyte. Although the quality of the oocyte is important, as the quality of the developing embryo is ultimately dependent on the quality of gametes from which it is derived, it provides limited information regarding true embryo developmental potential and eliminates the impact of the male gamete on development.
3. Not all fertilized oocytes are normal, and therefore a percentage always exists that is not destined to establish a pregnancy or go to term. Factors contributing to embryonic attrition include an insufficiency of stored oocyte coded gene products, and a failure to activate the embryonic genome [188]. The culmination of this is that many abnormal embryos arrest during development *in vitro*. So, by culturing embryos to the blastocyst stage, one has already selected against those embryos with little if any developmental potential. Chromosomally abnormal human embryos can reach the blastocyst stage *in vitro* [189], and so even though euploid embryos are more likely to form blastocysts than their aneuploid siblings [190], and the frequencies of aneuploidy are significantly less at the blastocyst stage compared with cleavage stage embryo [191] blastocyst culture alone cannot be used as the sole means in identifying chromosomally abnormal embryos.
4. Uterine contractions have been negatively correlated with embryo transfer outcome, possibly by the expulsion of embryos from the uterine cavity [192]. Uterine junctional zone contractions have been quantitated and found to be strongest on the day of oocyte retrieval [193]. All patients exhibited such contractions on day 2 and 3 after retrieval, but contractility decreased and was barely evident on day 4. It is therefore feasible that the transfer of blastocysts on day 5 is, by default, associated with reduced uterine contractions and therefore there is less chance for embryonic expulsion and loss [194].

5. Trophectoderm biopsy and analysis enables the removal of more cells compared to cleavage stage embryos which facilitates the use of newer technologies such as next generation sequencing [195, 196]. It has been reported that cleavage stage biopsy for PGT actually negatively impact cycle outcomes [197], and that trophectoderm biopsy is less invasive than cleavage stage embryos for preimplantation genetic screening [198].

The potential disadvantage of extended embryo culture in a program where only blastocyst culture and transfer is offered is the possibility that a patient will not have a morula or blastocyst for transfer. Certainly there has been an increase in the percentage of patients who do not have an embryo transfer from 2.9% on day 3 to 6.7% on day 5 in one clinic [177], and from 1.3% on day 3 to 2.8% on day 5 in another [176]. Interestingly, in spite of the increase in patients not having an embryo transfer, there was a significant increase in pregnancy rate per retrieval with blastocyst culture, due to a significant increase in implantation rates.

There is significant evidence to show that in many laboratories blastocyst transfer can be more successful than cleavage stage transfer [9, 199–201]. A model previously developed to determine which patients should have SET, showed that pregnancy outcome was more favourable with day 5 than day 3 transfer [202]. Along with the published prospective randomized trials, there are retrospective studies that have concluded that day 5 transfer exhibits significant benefits for human ART in both non-selected and specific patient populations [176, 177, 203]. However, this has not been universal, and differences between outcomes likely due to the interactions of the components that have already been considered, from ovarian stimulation, culture media and system, oxygen levels, training levels, and numbers of embryologists, along with quality control.

For patients having oocyte donation, blastocyst culture and transfer is the most effective course of treatment. Oocytes from donors generally represent a more viable cohort of gametes, as they tend to come from young fertile women. Embryos derived from oocyte donors tend to reach the blastocyst stage at a higher frequency than those from IVF patients and be of higher quality. It is possible to attain an implantation rate of >65% when transferring blastocysts to recipients whose mean age is over 40 (Table 15.3) [204]. Such data not only reflect the competency of modern embryo culture systems, but emphasize the need to move to SETs, especially when performing blastocyst transfer [205].

Towards single embryo transfer

Several reviews have discussed the development of scoring systems used in clinical IVF and their significance in identifying the most viable embryo(s) for transfer [206–208] (see also Chapter 16). Certainly, with newer types of embryo culture media, implantation rates are increasing whether embryos are transferred at the cleavage stage or blastocyst. It is envisaged that for most patients, blastocyst culture and transfer will be the most effective means of being able to transfer a single embryo while maintaining high pregnancy rates, as it is evident that blastocyst score is highly predictive of implantation potential. In a prospective randomized trial of one versus two blastocysts transferred in patients with 10 or more follicles, the data in indicate that it is possible to transfer a single blastocyst and obtain an ongoing pregnancy rate of 60% [205] (Figure 15.5). Subsequent trials of single blastocyst transfer versus cleavage stage embryo transfer have confirmed the higher implantation rate of the later stage embryo. It has also been established that fetal loss is significantly less following blastocyst transfer [200].

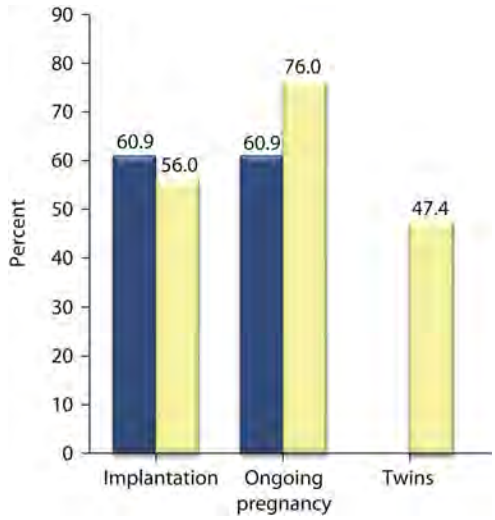

FIGURE 15.5 IVF outcome following the transfer of either one or two blastocysts. Blue bars represent the transfer of a single blastocyst (Group I), yellow bars represent the transfer of two blastocysts (Group II). Implantation and pregnancy rates were not statistically different between the two groups of patients. There were no twins in Group I in contrast to 47.4% twins in Group II. The biochemical pregnancy rate was equivalent between the two groups (Group I, 12.5%; Group II, 5%). (From [205], with permission.)

Cumulative pregnancy rates per retrieval: The significance of cryopreservation

The introduction of blastocyst culture was met with much speculation as not all laboratories were able to cryopreserve blastocysts that were not transferred. However, clinical data following blastocyst vitrification are encouraging. It has now been demonstrated that the move to blastocyst vitrification is associated with a significant increase in clinical pregnancy (50% increase) and live birth rates (40% increase) compared with those obtained with slow freezing [209]. Consequently, cumulative pregnancy data for cleavage- and blastocyst-stage embryos must be re-examined and be based upon cycles where vitrified blastocysts were utilized [143]. The latter has been reported to result in pregnancy rates and outcomes equivalent to, or even greater than, fresh transferred blastocysts [209–211]. Furthermore, the ability of a given culture system to support embryo cryo-survival is of utmost significance, with media containing hyaluronan conferring great advantage in this regard [50, 104].

Practical aspects of embryo culture

In the earlier editions of this Textbook, a section on how to prepare culture dishes was included. However, with the move to more tailor-made devices to incubate embryos, it is beyond the scope of this text to consider all dish preparation techniques which are now clearly specified by each device manufacturer. However, the basic principles outlined earlier in this chapter apply, specifically with regards to being mindful of media evaporation during dish set up, and the need to prepare a minimum number of dishes at a time. Furthermore, care must be paid to the temperature of all heated stages, the temperatures of which need to be calibrated under conditions of use, i.e. the temperature needs to be measured in the medium drop under oil, and not the actual surface of the heating stage as temperature is lost between the actual stage and the inside of the dish. Be mindful that different dish designs can affect temperature readings

depending on whether they have a lip on the bottom of the dish as this can create an air pocket and will therefore be at a different temperature to a dish whose bottom side is in direct contact with the stage. Finally, it is important to ensure accurate and gentle pipetting of the gametes and embryos themselves. It is essential to move embryos in the smallest possible volumes, made possible by using a pipette whose internal diameter is just slightly larger than the embryonic stage (never use a pipette whose inner diameter constricts the embryo). Pipetting should be slow and at no time should the embryo be moved rapidly up into a pipette, as this will increase shear stress which can have an adverse effect on development [212].

Quality control

The type of quality control used in media preparation is an important consideration when choosing a supplier, and in order for the reader to make informed decisions with regards to the types of testing offered by media providers. Establishing an appropriate quality control system for the IVF laboratory is a prerequisite in the establishment of a successful laboratory (Chapter 2). The types of bioassays conducted for this have been the focus of much discussion [213]. In reality there is no perfect model for the human, save for the very patients we treat. Consequently, it is important to understand the limitations of the assays performed and to use data obtained from bioassays in an appropriate fashion. Quality control should not be limited to the culture media used but should include all contact supplies and gases used in an IVF procedure. The bioassay favoured is the culture of pronucleate mouse oocytes in protein-free media. There has been a lot of conflicting data regarding the use of the mouse embryo assay (MEA), but by adjusting conditions, one can not only increase the sensitivity of the assay but can also quantitate quality with it.

First of all, when using the MEA, the stage at which the embryo is cultured from has an impact on development. Mouse embryos collected at the pronucleate stage do not tend to fair as well in culture as those collected at the 2-cell stage, consistent with their heightened sensitivity to stress. Second, the strain of mice is important. Embryos from hybrid parents have a decided advantage in culture, and do not represent the diverse genetic background one is dealing with in an infertility clinic. Therefore, a random bred strain of mice provides greater genetic diversity [214]. Third, the embryo cultures should be performed in the absence of protein, as protein has the ability to mask the effects of any potential toxins present. Reports that mouse embryos can develop in culture in medium prepared using tap water [215, 216] should be interpreted carefully after considering the strain of mouse, types of media used and the supplementation of medium with protein. Silverman et al. [215] used Ham's F-10. This medium contains amino acids, which can chelate any possible toxins present in the tap water, e.g. heavy metals. George et al. [216], included high levels of BSA in their zygote cultures to the blastocyst. Albumin can chelate potential embryo-toxins and thereby mask the effect of any present in the culture medium [217, 218]. Furthermore, all such studies used blastocyst development as the sole criterion for assessing embryo development. Blastocyst development is a poor indicator of embryo quality and does not accurately reflect developmental potential [74]. Therefore, rates of development should be determined by scoring the embryos at specific times during culture. Key times to examine the embryos include the morning of day 3 to determine the extent of compaction, the afternoon of day 4 to determine the degree of blastocyst formation and the morning of day 5 to assess the initiation of hatching [213]. This latter approach can now be readily applied through the utilization of time-lapse microscopy [219].

Finally, the embryos that form blastocysts in a given time, typically on the morning of day 5, should have their cell numbers determined, as blastocyst cell number is a good indicator of subsequent development potential. When new components of certain culture media could affect the development of the inner cell mass directly, such as essential amino acids, a differential nuclear stain should be performed in order to determine the extent of ICM development. Using such an approach it is possible to identify potential problems in culture media before they are used clinically. In our experience around 25% of all contact supplies fail such pre-screening [213]. Although some of the contact supplies that fail the bioassay are not outright lethal, they do compromise embryo development. If undetected this would result in reduced clinical pregnancy rates. Consequently, this helps to explain periodic changes in clinical pregnancy rates and emphasizes the significance of an ongoing quality control program. The majority of products on the market are now prescreened for embryo toxicity. However, it is worth noting that not all testing is the same and that it is worth understanding the sensitivity of the assay used before introduction of an item into the laboratory. However, irrespective of the testing, all supplies should be tracked as they enter the laboratory to confirm efficacy for human embryos.

Future developments in embryo culture systems

Antioxidants

A subject already touched upon in this chapter is the toxicity of oxygen, induced in part through the induction of reactive oxygen species. As a result of the growing data on the pathologies induced by atmospheric oxygen, there has been a resurgence of interest in the role of antioxidants in supporting IVF and embryo development (reviewed by Gardner and Truong [220]). Rather than focusing on individual antioxidants, recent works have concentrated on the efficacy of groups of antioxidants, a more physiologically approach. Truong and colleagues, working with the mouse model, were able to establish that a combination of alpha-lipoic acid (5 uM), acetyl-carnitine (10 uM) and N-acetyl-L-cysteine (10 uM) was highly effective at protecting the developing embryo from oxidative stress at both 5% and 20% oxygen, culminating in blastocyst cell numbers and an increase in viability post transfer [172]. Subsequent studies determined that this group of three antioxidants was also effective at increasing both IVF and vitrification outcomes [221, 222], and that the gene expression of the resultant fetuses and placentae was closer to *in vivo* developed controls when antioxidants were present for embryo culture and for vitrification [142]. These three antioxidants were then evaluated in a randomized sibling oocyte study, in which it was shown that ongoing pregnancies were increased in patients 35 to 40 years of age [223]. Subsequent larger clinical studies will help to establish the roles of antioxidants in human ART.

Growth factors and cytokines

Growth factors and cytokines are present in the fluids of the human female reproductive tract, with increasing abundance in

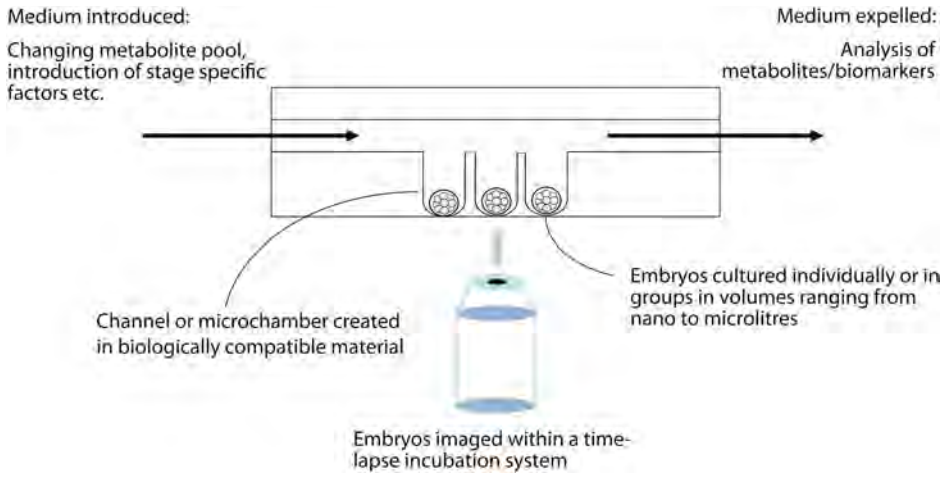

FIGURE 15.6 Schematic of an embryo perfusion culture system. Culture media are continuously passed over the embryo(s). The composition of the culture media can be changed according to the specific requirements of each stage of embryonic development. Toxins such as ammonium are not able to build up and impair embryo development, while more labile components of the culture system are not denatured. Further, media can be sampled in real time to quantitate embryo physiology. (Modified from [40].)

the uterus [224, 225], and have been shown to have effects on animal embryo viability [226], and yet they are conspicuously absent from clinical embryo culture media. An exception to this is a study on the effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) [227]. However, it was reported that GM-CSF only had a beneficial effect when the levels of HSA were reduced in the medium, an observation also previously reported in the mouse model [228]. Similar to antioxidants, more recent works have focused on the efficacy of groups of growth factors, as opposed to the inclusion of individual factors [229, 230]. While it has been shown that such an approach can improve embryo development *in vitro* and subsequent fetal development, subsequent analysis of both fetal and placental gene expression following embryo transfer in the mouse, revealed aberrant transcription profiles compared to embryo developed *in vivo* [230]. Hence, although there was a positive effect in culture, the normalcy of the resultant fetuses and subsequent developmental programming following culture in the presence of growth factors and cytokines is brought into question. Further in-depth works on animal models, including the long-term follow-up of offspring, are therefore warranted before the addition of such factors to human embryo culture media can be considered safe.

Perfusion culture

As discussed previously, there is nothing physiological about the physical conditions in which embryos are cultured. Rather than a static drop of medium, the future may engage perfusion culture systems, enabling the embryo to be exposed to a flux of nutrients and factors (Figure 15.6) [40, 231, 232] (Chapter 28 by Goss et al.). This latter approach has the advantage of being able to expose embryos to numerous gradients and fresh media throughout development. Furthermore, samples of medium can be taken and analysed for carbohydrates [233], amino acids [234], and other factors related to implantation potential post transfer [7, 39] (Chapter 16 by Sakkas and Gardner). Although this concept has been considered for several decades, the available technologies were not sufficiently developed in order for this to be evaluated. With recent advances in 3D printing and 2 photon polymerization, it is now possible to fabricate devices capable of perfusing nanolitre volumes of culture media over preimplantation embryos during their culture [235] (see Chapter 28 by Goss et al.). Consequently, in the next few years we shall be able to fully evaluate perfusion culture in human ART.

Conclusions

In this chapter the complexities of human embryo culture have been considered, and advances in culture technologies discussed. Rather than perceiving embryo culture as an optimized procedure, it should be considered as continuously improving process as we learn more about the preimplantation human embryo and its environment *in vivo* [7]. In combination with the introduction of new technologies, this will ultimately lead to greater efficacies and efficiencies while also paving the way to automation of several key laboratory processes [236]. Diligent monitoring and reporting of pregnancy outcomes and consideration of cumulative pregnancy rates per cycle will help in the continued improvement and evaluation of assisted conception. As more reports on IVF outcomes become available, it is essential that when interpreting findings careful attention is paid to precise the conditions used by clinics, given the number of variables associated with laboratory processes which can affect outcomes [237] (Table 15.4). This is especially important for oxygen concentration, which as discussed in detail, has the capacity to affect the embryo in a number of ways. Indeed 20% oxygen can compromise an otherwise safe and effective culture system, which in turn will lead to erroneous conclusions with regards to clinical outcomes [143].

TABLE 15.4 Effects of Laboratory Conditions on Human Embryo Phenotype

IVF Laboratory Conditions	Observed Effects on Human Preimplantation Embryo Phenotype
Amino acids	Increases the rate of embryo development and blastocyst formation
Ammonium	Decreases cleavage rate and metabolism; decreases blastocyst formation and alters gene expression
Carbohydrates	Stage specific response to nutrients. Gradients of key nutrients such as lactate will regulate the intracellular redox
Serum	Sequestration of lipids resulting in an altered physiology and possible effects on gene expression and imprinting
Oxygen[a]	Atmospheric oxygen decreases cleavage times, decreases blastocyst formation, reduce cell number and further it alters embryonic metabolism, the transcriptome and epigenome.
Embryo density/single culture	Affects blastocyst formation, embryo morphology and pregnancy rate
Microwells	Increase in blastocyst formation, morphology scores, and cells in ICM
Undisturbed culture	Current data contradictory
Air Quality	Filtered air increases blastocyst formation and morphology scores, and live birth rate
Oil	Poor oils reduce morphological quality on Day 3
Osmolality	Hypo-osmotic conditions induce release of osmolytes and activation of anion channels
pH	Oocytes exposed to acidic or alkaline environment are less likely to reach first cleavage and have higher fragmentation
Temperature	Fluctuations are detrimental to oocyte spindle stability and fertilization. Reduced temperature during embryo culture results in fewer cells and blastocyst formation.

Note:

[a] Not only does atmospheric oxygen have its own direct negative effects on several key processes within the embryo, it also increases the susceptibility of the embryo to a second stressor. Examples of this include individual culture where development is significantly impaired in the presence of atmospheric oxygen, and the ability of the embryo to regulate against ammonium toxicity. In the latter case, atmospheric oxygen reduces the ability of an embryo to utilize amino acids and to transaminate ammonium.

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16

EMBRYO SELECTION THROUGH THE ANALYSIS OF MORPHOLOGY AND PHYSIOLOGY

Denny Sakkas and David K. Gardner

Introduction

Worldwide the utilization of assisted reproductive technologies (ART) continues to increase annually. In 2006, more than one million cycles were registered in the international report on ART monitoring [1]. Current data indicate that more than two and a half million ART cycles are being performed each year, with a total of over eight million babies born worldwide since the birth of Louise Brown [2–4]. Well, over a million treatment cycles are initiated annually in the United States, Europe, Australia, and New Zealand alone [1–3, 5]. This increasing trend of ART utilization has been driven by the steady improvement in delivery rates, improved access to care in many areas, and the relative ineffectiveness of other treatment options. The proportion of infants after ART in Europe, the United States, and Australia/New Zealand [6] now ranges from 1.9% to 4.1% of all children born [3, 7].

Historically acceptable success rates through in vitro fertilization (IVF) were attained, in many cases, only through the simultaneous transfer of multiple embryos. However, this trend has changed dramatically. In the United States, an average of 2.8 embryos were transferred in women <38 years of age in 2003 compared to 1.2 embryos per patient in 2019 [8]. A further trend has been a shift to transferring embryos after cryo-storage into a more receptive uterine environment, which has also led to lower numbers of embryos transferred [8].

The risks to both mother and baby related to multiple gestations are well documented and include maternal hypertension, preterm delivery, low birth weight, and a dramatic increase in the relative risk for cerebral palsy (reviewed by [9–12]). These complications lead to a higher incidence of medical, perinatal, and neonatal complications and a tenfold increase in healthcare costs compared to a singleton delivery [13]. Decreasing the prevalence of multiple gestations in IVF can only be achieved by the transfer of a single embryo.

In many countries, including Norway, Sweden, Denmark, Belgium, England, Italy and Germany, legal restrictions/medical guidelines have been implemented for several years that govern the number of embryos that can be transferred in a given IVF cycle. For example, in most Scandinavian countries and Belgium, the government has set a legal limit of single embryo transfer (i.e. only one embryo to be transferred per cycle) for specific patient groups, while many other European countries have restricted the number of transferred embryos to a maximum of two. In other parts of the world, where no legal restrictions exist, the onus is on the individual clinic (along with the patient) to decrease the number of embryos transferred so that an acceptable balance can be achieved between the risks associated with multiple gestations and "acceptable" pregnancy rates. In Australia and New Zealand, this was achieved by clinicians and patients willingly shifting to single embryo transfer, with the proportion increasing from 69.7% in 2009 to 79.2% in 2013 to 93% in 2020 [6]. Of note, in the USA, the Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology have now stipulated under what circumstances single embryo transfer should take place [14]. In China, the number of embryos transferred has also shifted to just under an average of two per transfer [15]. Current indications are that in the future all countries currently lacking legislation will be compelled via legal, financial, and/or moral obligation to restrict the number of embryos transferred in order to minimize the risk of multiple gestations.

A major issue in limiting the number of embryos transferred remains the apparent inability to accurately estimate the reproductive potential of individual embryos within a cohort of embryos using the existing selection techniques, which largely depends upon morphological evaluation. Faced with the scenario that we, the worldwide IVF community, will in the future have to select only one or, at most, two embryos for transfer, will force us to make certain choices. There has also been a debate on the value of milder stimulation protocols that generate a lower number of eggs at collection. The generation of a smaller number of oocytes has been argued to lead to a greater percentage of viable embryos within a given cohort [16, 17]. Contrary to this argument, a higher egg yield has been shown to improve cumulative live birth rates [18], and can provide two or more live births with just one stimulation cycle in almost a quarter of patients [19–24]. With the implementation of vitrification, the fear of loss of embryo quality after cryopreserving has also been largely removed. The scenario of creating more embryos and performing a frozen embryo transfer will place a greater onus on improving the selection process for defining the quality of individual embryos so that the ones we choose for transfer are more likely to implant, thereby significantly decreasing the time to pregnancy. This chapter will discuss several strategies in selection criteria that will help us achieve this second choice.

Morphology as an assessment tool

For more than 30 years, morphological assessment has been the primary means for embryologists to select which embryo(s) to replace. From the early years of IVF, it was noted that embryos that cleave faster, and those of better morphological appearance, were more likely to lead to a pregnancy [25, 26]. Morphological assessment systems subsequently evolved over the past three decades, and in addition to the classical parameters of cell number and fragmentation, numerous other characteristics have been examined, including pronucleate oocyte morphology, early cleavage to the 2-cell stage, top-quality embryos on successive days, and various forms of sequential assessment of embryos (see reviews [27, 28]). In addition, the ability to culture and assess blastocyst stage embryos has also significantly improved embryo

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selection on the basis of morphology [29]. Although morphology has proved a difficult target to marry with viability, it does provide us a gross overall vision on key milestones an embryo should obtain at specific times [30]. This has been better appreciated in the past few years, when we have also seen the advent of commercially available video-imaging technologies which bring new light to how we interpret and use the morphological features of the embryo (see Chapter 17 and [31]). Here we briefly describe some of the historical papers that examined key morphogenic events and the key times at which they should take place in the laboratory.

The pronucleate oocyte

The many transformations that take place during the fertilization process make this a highly dynamic stage to assess. The oocyte contains the majority of the developmental materials, maternal mRNA, for ensuring that the embryo reaches the 4- to 8-cell stage. In human embryos, embryonic genome activation has been shown to occur between the 4- and 8-cell stages [32]. The quality of the oocyte, therefore, plays the lead role in determining early embryo development and subsequent viability.

A number of studies postulated that embryo quality can be predicted at the pronucleate oocyte stage. Separate studies by Tesarik and Scott [33, 34] concentrated on the predictive value of the nucleoli. Tesarik and Greco [34] proposed that the normal and abnormal morphology of the pronuclei were related to the developmental fate of human embryos. They retrospectively assessed the number and distribution of nucleolar precursor bodies (NPB) in each pronucleus of fertilized oocytes that led to embryos that implanted. The characteristics of the zygotes were then compared to those that led to failures in implantation. The features that were shared by zygotes that had 100% implantation success were (i) the number of NPB in both pronuclei never differed by more than three, and (ii) the NPB were always polarized or not-polarized in both pronuclei but never polarized in one pronucleus and not in the other. Pronucleate oocytes not showing this criteria were more likely to develop into pre-implantation embryos that had poor morphology and/or experienced cleavage arrest. The presence of at least one embryo, which had shown the preceding criteria at the pronuclear stage in those transferred, led to a pregnancy rate of 22/44 (50%) compared to only 2/23 (9%) when none were present.

A further criterion of pronucleate oocytes that may affect embryo morphology is the orientation of pronuclei relative to the polar bodies. Oocyte polarity is clearly evident in non-mammalian species. In mammals, the animal pole of the oocyte may be estimated by the location of the first polar body, whereas after fertilization, the second polar body marks the embryonic pole [35]. In human oocytes, a differential distribution of various factors within the oocyte has been described and anomalies in the distribution of these factors, in particular the side of the oocyte believed to contain the animal pole, are thought to affect embryo development and possibly fetal growth [36, 37]. Following from this hypothesis, Garello et al. [38] examined pronuclear orientation, polar body placement, and embryo quality to ascertain if a link existed between a plausible polarity of oocytes at the pronuclear stage and further development. The most interesting observation involved the calculation of angle β (Figure 16.1), which represented the angle between a line drawn through the axis of the pronuclei and the position of the furthest polar body. It was determined that as the angle β increased there was a concurrent decrease in the morphological quality of pre-implantation stage human embryos. Hence, it was postulated that the misalignment of the polar body might be linked to cytoplasmic turbulence disturbing the delicate polarity of the zygote. To this day, the question of polarity in the oocyte and its importance in influencing embryo viability is still not well understood [39].

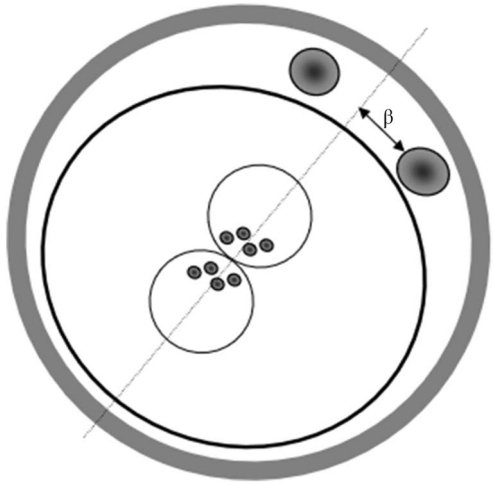

FIGURE 16.1 Ideal features shared by pronuclear embryos that have high viability as described by Tesarik and Greco [32], Garello et al. [37], and Scott and Smith [39]: (i) the number of nucleolar precursor bodies (NPB) in both pronuclei never differed by more than 3; (ii) the NPB are always polarized or not-polarized in both pronuclei but never polarized in one pronucleus and not in the other; (iii) the angle from the axis of the pronuclei and the furthest polar body is less than 50°.

In a further study, Scott and Smith [40] devised an embryo score on day 1 on the basis of alignment of pronuclei and nucleoli, the appearance of the cytoplasm, nuclear membrane breakdown, and cleavage to the 2-cell stage. Patients who had an overall high embryo score (≥15) had a pregnancy and implantation rate of 34/48 (71%) and 49/175 (28%), respectively, compared to only 4/49 (8%) and 4/178 (2%) in the low embryo score group. The use of pronuclear scoring was extensively reviewed by Scott [41]. The timing of pronuclear events has been confirmed to be correlative to implantation potential by video imaging. Aguillar et al. [42] showed that the timings at which second polar body extrusion (3.3–10.6 hours), pronuclear fading (22.2–25.9 hours), and length of S-phase (5.7–13.8 hours) occurred were all linked successfully to embryo implantation. The same group also confirmed that the method of fertilization, intracytoplasmic sperm injection (ICSI) or routine IVF, was also important in determining how these parameters should be evaluated [43]. Interestingly, these features may still provide some insights into viability assessment even with the advent of morphokinetics [44]. A recent study did however strengthen the concept that the closer an embryo is observed to the time of implantation the more relevant the information in relation to viability. Ezoe et al. [44] found that non-juxtaposition and asynchronous pronuclear breakdown was associated with abnormal mitosis at the first cleavage and impaired pre-implantation development. However, embryos displaying abnormal pronuclear breakdown also formed blastocysts which led to live births. They suggested blastocyst transfer as a more appropriate culture strategy.

Cleavage stage embryos

Although the use of blastocyst stage culture has been more widely accepted, selection at the cleavage stage for transfer, based on cell number and morphology [25] is still prevalent. In some cases, this is still driven by legal restrictions in certain countries. For example, the German embryo protection law, passed in 1991, stipulates that no more than three embryos can be created per cycle of IVF and all three, regardless of their quality, must be transferred. In relation to assessment of cleavage-stage embryos, some of the key studies were originally presented by Gerris et al. [45] and Van Royen et al. [46], who employed strict embryo criteria to select single embryos for transfer. These did not however differ greatly to papers published in the 1980s by Cummins et al. [25] who also described key cleavage events linked with viability. What constitutes a "top" quality embryo? These "top" quality embryos had the following characteristics: four or five blastomeres on day 2 and at least seven blastomeres on day 3 after fertilization, absence of multi-nucleated blastomeres, and <20% of fragments on day 2 and day 3 after fertilization. When these criteria were utilized in a prospective randomized clinical trial comparing single and double embryo transfers, it was found that in 26 single embryo transfers where a top-quality embryo was available an implantation rate of 42.3% and ongoing pregnancy rate of 38.5% was obtained. In 27 double embryo transfers, an implantation rate of 48.1% and ongoing pregnancy rate of 74% was obtained. A larger study analysing the outcome of 370 consecutive single top-quality embryo transfers in patients younger than 38 years for pregnancy showed that the pregnancy rate after single top-quality embryo transfer was 51.9% [47].

The majority of studies that have used and report embryo selection criteria on the basis of cell number and morphology do so by stating that embryos were selected on day 2 or day 3. As discussed by Bavister [48], one of the most critical factors in determining selection criteria was to ascertain strict time points to compare the embryos. Sakkas and colleagues therefore used cleavage to the 2-cell stage at 25 hours post insemination or microinjection as the critical time point for selecting embryos [49–51]. In a larger series of patients, it was found that 45% of patients undergoing IVF or ICSI have early cleaving 2-cell embryos. Patients who have early cleaving 2-cell stage embryos allocated for transfer on day 2 or 3 have significantly higher implantation and pregnancy rates [49]. Furthermore, nearly 50% of the patients who have two early cleaving 2-cell embryos transferred achieve a clinical pregnancy (Figure 16.2). The most convincing data supporting the usefulness of early cleaving 2-cell embryos is that provided by single embryo transfer [52, 53]. In one study, Salumets et al. [52] showed that when transferring single embryos a significantly higher clinical pregnancy rate was observed after transfer of early cleaving (50%) rather than non-early cleaving (26.4%) embryos. The embryos that cleave early to the 2-cell stage have also been reported to have a significantly higher blastocyst formation rate [54, 55]. Another study by Guerif et al. [54] reported the sequential growth of 4042 embryos individually cultured from day 1 to day 5 or 6. Interestingly, early cleavage and cell number on day 2 were the most powerful parameters to predict the development of a good morphology blastocyst at day 5. Video imaging has aided in refining these timing events and now numerous algorithms exist [56, 57] which help incorporate both cleavage and timing in predicting both blastocyst development and implantation potential [58, 59].

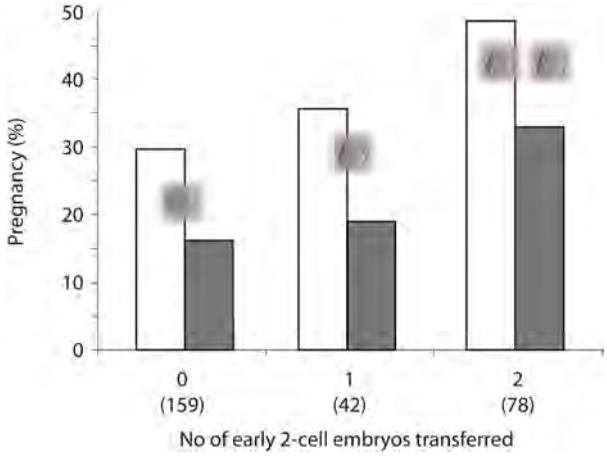

FIGURE 16.2 The percentage of clinical pregnancies (light columns) and implantation rate (dark columns) in relation to whether patients had zero, one, or two early cleavage embryos transferred. The numbers in parentheses indicate the number of cycles per group.

Morulae stage embryos

One somewhat overlooked stage has been the morula stage. Why this has not been used as an assessment tool is interesting but mostly stems from a need to not over-observe embryos and lack of definitive historical morphological assessment during this stage. With the introduction of time-lapse analysis, we can now readily visualize and analyse key morphogenic events around the time at which the first epithelium of the conceptus is formed. Studies now indicate that a day 4 scoring system could be successfully adopted and implemented [60] and may provide SET rates similar to day 5 SETs [61]. The adoption of such a strategy has however still not been thoroughly evaluated.

Development to the blastocyst stage

Blastocyst stage transfer has become standard clinical care in several countries, due to a number of factors. These include: the commercial availability of sequential, one-step, and time-lapse culture media; improvements in blastocyst cryopreservation made possible through vitrification; [62–64] and finally the move to the biopsy of the trophectoderm for PGT-A [31, 65–68].

The quality of blastocyst obtained is however of critical importance. As with the scoring of embryos during the cleavage stages, timing and morphology are key in selecting the best blastocyst. The scoring assessment for blastocysts devised by Gardner and Schoolcraft [69] is one of the most widely adopted. In effect, even the Alpha Scoring System is a numerical interpretation of the Gardner scale [70]. The Gardner scoring system is based on the expansion state of the blastocyst and on the consistency of the inner cell mass (ICM) and trophectoderm cells (Figure 16.3). Examples of high-quality blastocysts are shown in Figure 16.4. Using such a grading system it was determined that when two high-scoring blastocysts (>3AA), i.e. expanded blastocoel with compacted ICM and cohesive trophectoderm epithelium are transferred, a clinical pregnancy and implantation rate of >80% and 69% can be attained [71]. When two blastocysts not achieving these scores (<3AA) are transferred, the clinical pregnancy and implantation rate are significantly lower, 50% and 33%, respectively [72].

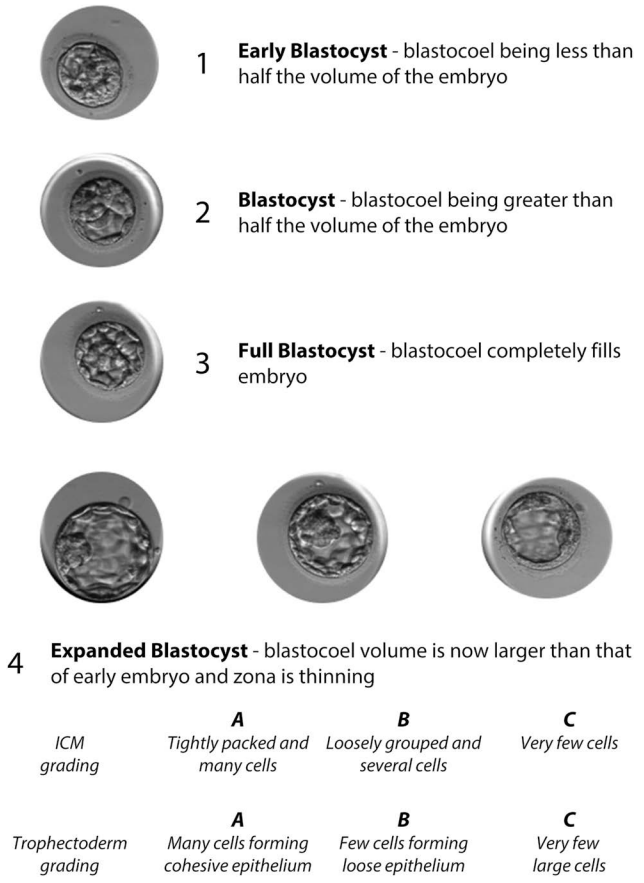

FIGURE 16.3 The blastocyst grading system. (Modified from Gardner and Schoolcraft [69].)

Although reduced from the values obtained with top-scoring blastocysts, it is evident that early blastocysts on day 5 still have high developmental potential.

More recent detailed analyses of whether the ICM or trophectoderm provides greater predictive weight for embryo selection concluded that the predictive strength of the trophectoderm grade was greater compared to the ICM for selecting the best blastocyst for embryo replacement [73]. It has been suggested that even though ICM is important, a strong trophectoderm layer is essential at this stage of embryo development, allowing successful hatching and implantation. This has subsequently been validated by a number of studies [74–78] which all highlighted the need for a strong trophectoderm grading in relation to pregnancy. Interestingly, one study found that a poor ICM grading was also related to higher miscarriage rates [79].

Van den Abbeel and colleagues [79] have also provided data on the importance of the ICM grade when assessing the blastocyst, reporting that all three parameters of the blastocyst (degree of expansion, ICM, and TE quality) were significantly associated with pregnancy and live birth rates. It was further determined that transfer of blastocysts with an "A" grade ICM reduces the incidence of pregnancy loss [79], and that ICM grade is positively associated with birth weight [80]. Physiologically, the TE and ICM do not exist in isolation, but rather are a functional unit. Although the TE creates a unique environment for the ICM by the synthesis of blastocoel fluid, it is the ICM itself that regulates the proliferation and activity of the trophectoderm in the mouse blastocyst [81–83]. Hence, it appears prudent to utilize both the ICM and TE grades in decisions regarding the fate of an embryo. Interestingly, it was also recently shown that a blastocyst with a low overall grading was associated with a higher chance of female baby (48% vs 42%, adjusted OR = 1.26 [1.13, 1.39]) and a higher rate of caesarean section (C-section; 71% vs 68%, adjusted OR = 1.15 [1.02, 1.29]) [84]. Finally, it is also evident that even poor-grade blastocysts (CC) have potential, although greatly reduced when compared to high-quality blastocysts [85].

In addition to the ICM and TE grade the timing of blastocyst formation is also crucial, in particular when performing fresh transfers [86]. The timing of blastocyst formation however is less important when considering frozen transfers. In theory, achieving the blastocyst stage at day 5 suggests that they may be of higher quality. Recent retrospective cohort studies using vitrification have demonstrated that the live birth rate may be slightly higher with day 5 versus day 6 vitrified-warmed blastocysts [73, 87, 88]. It is also clear now that even blastocysts forming on day 7 have respectable live birth rates and that when frozen and transferred back to a re-synchronized uterus they can add significantly to a patient's chance of success [89, 90].

Some groups have also attempted to correlate blastocyst rates and quality with overall ploidy status of the embryo. Interestingly, identifying euploid embryos by PGT-A appeared to override blastocyst morphological grade and day of cryopreservation, as regardless of grade their live birth rates were not significantly

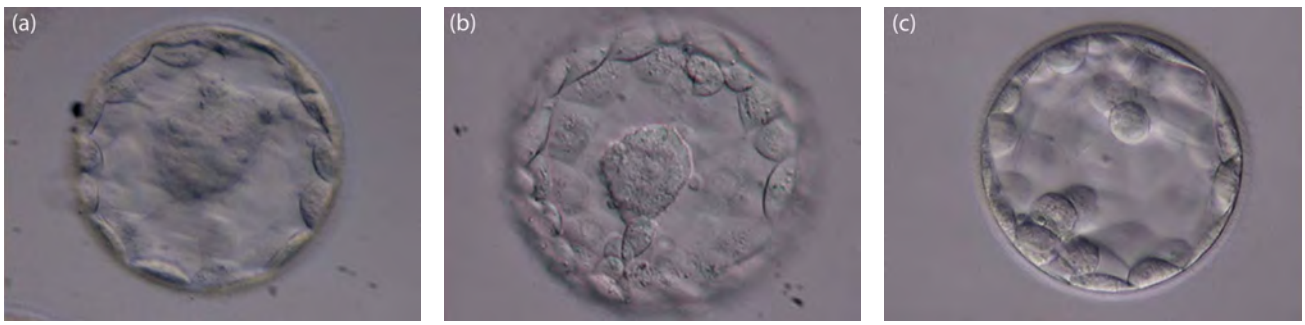

FIGURE 16.4 Day 5 human blastocysts. Using the grading system reported by Gardner and Schoolcraft [69]. Blastocysts in (a) and (b) would both score 4AA; the embryo in (c) would only score 4CA due to the apparent absence of an ICM, in spite of the development of an excellent trophectoderm.

different [88]. Predicting the euploid blastocyst by morphology and time-lapse has become a particular goal but has not been conclusive. In one study, Campbell and colleagues reported that the timing of formation of the blastocoel was delayed in aneuploid embryos [91]. Time to the start of blastulation of <100 hours after insemination and the morphokinetic scoring system used in the time-lapse group were independently associated with implantation. The association between cleavage parameters and prediction of aneuploidy warrants further study [92]. Recently, the use of artificial intelligence to examine static images of blastocysts has shown promise [93]. In this study a total of 1231 embryo images were classed as good prognosis if euploid and implanted or poor prognosis if aneuploid and failed to implant. An accuracy of 0.70 was obtained using an embryo ranking artificial intelligence (AI) algorithm, with positive predictive value of 0.79 for predicting euploidy. The use of AI, in reference to embryo selection, is providing some exciting promise with high predictability [94] (Chapters 18 and 19).

A strategy for selecting the best embryo by morphology

The preceding selection criteria have all shown that they generate some benefit in identifying individual embryos that have a high viability. Curiously, one thing that video imaging seems to be teaching us is to not only investigate the things that go right but also the things that go wrong. Although video imaging has aimed to develop selection algorithms looking for positive selection features related to embryo implantation potential, it has also shown us that numerous events can be used to deselect embryos from the transfer pool. One of the most evident deselection events seems to be direct cleavage to the 3-cell stage [95].

How do we implement a strategy for selecting a single embryo when we have many embryos to choose from? A few schools of thought are now being adopted for embryo selection. Previously, it was suggested that a multiple-step scoring system that encompasses all the preceding criteria would be the best approach. The use of sequential scoring systems has been shown to be beneficial by a number of authors [46, 55, 96]. The advent of machine learning with time-lapse has superseded this manual process, as all the preceding criteria can be better integrated in an automated fashion.

18–19 hours post insemination/ICSI (Figure 16.1): Identification of two pronuclei embryos. The pronuclei are examined for:

a. symmetry
b. the presence of even numbers of NPB
c. the positioning of the polar bodies

25–26 hours post insemination/ICSI (Figure 16.5):

a. embryos that have already cleaved to the 2-cell stage
b. zygotes that have progressed to nuclear membrane breakdown

42–44 hours post insemination/ICSI (Figure 16.5):

a. number of blastomeres should be greater or equal to four
b. fragmentation of less than 20%
c. no multi-nucleated blastomeres

66–68 hours post insemination/ICSI (Figure 16.5):

a. number of blastomeres should be greater or equal to eight
b. fragmentation of less than 20%
c. no multi-nucleated blastomeres

116–118 hours post insemination/ICSI (Figures 16.3 and 16.4):

a. the blastocoel cavity should be full
b. ICM should be numerous and tightly packed
c. trophectoderm cells should be numerous and cohesive

Which of the preceding criteria will prove to be the most important? Or will they all be needed? In brief, AI has refined sequential embryo assessment but still relies on the preceding criteria so as to decipher the most impactful hurdles of development. At every step an embryo is effectively given a positive mark when it reaches the ideal criteria of a certain stage. It would however be possible that an embryo may not pass one step but would pass the hurdle at a following step. The embryo or embryos attaining the best criteria at each

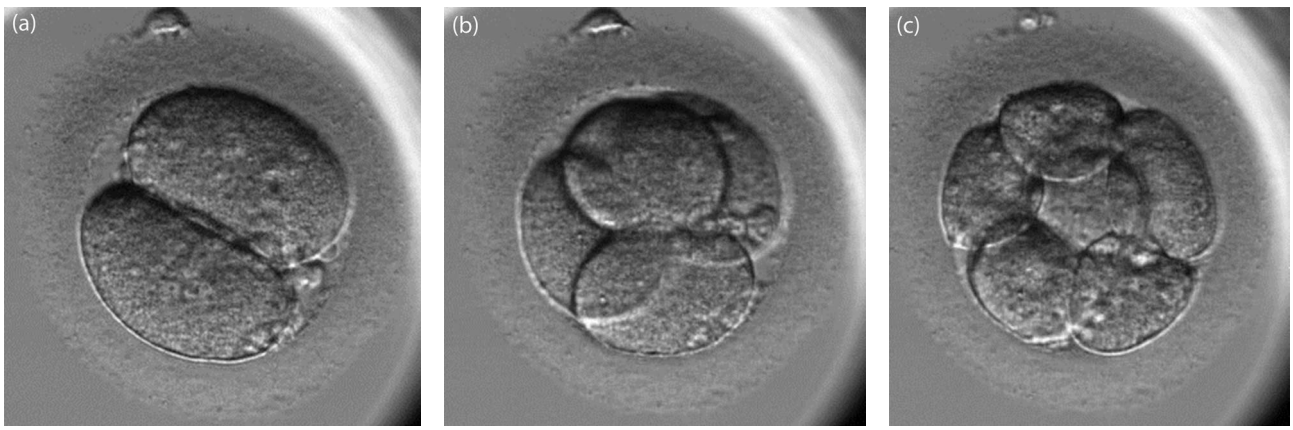

FIGURE 16.5 Ideal features of embryos scored at (a) 25–26 hours, (b) 42–44 hours, and (c) 66–68 hours post insemination/ICSI. For greater details on the scoring criteria, see Sakkas et al. [49], Shoukir et al. [51] and Van Royen et al. [46].

step would therefore be the ones that would be selected for transfer (Figure 16.6). For example, if we are attempting to transfer a single embryo to a patient, the following scenario could be envisaged. An embryo may not pass any of the earlier hurdles but still form a high-grade blastocyst on day 5. If this were the most successful of the cohort of embryos then this would be the one selected. If, however, six blastocysts were observed on day 5, all of equally high grade, then the blastocyst that had achieved the most positive scores at each of the previous hurdles could be transferred. If the shortened protocol was used and only day 2 was the previous score then the best-looking day 2 embryos would be ranked as better. Furthermore, patients who have low numbers of embryos, and will have transfer on day 2 or 3, could be assessed using the initial criteria and the embryo that passed the initial hurdles would be selected. Proposed schedules of embryo selection are given in Figure 16.6, taking into account different strategies or assessment criteria. It is important to note that to-date the strongest criteria of selection appear to be the selection of a high-quality blastocyst on day 5 of development [54, 71]. Since we first developed and advocated this approach to embryo selection in [97] and then developing it further to include a weighted score for each stage [98], such data has been incorporated into many algorithms for use with time-lapse microscopy, to facilitate both embryo deselection and selection.

One of the perennial questions that still remains is whether cleavage or blastocyst transfer is performed. Even though we have the choice of assessing multiple stages by either repeated manual or time-lapse assessments, many groups have looked at actually minimizing their assessment of embryos and culturing all embryos directly to the blastocyst stage (Figure 16.6). This has coincided with a shift in general opinion that blastocyst morphology can potentially provide stronger evidence of viability [73] and also the data consistently indicating that single embryo transfer is more likely to be implemented with blastocyst rather than cleavage transfer [99, 100]. This would possibly involve scoring fertilization on day 1, and then leaving embryos in culture until day 5, 6, and 7 when they are assessed for transfer or cryopreservation at the blastocyst stage. For example, the clinic may want to set a limit on how many fertilized embryos they need to continue for blastocyst culture (Table 16.1). Data from Boston IVF indicates that a patient <40 years of age with at least four fertilized embryos has over an 80% chance of having a blastocyst for transfer or cryopreservation. Some argue that even identifying blastocysts where the 2PN have not been observed is valuable and that any blastocyst could be included in the pool of usable embryos [101].

The following strategies would be available for most laboratories. Firstly, standard assessment of embryos adopting all or some of the aforementioned morphological criteria. A practical issue for performing such a selection process is that embryos need to be cultured in individual drops. This may remove any necessary benefits of culturing embryos in groups [102–104]. Group culture could

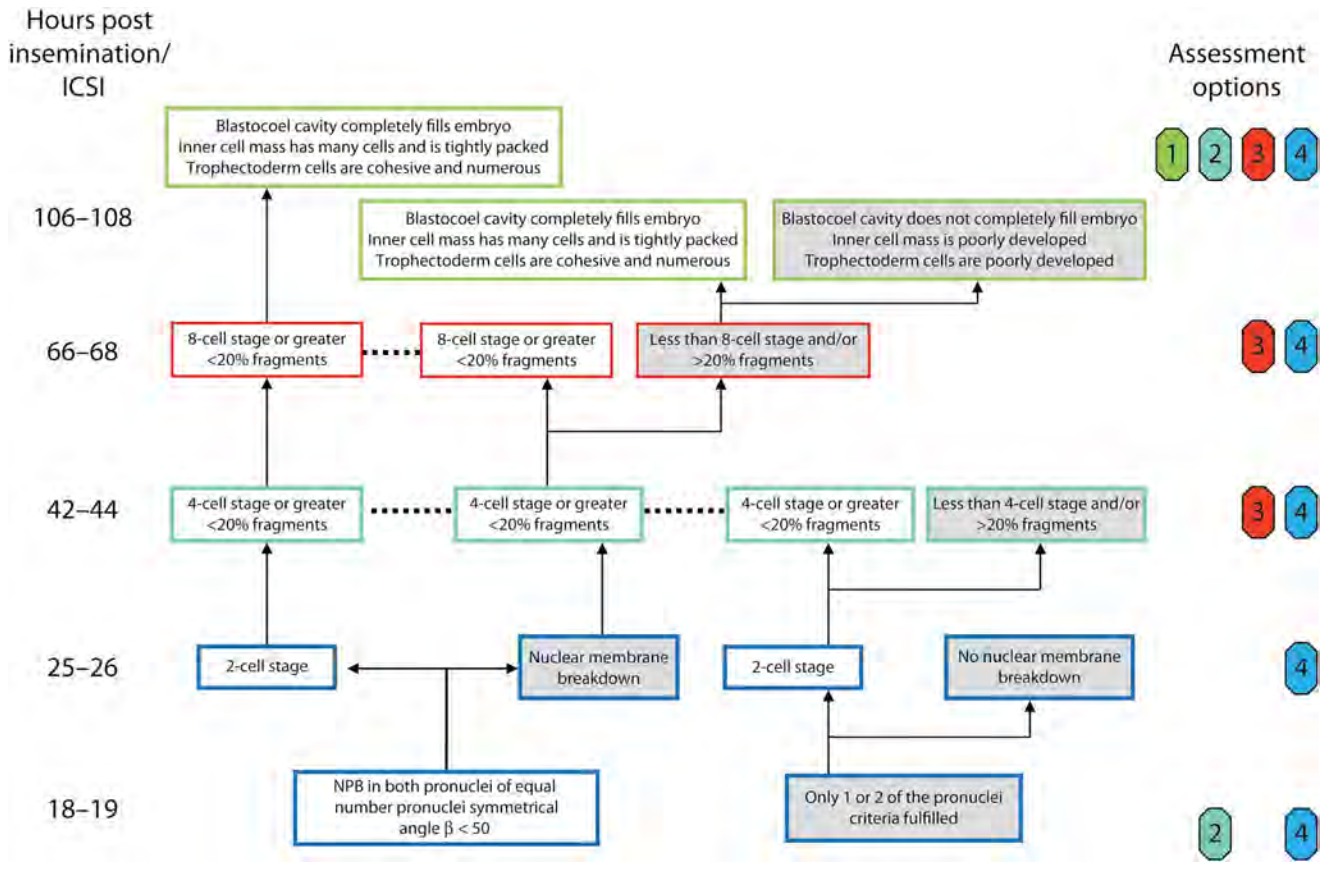

FIGURE 16.6 A strategy for selecting a single embryo for transfer using different morphological assessment options. Number 1 would entail a single assessment only at the blastocyst stages (green); number 2 (light blue) would allow triaging of patients on day 1 followed by assessment at the blastocyst stage; number 3 (red) would allow triaging of patients on day 2 and/or 3 followed by assessment at the blastocyst stage. Number 4 (dark blue) would allow triaging of patients on each day 1, 2, and 3 followed by assessment at the blastocyst stage.

TABLE 16.1 The probability of obtaining at least 1–6 blastocysts for transfer or cryopreservation in relation to having three, four, five, or six fertilized two pronuclear embryos.

	NUMBER OF 2PN FERTILIZED	AT LEAST					
		1	2	3	4	5	6
AGE GROUP (YEARS)		BLASTOCYST(S)					
<35	6	96.11%	81.11%	57.96%	35.00%	14.26%	3.15%
	5	94.41%	76.40%	45.41%	8.83%	7.57%	
	4	92.66%	67.09%	37.74%	23.90%		
	3	77.63%	42.98%	11.84%			
35–37	6	91.82%	77.27%	49.32%	26.59%	10.91%	2.05%
	5	92.98%	73.68%	39.91%	17.98%	5.04%	
	4	87.61%	56.88%	22.48%	4.82%		
	3	78.16%	42.32%	11.60%			
38–40	6	93.89%	77.02%	46.70%	21.52%	7.58%	1.71%
	5	89.50%	67.06%	36.04%	14.32%	1.91%	
	4	82.70%	51.08%	19.46%	3.51%		
	3	76.74%	36.86%	9.06%			
	6	82.43%	37.24%	10.88%	3.35%	0.42%	0.00%
>40	5	68.84%	27.90%	6.16%	1.45%	0.36%	
	4	66.06%	24.55%	3.25%	0.36%		
	3	52.63%	14.57%	3.24%			

Note: The data are from an analysis of over 20,000 fertilized embryos left for culture to the blastocyst stage. The green shade shows when the chance is >90% and yellow shade shows when the chance is between 80% and 90%.

be performed, however; as embryos reach certain milestones they could be triaged to a subsequent embryo culture drop. A further practical issue when embryos need to be observed more often is that using a drop culture system under oil with adequate heating control of all microscope stages will greatly reduce pH, osmolality, and temperature fluctuations [105]. Fortuitously, the move to commercialize real-time imaging of embryos has now placed multiple embryo assessment procedures closer to a practical reality, removing any concerns related to constant visualization of the embryos away from the incubator [56, 57, 105]. The further development of this type of imaging system is covered in Chapters 17 to 19. However, the scoring regimens described in detail in this chapter will serve all those clinical laboratories that do not have access to time-lapse analysis. A second approach is to minimize observations and culture directly to the blastocyst stage. As mentioned earlier, this can be performed on all patients [106] or on patients that reach certain milestones such as a specific number of fertilized or cleaved embryos (Table 16.1).

It is evident that with improved culture conditions, together with suitable grading systems, it is possible to dramatically increase implantation rates, decrease the number of embryos transferred, and increase the live birth rate. However, this approach raises two issues; if the laboratory in question is not performing blastocyst transfer, then it cannot rely on advanced grading systems, and, secondly, morphology will only tell us a limited amount about the physiological status of the embryo. The rest of this chapter is, therefore, devoted to the application of novel tests of embryonic function. It is assumed that such tests must be non-invasive for the adoption in clinical use. Therefore, methods that can be considered as semi-invasive, i.e. those that involve embryo biopsy prior to cell analysis are not considered here and are discussed in Chapters 13 and 25.

Beyond embryo morphology: The non-invasive quantification of embryo physiology

A number of quantitative techniques have been trialled which attempt to monitor the uptake of specific nutrients by the embryo from the surrounding medium, and to detect the secretion of specific metabolites and factors into the medium (Figure 16.7). Such approaches have strived to measure changes in culture media and fulfil the following three key criteria so that they can be applicable in IVF clinics.

1. They must have the ability to measure the change without damaging the embryo.
2. They must have the ability to measure the change quickly (this requirement may however be lower, as the success of vitrification and move away from fresh transfers [107] may circumvent the need for a rapid test).
3. They must have the ability to measure the change consistently and accurately.

The analysis of metabolite levels within spent embryo culture media fulfils the preceding criteria, and has been one method examined to augment the analysis of embryo morphology as a means of embryo selection. Three approaches have been evaluated: analysis of carbohydrate utilization, the turnover of amino acids, and the analysis of the embryonic metabolome. The first two approaches could be considered analysis of the activity of specific metabolic pathways, whereas analysis of the metabolome should be considered as the systematic analysis of the inventory of metabolites that represent the functional phenotype at the cellular level.

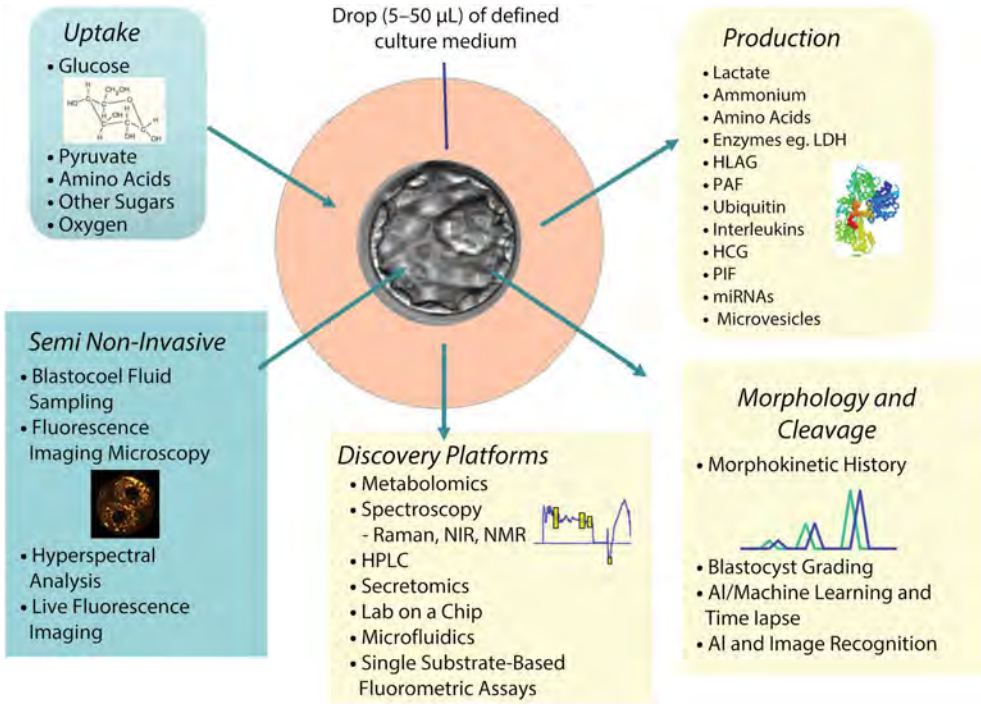

FIGURE 16.7 Options for the non-invasive analysis of human embryo nutrient consumption and metabolite/factor production. Individual blastocysts are incubated in 5.0 to 50.0 µL volumes of defined medium. Serial or end point samples of medium can then be removed for analysis and an indirect measurement of metabolic pathways can be ascertained by measuring uptake or production of various factors or using discovery platforms. Non-invasive platforms can also include current morphology and cleavage criteria using static or time-lapse measurements. Semi-non-invasive platforms are also under development using novel microscopy platforms or examining the blastocoelic fluid.

Depending upon the technology employed to analyse the metabolome, one does not necessarily obtain identification of specific metabolites, but rather one is able to create an algorithm that relates to cell function and hence to potential viability.

Analysis of carbohydrate utilization

A relationship between metabolic activity and embryo development and viability has been established over several decades [108]. As early as 1970, Menke and McLaren revealed that mouse blastocysts developed in basic culture conditions lost their ability to oxidize glucose [109]. This initial observation was followed by several studies that elucidated changes in embryo metabolism associated with loss of developmental capacity *in vitro* [reviewed by [110]]. In 1980, Renard et al. [111] observed that day-10 cattle blastocysts which had an elevated glucose uptake developed better, both in culture and *in vivo*, after transfer than those blastocysts with a glucose uptake below this value. In 1987, using the then relatively new technique of non-invasive micro-fluorescence, Gardner and Leese [112] measured glucose uptake by individual day-4 mouse blastocysts prior to transfer to recipient females. Those embryos that went to term had a significantly higher glucose uptake in culture than those embryos that failed to develop after transfer. This work was then built on by Lane and Gardner [113], who showed that glycolytic rate of mouse blastocysts could be used to select embryos for transfer prospectively. Morphologically identical mouse blastocysts with equivalent diameters were identified, using metabolic criteria, as "viable" prior to transfer and had a fetal development of 80%. In contrast, those embryos that exhibited an abnormal metabolic profile (compared to *in vivo* developed controls) developed at a rate of only 6%. Clearly, such data provides dramatic evidence that metabolic function is linked to embryo viability (Figures 16.8a and b), and that perturbations in relative activity of metabolic pathways is associated with loss of cell function, leading to compromised development post transfer.

Analysis of the relationship between human embryo nutrition and subsequent development *in vitro* was undertaken by Gardner et al. [114], who determined that glucose consumption on day 4 by human embryos was twice as high in those embryos that went on to form blastocysts. Subsequently, Gardner and colleagues [115] went on to confirm a positive relationship between glucose uptake and human embryo viability on day 4 and day 5 of development (Figure 16.9). Furthermore, the data generated indicate that differences in nutrient utilization differ between male and female embryos, a phenomenon previously documented in other mammalian species [116, 117]. A subsequent analysis of more than 200 human blastocysts again confirmed that those embryos that go on to form a pregnancy consume significantly more glucose than those blastocysts which failed [118].

Currently making accurate analysis of nutrient uptake by individual embryos is performed using non-commercial fluorescence assays, which have been limited to just a few laboratories worldwide. The widespread implementation and subsequent validation of this approach should be made possible through the development of chip-based devises capable of quantitation accurately sub-microlitre volumes of medium [119–121].

Analysis of amino acid utilization

In studies on amino acid turnover by human embryos, Houghton et al. [122] determined that alanine release into the surrounding medium on day 2 and day 3 was highest in those embryos that did

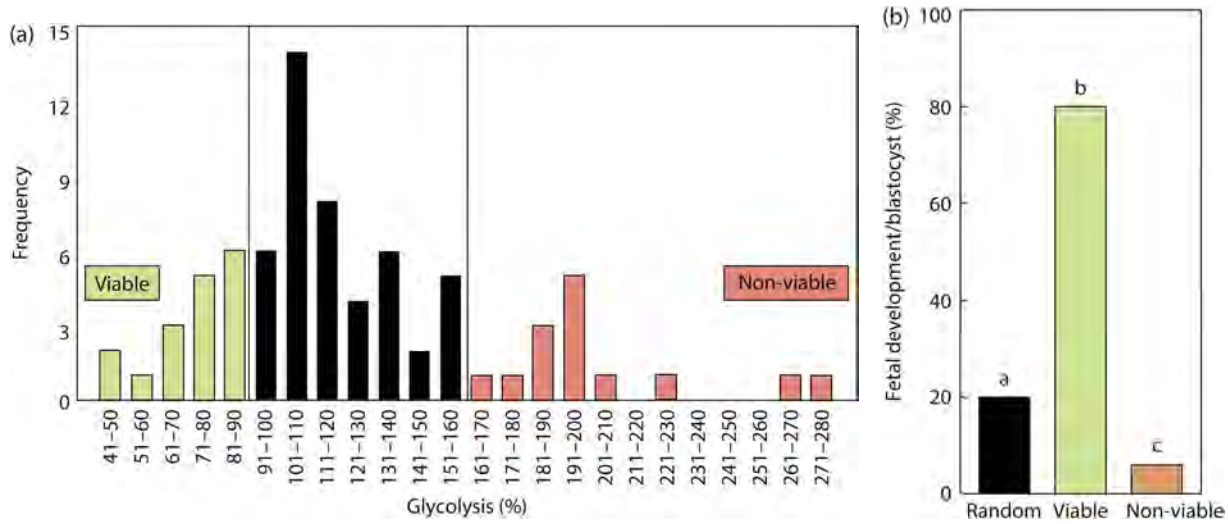

FIGURE 16.8 (a) Distribution of glycolytic activity in a population of 79 morphologically similar mouse blastocysts cultured in medium DM1. The lowest 15% of glycolytic activity (<88%) were considered viable, while the highest 15% of the range (>160%) were deemed non-viable. (Adapted from [113].) (b) Fetal development of mouse blastocysts selected for transfer according to whether they were considered viable or non-viable using glycolytic activity as a biochemical marker. On each day of the experiment, a selection of blastocysts was transferred at random, along with those selected as either viable or non-viable. Different superscripts [a,b,c] indicate significantly different populations ($P < 0.01$).

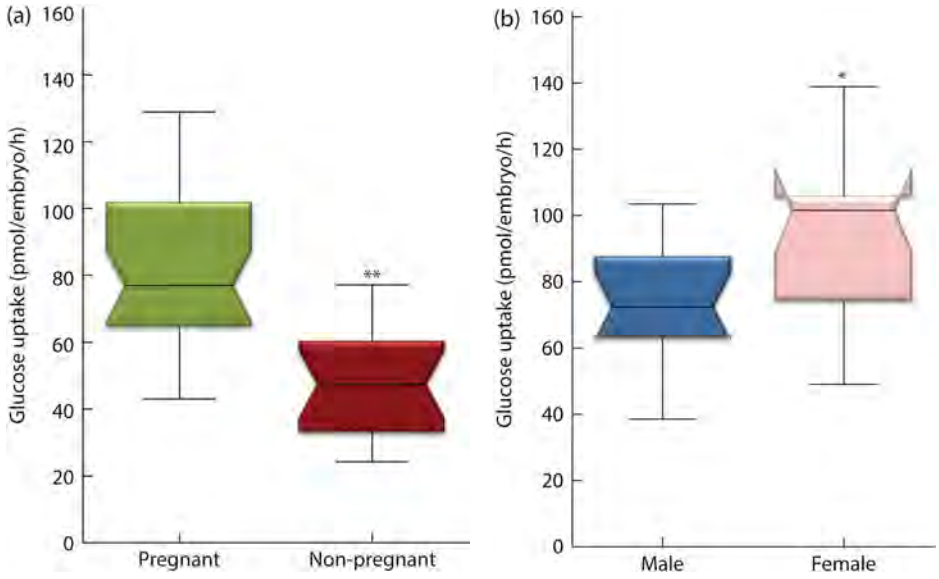

FIGURE 16.9 Relationship between glucose consumption on day 4 of development and human embryo viability and embryo sex. (a) Glucose uptake on day 4 of embryonic development and pregnancy outcome (positive fetal heart beat). Notches represent the confidence interval of the median, and the depth of the box represents the interquartile range (50% of the data); whiskers represent the 5% and 95% quartiles. The line across the box is the median glucose consumption. **, significantly different from pregnant ($P < 0.01$). (b) Glucose uptake by male and female embryos on day 4 of development. Notches represent the confidence interval of the median, and the depth of the box represents the interquartile range (50% of the data); whiskers represent the 5% and 95% quartiles. The line across the box is the median glucose consumption. *, significantly different from male embryos ($P < 0.05$). (Adapted from [115].)

not form blastocysts. Subsequently, Brison et al. [123] reported changes in concentration of amino acids in the spent medium of human zygotes cultured for 24 hours in an embryo culture medium containing a mixture of amino acids using high performance liquid chromatography. It was found that asparagine, glycine, and leucine utilized in the 24 hours following fertilization were significantly associated with clinical pregnancy and live birth following day 2 embryo transfer. Further analysis also revealed an association with aneuploidy and embryonic sex with amino acid turnover [124]. Recent works have further revealed that amino acid consumption by human blastocysts is related to their grade, morphokinetics, and AI ranking [118].

Ongoing studies in this area could help to identify which amino acids at each stage of development is linked with subsequent viability. Recent animal studies have revealed how dynamic the use of amino acids is and how uptake can be affected by other aspects of the culture system, such as oxygen and the accumulation of ammonium through the spontaneous breakdown and metabolism of amino acids [125, 126]. Consequently, data on the use of nutrients needs to be carefully interpreted with regards to the conditions under which the embryos were developed.

Metabolomics

Evolving metabolomics technologies may allow us in the future to measure multiple factors in embryo culture media. Initial and encouraging metabolic studies of embryos indicated that embryos that result in pregnancy are different in their metabolomic profile compared to embryos that do not lead to pregnancies [127]. Investigation of the metabolome of embryos, as detected in the culture media they grow in, using targeted spectroscopic analysis and bioinformatics did show differences in some initial proof of principle studies [127].

Although a series of preliminary studies [128–131] showed a benefit of metabolomics-related techniques, they were largely based on retrospective studies and performed in a single research laboratory as distinct from a real clinical setting. The subsequent randomized clinical trials comparing standard morphological techniques for embryo selection versus using the near infrared (NIR) system to rank embryos within a cohort that had good morphology and were being selected for either transfer or cryopreservation failed to show compelling benefits [128, 131]. More recently, Pais et al. [132] examined the secretome of human embryos using Matrix-assisted laser desorption/ionization-time of flight (MALDI-ToF) mass spectrometry. They applied bioinformatic analysis to identify specific spectra of euploid and aneuploid embryo secretome signatures and were able to differentiate between genotypes with a sensitivity of 84% and a false positive rate of 18%.

Similarly, although Katz-Jaffe et al. [133–135] revealed that the proteome of individual human blastocysts of the same grade differed between embryos, and also identified a number of secreted protein markers that could be used to identify the best embryo, a relationship between such biomarkers and subsequent viability has yet to be validated prospectively.

Although the use of metabolomic and proteomic platforms has yet to be proven and employed clinically, analysis of embryo function for its own sake can greatly enhance our understanding of embryo development and hence such approaches could ultimately assist in defining parameters than can be used in embryo selection. To this end, an analysis of the relationship between the morphokinetics development of embryos and their metabolic activity has been undertaken.

When morphometrics and metabolic analysis collide

Using a mouse model to analyse the relationship between key morphometric and metabolic data from individual IVF-derived embryos, Lee and colleagues determined that blastocysts developing from those embryos exhibiting early cleavage (and hence presumed to have a higher viability) possessed a metabolic profile of increased glucose uptake and reduced rates of glycolysis (and hence exhibiting metabolic characteristics of enhanced viability) [136]. Furthermore, it was observed that blastocysts developed from embryos with early cleavage also consumed more aspartate, potentially reflecting a more active malate-aspartate shuttle, which has been implicated in the regulation of blastocyst metabolism and viability [137, 138]. Together, such data generate renewed excitement regarding the potential of non-invasive quantification of embryo physiology to assist in the selection of the most viable embryo for transfer, and the potential of combining two independent means of assessing the pre-implantation embryo to improve the accuracy of selection.

Non-invasive fluorescence microscopy

In recent years a greater focus has been made on implementation of fluorescence microscopy to investigate embryo metabolism. Two techniques have been examined, Fluorescence Lifetime Imaging Microscopy (FLIM) and Hyperspectral Microscopy. Both technologies rely on autofluorescence, which is the natural emission of light by biological structures such as mitochondria and lysosomes. The most commonly observed autofluorescencing molecules are NADPH and flavins; the extracellular matrix can also contribute to autofluorescence because of the intrinsic properties of collagen and elastin. Using FLIM, it was determined [139] that the metabolic state of human blastocysts continuously varies over time. Although it was possible to identify metabolic variations between blastocysts in relation to day of development and developmental expansion stage, their morphological grade was not related to metabolic state. Interestingly, substantial metabolic variations between blastocysts from the same patients were observed. Furthermore, there was significant metabolic heterogeneity within individual blastocysts, including between the ICM and the trophectoderm. Both FLIM and hyperspectral microscopy have also provided evidence that they can distinguish between euploid and aneuploid embryos [140, 141].

The non-invasive nature of both these microscopy techniques and the aforementioned observations combined give merit to the intricate timing of metabolic shifts in human embryos and how understanding these changes has great potential to improve our ability to distinguish between viable and non-viable embryos.

Other specific factors

Other techniques have also been reported to measure metabolic parameters in culture media; however, they have yet to be tested in a clinical IVF setting. These include the self-referencing electrophysiological technique, which is a non-invasive measurement of the physiology of individual cells and monitors the movement of ions and molecules between the cell and the surrounding media [142, 143]. Another technique using a probe was initially developed by Unisense to non-invasively measure oxygen consumption of developing embryos. Interestingly, although this technology was shown to correlate with bovine blastocyst development it was less successful in predicting mouse embryo development [144, 145].

A number of studies have also investigated the assessment of secreted factors in the embryo culture media (Figure 16.7) and correlated them with better embryo development and pregnancy rates. One such factor is soluble HLA-G [146, 147], which is believed to protect the developing embryo from destruction by the maternal immune response. Soluble HLA-G has been found in media surrounding the early embryo and a number of papers have also reported that its presence correlates with the improved pregnancy potential of an embryo [148–150]. However,

some studies have raised some serious concerns regarding the use of HLA-G production as a marker of further developmental potential [151–153], and prospective clinical trials are needed to further evaluate this parameter. Included in the studies examining secretion of factors in the media by embryos are numerous papers examining the secretion of platelet-activating factor (PAF). The clinical utility of PAF in an IVF setting has also yet to be stringently examined (see review by O'Neill [154]). Other factors are currently under investigation, including one called the pre-implantation factor (PIF), which has been reported to provide some indication of embryo viability when measured and to possibly improve embryo quality when placed in embryo culture media [155]. Numerous other candidates have also been postulated and tested, including human chorionic gonadotropin [156, 157] and interleukin 6 [158]. All these molecules could also benefit from novel single substrate-based fluorometric assays that are currently being optimized [159].

It is beyond doubt that markers do exist in the spent embryo culture media indicative of viability. The most advanced current techniques are AI coupled with time-lapse or static blastocyst images (see Chapters 18 and 19) and the analysis of cell-free DNA in embryo culture media [160] to assess chromosomal copy numbers. The major benefits of a non-invasive fluorescence technology is the fact that the technology visualizes the whole embryos, and the time taken to assess the samples is relatively short, making it possible to perform the analysis just prior to fresh embryo transfer or freezing. Many research groups around the world are still attempting to make this a reality for the IVF clinic.

Summary

Analysis of embryo morphology and the development of suitable grading systems have greatly assisted in the selection of human embryos for transfer. However, it is proposed that in the near future embryo selection will also be significantly aided by the non-invasive analysis of embryo physiology and function, using approaches that better quantify embryo metabolism. The addition of such technologies will be of immense value in helping both clinicians and embryologists to more confidently select the most viable embryos within a cohort and making the need to transfer more than one embryo a thing of the past.

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17

EVALUATION OF EMBRYO QUALITY

Time-Lapse Imaging to Assess Embryo Morphokinesis

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Introduction

In vitro fertilization (IVF) programs are coming closer every day to the goal of reducing multiple pregnancies while maintaining good clinical results. The transfer of a single embryo progressively became a reality specifically in developed countries, and this is the result of major improvements in different areas. From a clinical point of view, two major achievements are worth mentioning: first, physicians have learned to handle the stimulation drugs that are purer, more powerful, and more comfortable for the patient; and, second, an increased knowledge of the pathophysiology of ovarian hyperstimulation syndrome has made the frequency of this syndrome almost anecdotal. On the other hand, concerns about the "epidemic" of multiple gestations have raised awareness of the risks not only to the mother (gestational diabetes, hypertension, and anaemia) but also to the babies—extreme prematurity, low birth weight, children with neurological damage, and so on—not to mention the psychological burden and suffering of the parents and the tremendous health costs that it entails. From the laboratory point of view, several achievements are worth mentioning as well: studies on embryo metabolism have led to the formulation of suitable culture media. In the early 1990s, the introduction of intracytoplasmic sperm injection (ICSI) revolutionized the treatment of male infertility and genetic screening became the gold standard for the selection of aneuploid embryos. Vitrification came along, and preservation of fertility was no longer a utopia for modern women; the wave of the "omics" initiated an era of non-invasiveness for studying human embryos in the laboratory, and most recently in the last decade, the introduction of imaging systems, artificial intelligence (AI), and machine learning allowed us to assess embryos in a different way: through their morphokinetics and analysis of images and video captured during the early embryonic development.

Time-lapse technology

The success of an IVF treatment mainly depends on two factors, (i) optimal incubation environment and (ii) accurate embryo selection apart from other factors such as stimulation, endometrial preparation, etc. According to recent trend, the success of an IVF cycle is measured in terms of live birth instead of implantation. Traditional embryo assessment is based on time point evaluations. Through this approach, embryo categories are normally based on the number of blastomeres and nuclei, the percentage of fragments, cell symmetry, and the quality of the inner cell mass (ICM) and trophectoderm (TE). Even though great knowledge has been achieved through this approach, it has been demonstrated that embryo status can markedly change within a few hours [1–4]. In addition, inter- and intra-observer variabilities are commonly described problems [5], probably due to the subjective nature surrounding traditional morphological assessment [4, 6–10]. In theory, increasing the number of observations could provide better information on the development of the embryo and therefore improve its assessment [4, 11, 12]. However, increased handling and higher evaluation frequencies will expose the embryo to undesirable changes in temperature, humidity, and gas composition [11–13]; apart from these exposure to light is also harmful to embryos.

Time-lapse technology (TLT) represents a solution to this problem. In 1997, Payne et al. [14] developed time-lapse cinematography to manage intermittent observation of the process of oocyte fertilization. Later, the observation period was augmented while maintaining optimal culture conditions [2], and nowadays TLT allows the complete observation of the entire process of embryo development in the IVF laboratory. The two main advantages of these systems are (i) improved and stable culture conditions and (ii) the determination of objective and accurate markers, both quantitative and qualitative [4]. In addition, we should mention that there is reduced handling and human risk; minimization of culture media, gas, and oil; detection of abnormal events that would normally occur between observations; reduced inter- and intra-observer variability; and reduced numbers of hours needed by the embryologist in the laboratory [15].

In 2011, Meseguer et al. coined the term "morphokinetics," defined as the combination of the embryo appearance (morphology) and the timing in which cellular events occur. Morphokinetics has been introduced as a new concept to improve embryo selection. The use of this strategy could allow single-embryo transfers (SETs) without jeopardizing the overall IVF success [16], becoming very attractive, especially in European countries in which legislation is stricter about the number of embryos transferred [15].

Theoretically, the uninterrupted culture represents an improvement to the conventional incubator, where embryos are removed from the incubator for inspection at each time point for evaluation. There have been several studies on TLT in the last decade regarding blastocyst formation, blastocyst quality, pregnancy, implantation, live birth, aneuploidy, and other patient characteristics to measure the benefit and usefulness of the system through morphokinetic parameters and other observations which were not possible with conventional morphology and incubators. Apart from that, new knowledge and information has been acquired through this technology regarding early embryo development and the potential of non-invasive markers. Recently promising machine learning (ML) technology, part of AI and computer vision (CV), has overtaken the field in terms of creating selection algorithms.

Models on the market

There are different options of TLT available on the market. Some of them present all the items integrated into one single piece of equipment (e.g. Embryoscope®, Embryoscope+® [Vitrolife], Geri®, Geri+® [Genea Biomedx], Miri® TL [Esco Medical], and ASTEC™).

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Others offer the option of introducing a microscope inside an available incubator (e.g. Primo Vision® [Vitrolife] and the Eeva™ Test [Merck-Serono]). Different systems also provide different embryo selection algorithms and the capability to create one's own algorithm or use the algorithm from other algorithms available in the market. Table 17.1 describes the clinical and technical features of all the TLT available on the market [17].

Kinetic parameters (individual plus calculated)

As described in the proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group [18], Table 17.2 describes the morphokinetic "individual" variables.

Additionally, Table 17.3 defines "calculated" variables that represent a certain cell stage or cycle duration, and during the de-vitrification process we can also define the duration of re-expansion and completion of re-expansion (Figure 17.1).

Blastocyst development studies

After the first study by Payne et al., in 1997, on human embryos, TLT first appeared in the market in 2009, and only a few IVF laboratories adopted the system in starting because of cost. But now TLT is widely used in IVF laboratories. Several studies have been conducted since 2008 to associate the morphokinetics markers with good-quality embryos and the prediction of blastocyst formation (Table 17.4).

After TLT appeared in the market, the potential of technology shifted from mere observation of human embryos while in culture to a selection and prediction tool. With TLT, a substantial amount of novel information about embryo development is acquired. The challenge has been how to use this information. Many studies have tried to assess the embryo based on different potential kinetic markers, such as time interval between cell division, fertilization markers, cleavage features (e.g. direct cleavage, absent cleavage, chaotic cleavage, reverse cleavage, etc.), multinucleation, compaction, symmetry, fusion, fragmentation, collapse, expansion, uneven blastomere, hatching, etc., to determine the highest chances for achieving success. Most of the studies in the past have been done on EmbryoScope™; and a few studies on time-lapse incubators which can be integrated in conventional incubators, such as EEVA™ and PrimoVision™, as other time-lapse incubators like Geri™, Miri™, etc., were introduced later in the market.

In 2010, Wong et al. analysed kinetic parameters of 100 embryos that were cultured up to days 5 or 6 of development [19] and found out three predictors of blastocyst formation: P1—duration of the first cytokinesis (14.3 ± 6.0 minutes); P2—interval between the end of the first mitosis and the initiation of the second mitosis (11.1 ± 2.2 hours); and P3—the synchrony between the second and third mitosis (1.0 ± 1.6 hours). The authors concluded that embryo development to the blastocyst stage could be predicted with 94% sensitivity and 93% specificity after using parameters P1, P2, and P3. Embryos with one or more values outside these ranges were expected to arrest. The time of completion of the second and third mitosis was also analysed by Hashimoto et al., who observed that high-scoring blastocysts took significantly shorter times for these divisions [20]. Later Coticchio et al. observed faster fertilization kinetics was associated with better embryo development, and new kinetic parameters were defined: cytoplasmic halo appearance (tHaloA) → disappearance (tHaloD); halo appearance (tHaloD) → PN fading (tPNf); PN fading (tPNf) → first cleavage (t2); andmale PN appearance ($tPNa_m$) → male PN fading ($tPNf_m$) [21]. After the study from Wong et al., a lot of studies tried to examine different morphokinetic parameters linked to blastocyst formation and blastocyst quality based on selection and deselection model. A few algorithms have also been proposed by different groups, most of the algorithms proposed earlier were based on a hierarchical model.

In a retrospective cohort study, Cruz et al. [22] monitored 834 embryos and defined kinetic markers linked to embryo development (Figure 17.2). In the same year, Dal Canto et al. [23] observed different cleavage kinetic parameters linked to embryo development and found that early cleavage from 2- to 8-cell stage occurs progressively earlier in embryos with the ability to develop to blastocyst and expand.

In 2013, Chamayou et al. [24] reported time intervals of morphokinetic parameters identified as predictors of embryo competence. The authors concluded that day-3 embryos developed into viable blastocysts when their kinetic parameters met the following ranges: t1 (18.4–30.9 hpi [hours post insemination]), t2 (21.4–34.8 hpi), t4 (33.1–57.2 hpi), t7 (46.1–82.5 hpi), t8 (46.4–97.8 hpi), tC – tF (7.7–22.9 hpi), and s3 (0.7–30.8 hpi). In the same year, a study by Kahraman et al. found a statistically significant difference in kinetics parameters t2, t8, tM, tB, cc2, s2 between top-quality blastocyst and good-quality blastocyst versus poor-quality blastocyst [25].

A couple of prospective studies were performed in 2013. Kirkegaard et al. [28] analysed 571 embryos from good prognosis patients and reported three markers linked to high-quality blastocysts: duration of the first cytokinesis, duration of the 3-cell stage, and direct cleavage to the 3 cell, all of which had comparable predictive values but no connection to implantation results. The Wong et al. study was the origin of EEVA™ test, and later Conaghan et al. [29] conducted a two-phase multicentre study to develop and validate an algorithm to predict blastocyst formation. A total of 1727 embryos were monitored by automatic cell-tracking software. The time between cytokinesis 1 and 2 (P2) and the time between cytokinesis 2 and 3 (P3) turned out to be the strongest parameters in the prediction model. The results indicated a higher probability of usable blastocyst formation when both P2 and P3 were within specific cell division timing ranges (P2, 9.33–11.45 hours; P3, 0–1.73 hours) and a lower probability when either P2 or P3 were outside the specific cell timing ranges.

The Conaghan model, also known as EEVA I, was tested retrospectively by a different group using a set of 1519 transferred embryos with known clinical outcome [31]. According to the algorithm, embryos were classified as usable or non-usable based on EEVA high and EEVA low score. The difference in implantation rate between the usable group and the whole cohort was 30%, indicating that implantation rates could increase using this model. In addition, the percentage of non-usable embryos that resulted in implantation was 50.6%, raising concerns regarding the discarding of viable embryos. Even though the Conaghan model was developed for blastocyst formation and the end point of this study was clinical outcome, the authors expressed that an implanted embryo should derive from the usable embryo group and not from the non-usable group (or at least not in such high proportions). The possible explanation for these findings, according to the authors, could be that the model is based on narrow time intervals (Figure 17.3).

In 2015, Cetinkaya et al. [33] studied 17 kinetic markers in 3354 embryos cultured up to day 5. The parameters t8 – t5, cleavage

TABLE 17.1 Technical and Clinical Features Compared between the Time-Lapse Systems Available on the Market

Feature	System A	System B	System C	System D	System E	System F	System G
System	ASTEC™	Primo Vision™	ESD+®	ESD®	MIRI®	Geri®	Geri+®
Incubator	Integrated	Placed in conventional Incubators	Integrated	Integrated	Integrated	Integrated	Integrated
External dimensions (W × D × H mm)	382 × 590 × 219	220 × 80 × 110	550 × 600 × 500	603 × 560 × 435	805 × 585 × 375. 950 × 685 × 375	615 × 500 × 300	615 × 500 × 300
Specificculture dish	Single culture	Group culture	Group culture (shared medium)	Single culture	Single or group culture (shared medium)	Group culture (shared medium)	Group culture (shared medium)
Specification							
Number of focal planes	11 (max.)	3 to 11	11	Up to 17;typically 7	3 to 7	Up to 11	Up to 11
Time between acquisitions	15 min. (adjustable between 15 and 60 min.)	5 to 60 min.	10 min.	10 min. for 7 focal planes, 2 min. for a single focal plane	5 min.	5 min.	5 min.
Camera (megapixels)	1.3	5 (1 px/µm)	2.2 (3 px/µm)	1.3 (3 px/µm)	1.25	5	5
Type of microscopy	Oblique illumination	Brightfield (Hoffman modulation)	Brightfield (Hoffman modulation)	Brightfield (Hoffman modulation)	Brightfield	Brightfield	Brightfield/ darkfield
Embryo illumination for image	Red LED (623 nm)	Adjustable green LED (550 nm)	Red LED (630 nm)	Red LED (635 nm)	Red LED (635 nm)	Orange LED (591 nm)	Red LED (630 nm)
Time of light exposure	0.008 s	0.2 to 0.005 s	<0.02 s	<0.032 s	0.064 s	<0.005 s	<0.005 s; <0.009 s
Software							
Morphokinetics annotation	Yes, manual	Yes, manual, guided/ semi-automated	Yes, manual, guided/ semi-automated/ fully automated	Yes, manual, guided/ semi-automated	Yes, manual and automated	Yes, manual, semi-automated and automated	Yes, manual, semi-automated and automated
Predictive algorithm	/	Yes, or defined by user	Yes, or defined by user	Yes, or defined by user	Defined by user	Defined by user	Yes
Gaseous Condition							
Gas consumption		N/A	N_2: max 5L/h, Typical 2–3 L/h, CO_2: max 2 L/h, Typical 0.5 L/h	N_2: <10 L/h, Typical 3 L/h, CO_2: <1 L/h,	N_2: 3–5 L/h, CO_2: 1–2 L/h,	N_2 &CO_2: 3.6 L/h	N_2 &CO_2: 3.6 L/h
Type of gas	Built-in gas mixer	N/A	Integrated gas mixer	Integrated gas mixer	Built-in gas mixer; premixed not required	Premixed	Premixed
Recovery time (min)	Temperature: 10–12 Gas: 5–6	N/A	CO_2 <5; O_2 <3	CO_2 and temperature <5; O_2 <15	Temperature <1; gas <3	Temperature <1; CO_2 <3; humidity 4 h (for full recovery)	Temperature <1; CO_2 <3; humidity 4 h (for full recovery)

(Continued)

TABLE 17.1 Technical and Clinical Features Compared between the Time-Lapse Systems Available on the Market *(Continued)*

Feature	System A	System B	System C	System D	System E	System F	System G
Other							
Dry or humid culture system	Dry	N/A	Dry	Dry	Dry	Dry or humid, independently on each chamber	Dry or humid, independently on each chamber
pH monitoring	Possible	N/A	Specific pH validation dish	Possible	Built-in	Possible	Possible
Capacity	12 embryos/dish	16 or 9 embryos/dish	16 embryos/dish	12 embryos/dish	14 embryos/dish	16 embryos/dish	16 embryos/dish
	9 dishes/incubator	1 dish/inverted microscope	15 dishes/incubator	6 dishes/incubator	6 or 12 dishes/incubator	6 dishes/incubator	6 dishes/incubator
Electronic record system	Manually	Possible to integrate	Possible to integrate	Possible to integrate	Under development	Possible to integrate	Possible to integrate
Remote access to image	Yes	Yes	Yes	Yes	Yes	Yes	Yes

TABLE 17.2 Morphokinetics Individual Variables

t0	Time of IVF or mid-time of microinjection (ICSI/intracytoplasmic morphologically selected sperm injection)
tPB2	The second polar body completely detached from the oolemma
tPN	Fertilization is confirmed
tPNa	The appearance of individual pronuclei: tPN1a, tPN2a, tPN3a, etc.
tPNf	Time of pronuclei disappearance: tPN1f, tPN2f, etc.
tZ	Time of PN scoring
t2 to t9	Time to two to nine discrete cells
tTM	Trichotomous mitosis at different stages
tSC	The first evidence of compaction
tMf/p	End of compaction
	"f" corresponds to full compaction
	"p" corresponds to partial compaction
tSB	Initiation of blastulation
tByz	Full blastocyst
	"y" corresponds to morphology of ICM
	"z" corresponds to morphology of TE cells
tEyz	Initiation of expansion; first frame of zona thinning
tHNyz	Herniation; end of expansion phase and initiation of hatching
tHDyz	Fully hatched blastocyst

TABLE 17.3 Morphokinetics Calculated Variables

VP	tPNf – tPNa	Pronucleus (PN) duration
ECC1	t2 – tPB2	Duration of first cell cycle
ECC2	t4 – t2	Duration of second cell cycle
cc2		Duration of single blastomere second cell cycle: cc2a = t3 – t2; cc2b = t4 – t2
cc3		Duration of single blastomere third cell cycle: cc3a = t5 – t4; cc3b = t6 – t4; cc3c = t7 – t4; cc3d = t8 – t4
ECC3	t8 – t4	Duration of third cell cycle
s2	t4 – t3	Synchronization of cell divisions
s3	t8 – t5	Synchronization of cleavage pattern
Dcom		Duration of compaction tMf – tSC (full compaction); tMp – tSC (partial compaction)
dB	tB – tSB	Duration of blastulation
Dexp	tHN – tE	Duration of blastocyst expansion
Dcol	tBCend(n) – tBCi(n)	Duration of blastocyst collapse "n" is number of episodes of collapse and re-expansion
dre – exp tre – exp end(n) – tre–expi(n)		Duration of re-expansion
dHN	tHN – tHD	Duration of herniation
tRE		Time of the start of re-expansion
tCRE		Time of completion of re-expansion

synchronicity from four to eight cells (CS4–8), and cleavage synchronicity from two to eight cells (CS2–8) were found to be good indicators. In particular, CS2–8, defined as ([t3 – t2] + [t5 – t4])/(t8 – t2), was selected as the best predictor on day 3 for blastocyst formation and quality (area under the curve [AUC] = 0.786).

Yang et al. [34] took a different approach and developed a study to describe different types of abnormal divisions and how they may affect the developmental potential of the embryo. Seven types of divisions within two categories were defined according to the impact caused on blastocyst development. Category 1(minor abnormality) consisted of divisions with low impact on the development potential: normal division, uneven blastomere formation, and appearance of big fragments. Category 2 (major abnormality) consisted of divisions with high impact on embryo development: direct cleavage, fragmentation, developmental arrest, and disordered division. By taking this into consideration,

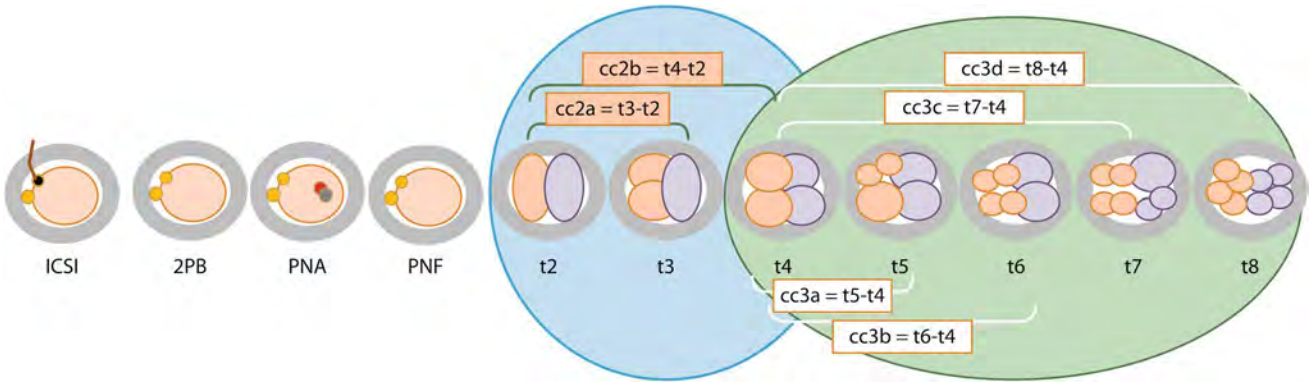

FIGURE 17.1 Graphical representation of kinetic variables up to the 8-cell stage. *Abbreviations*: ICSI, intracytoplasmic sperm injection; 2PB, two polar bodies; PNa, pronucleus appearance; PNf, pronucleus fading.

TABLE 17.4 Studies Associating Blastocyst Formation with Kinetic Markers

Author	Study Design	Embryos (n)	Embryo Origin	Time-Lapse System	Predictive Marker Identified
Lemmen et al. [26]	Retrospective study	102	IVF/ICSI cycles	Nikon Diaphot 300 microscope with camera in a closed system	T2, PN breakdown
Wong et al. [19]	Retrospective study	100	Supernumerary frozen 2PN	Modified Olympus IX-70/71; CKX-40/41	First cytokinesis, P2 and P3
Hashimoto et al. [20]	Experimental study	80	Donated human embryos for research	Biostation CT	Durations of second (t4 – t3) and third mitotic divisions (t8 – t5)
Hlinka et al. [27]	Retrospective study	180	Clinical IVF routine	Primovision	c2, c3, and c4; i2, i3, and i4
Cruz et al. [22]	Retrospective cohort study	834	Oocyte donation cycles	EmbryoScope	t4, t5, s2, DC3 cells, and tM; UN2 cells
Dal Canto et al. [23]	Retrospective cohort study	459	Clinical IVF routine	EmbryoScope	t3, t4, t5, t6, t7, t8, t3 – t2, t4 – t3, t4 – t2, t8 – t4, t8 – t5
Kahraman et al. 2013 [25]	Randomized controlled trial	406	Clinical IVF routine	Embryoscope	cc2, s2, t2, t8, tM, tB
Chamayou et al. [24]	Retrospective study	224	Fresh oocyte ICSI treatments	EmbryoScope	t1, t2, t4, t7, t8, tC – tF, and s3
Kirkegaard et al. [28]	Prospective cohort study	571	Fresh oocyte ICSI treatments	EmbryoScope	First cytokinesis, t3, and DC3 cells
Conaghan et al. [29]	Prospective multicentre cohort study	1233	Fresh oocyte ICSI treatments	Eeva	P2 and P3
Wirka et al. [30]	Retrospective multicentre cohort study	651	Clinical IVF routine	Eeva	AS, $A1^{cyt}$, CC, AC
Kirkegaard et al. [31])	Retrospective multicentre study	1519	Fresh oocyte ICSI treatments	EmbryoScope	t3 – t2, t4 – t3
Desai et al. [32]	Retrospective study	648	ICSI cycles	EmbryoScope	tPNf, t2, t4, t8, t9+, tM, tSB, tB, tEB, cc2, s1, s2, s3, t5 – t4, t5 – t2
Cetinkaya et al. [33]	Retrospective observational cohort study	3354	Clinical IVF routine	EmbryoScope	CS2
Yang et al. [34]	Prospective observational study	345	Metaphase I donated for research	Primo vision	Cleavage patterns
Milewski et al. [35]	Retrospective observational study	432	Fresh oocyte ICSI treatments	EmbryoScope	t2, t5, cc2, and SC

(Continued)

TABLE 17.4 Studies Associating Blastocyst Formation with Kinetic Markers *(Continued)*

Author	Study Design	Embryos (n)	Embryo Origin	Time-Lapse System	Predictive Marker Identified
Storr et al. [36]	Prospective cohort study	380	Fresh oocyte ICSI treatments	Embryoscope	s3, t8, and tEB
Motato et al. [37]	Retrospective study	7483	Clinical IVF routine	EmbryoScope	tM; t8 – t5
Mizobe et al. [38]	Cohort Study	791	ICSI/IVF	EmbryoScope	Fragmentation based
Liu et al. 2016 [39]	Retrospective cohort study	270	ICSI	EmbryoScope	s2, t5 – tPNf
Zhan et al. 2016 [40]	Retrospective observational study	21261	ICSI/IVF cycles	Embryoscope	DUC
Mizobe et al 2018 [41]	Cohort Study	948	ICSI treatment	EmbryoScope	EC, HS
Coticchio et al. 2018 [21]	Retrospective cohort study	500	ICSI treatment	EmbryoScope	t2 – tPNf, $tPNa_m$ – $tPNf_m$, tPNf – tHaloA, tHaloD – tHaloA
Fishel et al. 2018 [42]	Retrospective cohort study	843	ICSI/IVF treatment	Embryoscope	tSB, dB
Desai et al. 2018 [43]	Retrospective study	1478	ICSI cycles	Embryoscope	IDC, DUC, 2+ dysmorphism (MN, RC, IDC, DUC), tSB, tEB, tEB – tSB
Lagalla et al. 2020 [44]	Retrospective study	499	ICSI cycles	Embryoscope	FCM
Pennetta et al. 2021 [45]	Retrospective cohort study	780	ICSI/IVF treatment	Embryoscope	s2

Abbreviations: ICSI, intracytoplasmic sperm injection; IVF, *in vitro* fertilization; HS, high synchrony; FCM, full compaction; DUC, direct uneven cleavage; IDC, irregular chaotic division.

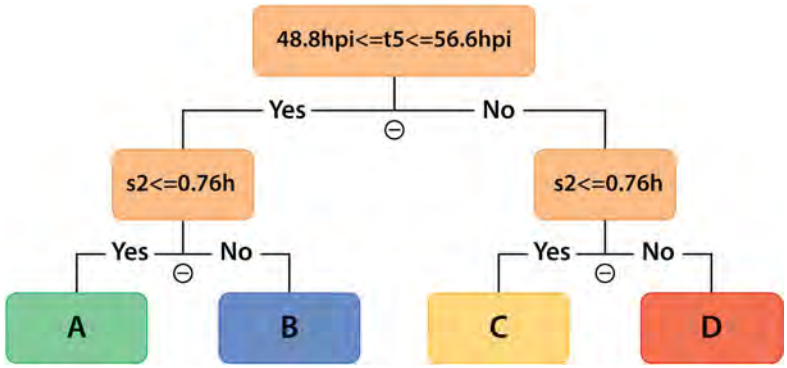

FIGURE 17.2 Blastocyst development algorithm (described by [22]). *Abbreviation*: hpi, hours post insemination.

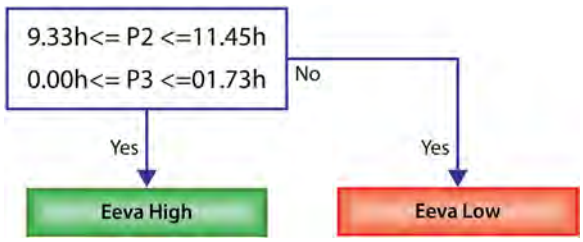

FIGURE 17.3 Original embryo categorization algorithm known as EEVA I. (Based on data from [29]).

a hierarchical classification model was developed based on the division patterns during the three initial embryo cleavages rather than on morphokinetic parameters as in previous studies. Day-3 embryos were then classified into six categories of A–F according to the number and category of the abnormal cleavages they had presented (Figure 17.4).

In a study by Milewski et al., the parameters t2, t3, t4, t5, cc2, and s2 were measured and differences were observed between embryos that reached the blastocyst stage and embryos that arrested. A total of 432 embryos were analysed. The resultant data for each parameter were divided into four intervals (C1–C4),

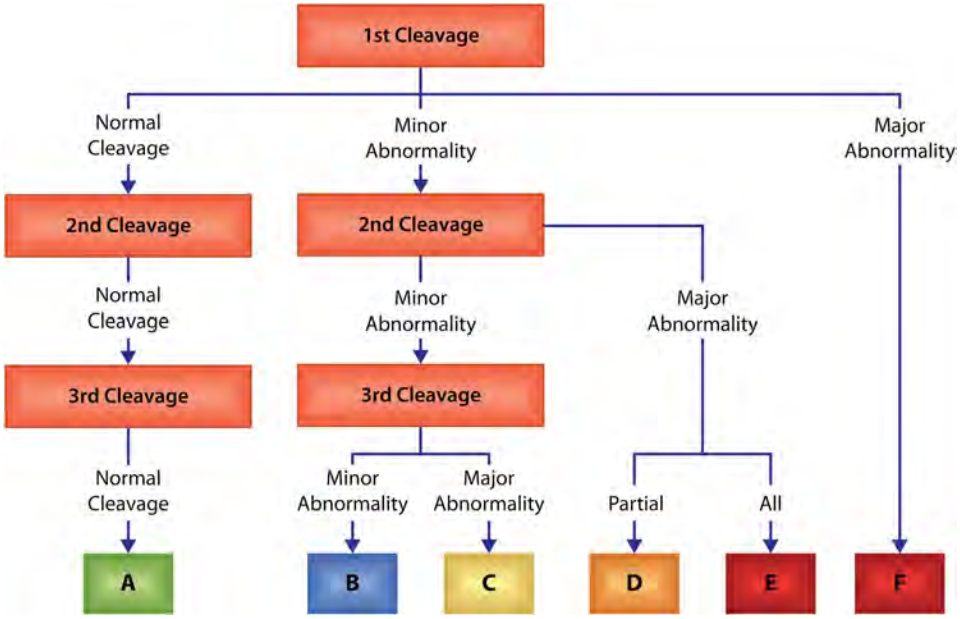

FIGURE 17.4 Yang algorithm for blastocyst development based on cleavage feature. (Based on data from [34].)

and score values were assigned in order to find out which parameter values corresponded to the highest blastocyst development rate. The highest ones generally belonged to compartments C3 and C2. The extreme compartments—C1 and C4—had the lowest rates. A univariate logistic regression analysis concluded that all the studied parameters were significantly associated with blastocyst development. However, after multivariate logistic regression, only the t2, t5, and cc2 parameters were considered and combined into a new parameter (SC), defined as the predictor of development to blastocyst [35].

Storr et al. recorded the timings of 380 blastocysts and found eight significant prediction markers of top-quality blastocysts: s3, t6, t7, t8, tM, tSB, tB, and tEB. Out of these potential predictors, s3 was identified as the one with the best individual discriminatory capacity before compaction (AUC = 0.585, 95% CI = 0.534–0.635), and tEB was identified as the best predictor regardless of embryo stage (AUC = 0.727, 95% CI = 0.675–0.775). By combining ts3, tEB, and t8, a model with higher discriminatory capacity for predicting top-quality embryos was proposed [36].

Motato et al., in 2016 [37], conducted a three-phase observational, retrospective, single-centre clinical study in which the authors describe the events associated with blastocyst formation and implantation based on the largest sample size ever described with time-lapse monitoring.

Phase 1 consisted of embryo scoring based on a classification tree to select embryos with higher blastocyst formation probabilities. The observed correlations between morphokinetic parameters and blastocyst formation were the basis for a proposed hierarchical classification procedure to select viable embryos with a high blastocyst formation potential. A detailed retrospective analysis of cleavage times was made for 7483 zygotes. A total of 17 parameters were studied and several were significantly correlated with blastocyst formation and implantation. The most predictive parameters for blastocyst formation were time of morula formation, tM (81.28–96.0 hours after ICSI), and t8 – t5 (≤8.78 hours) or time of transition of five-blastomere embryos to eight-blastomere embryos (Figure 17.5).

Phase 2 focused on the blastocysts transferred and implantation rate. Owing to a lack of a relationship between the

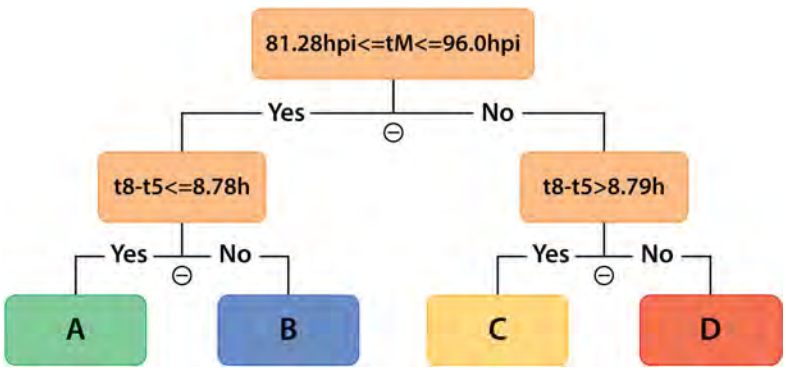

FIGURE 17.5 Algorithm for blastocyst development algorithm described by Motato et al. 2016.

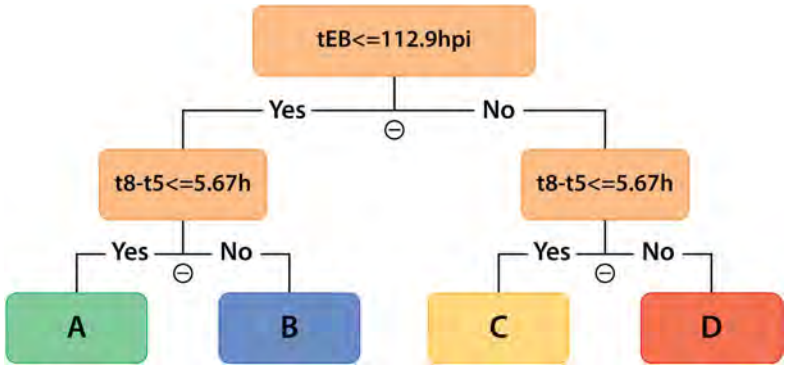

FIGURE 17.6 Implantation algorithm described by Motato et al. 2016.

previously described variables and implantation potential, the authors identified new variables by comparing transferred blastocysts (n = 383) that implanted with those that did not implant (n = 449). Once again, they analysed 17 morphokinetic parameters and identified the variables' time for expansion blastocyst, tEB (107.9–112.9 hours after ICSI), and t8 – t5 (5.67 hours after ICSI) as predicting blastocyst implantation, with a ROC value of 0.591 (95% CI = 0.552–0.630). Using these data, a hierarchical model representing a classification tree was proposed. The model subdivided blastocysts into four categories from A to D with higher or lower implantation rates (i.e. from 72.2% in category A to 39.7% in category D) (Figure 17.6). Phase 3 was the validation phase.

The authors concluded that the inclusion of kinetic parameters into score evaluations could improve blastocyst selection criteria and predict blastocyst formation with high accuracy. In addition, the proposed models classify embryos according to their probabilities of blastocyst stage and implantation. In 2018, Fishel et al. observed tSB and dB (tB – tSB) are linked to top-quality blastocyst formation [42]. A few other studies were performed by Mizobe et al. and observed t2, t4, and high synchrony are linked to blastocyst and top-quality blastocyst formation [38, 41]. In a recent study by Pennetta et al., s2 was linked to blastocyst development [45]. There were a few other studies that didn't find the significant differences in between the conventional incubator and TLT system in terms of blastocyst development [46, 47].

Implantation, pregnancy, and live-birth studies

In addition to blastocyst formation, the scientific community has also correlated kinetic markers to embryo implantation and live birth as an end point which is a main factor for a successful IVF cycle (Table 17.5).

Starting in 2008, Lemmen et al. [26] retrospectively compared time-lapse recordings of a small group of embryos transferred at the 4-cell stage that resulted in eight pregnancies. In this case, the authors observed that nuclei appearance in the first blastomere

TABLE 17.5 Implantation and Live Birth Studies

	Study Design	Total Number of Embryos	Embryo Origin	Time-Lapse System	Predictive Marker Identified/Utilized
Lemmen et al. (2008) [26]	Retrospective study	19	IVF/ICSI cycles	Nikon Diaphot 300 microscope with camera in a closed system	Nuclei appearance in the first blastomere
Meseguer et al. (2011) [1]	Retrospective study	247	ICSI cycles	EmbryoScope	t5, s2, cc2, UN 2 cell, MN 4 cell, DC 1–3 cells
Arazello et al. (2012) [50]	Prospective study	159	ICSI cycles	EmbryoScope	PN breakdown
Hlinka et al. (2012) [27]	Retrospective study	114	ICSI cycles	Primovision	c2, c3, and c4; i2, i3, and i4
Rubio et al. (2012) [51]	Multicentre retrospective study	5225 (1659 transferred)	IVF cycles from donated and autologous oocytes	EmbryoScope	DC 2–3 cells
Dal canto et al. (2012) [23]	Retrospective study	134	ICSI/IVF cycle	EmbryoScope	t8
Cruz et al. 2012 [22]	Retrospective study	120	ICSI Donor Oocyte	EmbryoScope	t5, s2
Freour et al. (2013) [52]	Retrospective analysis and prospectively collected database	191	ICSI cycles	EmbryoScope	t4 and s3

(Continued)

TABLE 17.5 Implantation and Live Birth Studies *(Continued)*

	Study Design	Total Number of Embryos	Embryo Origin	Time-Lapse System	Predictive Marker Identified/Utilized
Chamayou et al. (2013) [24]	Retrospective study	178	ICSI cycles	EmbryoScope	cc3
Kirkegaard et al. (2013) [28]	Prospective cohort study	84	ICSI cycles	EmbryoScope	None
Desai et al. 2014 [32]	Retrospective Study	105	ICSI cycles	EmbryoScope	tPNf, t2, t3, t5, t8, s1, and t5 – t2
Rubio et al. (2014) [48]	Prospective randomized control trial	775	ICSI cycles from donated oocytes	EmbryoScope	T5; s2; cc2; UN 2 cell; MN 4 cell; DC 1–3 cells
Aguilar et al. (2014) [53]	Retrospective cohort study	1448	ICSI cycles from donated oocytes	EmbryoScope	Time to 2PB; PF; length of S-phase
Basile et al. (2015) [54]	Retrospective multicentric study	1122	ICSI cycles from donated and autologous oocytes	EmbryoScope	cc2, t3, t5, UN 2 cell, MN 4 cell, DC 1–3 cells
Ergin et al. (2014) [55]	Retrospective Study	686	ICSI/IVF cycles	Embryoscope	MN
Vermileya et al. (2014) [56]	Retrospective multicentric study	331	IVF/ICSI cycles	EEVA	P2 and P3
Freour et al. (2015) [57]	Retrospective study	528	ICSI cycles	EmbryoScope	t5, s2, cc2, UN 2 cell, MN 4 cell, DC 1–3 cells
Siristatidis et al. (2015) [49]	Prospective Cohort study	239	ICSI cycles	PrimoVision	t2, cc2a, t3, s2, t4, cc3a, t5, s3, t8
Marcos et al. (2015) [58]	Retrospective cohort study	715	ICSI cycles	Embryoscope	Blastocyst contraction
Dominguez et al. (2015) [59]	Retrospective cohort study	28	ICSI cycles from donated oocytes	EmbryoScope	Cc2
Liu et al. (2016) [39]	Retrospective cohort study and prospective validation	336 (270 for study and 66 for validation)	IVF/ICSI cycles	Embryoscope	s2, t5 – tPNf, cells 68 hrs <8, abnormal cleavage
Adamson et al. (2016) [60]	Prospective concurrent cohort study		ICSI and IVF cycles from autologous oocytes	EEVA	P2 and P3
Wu. et al. [61]	Retrospective study	212	Clinical IVF routine	Primo Vision	tPNf, t2, t4, t4 – t3
Goodman et al. (2016) [62]	Prospective randomized control trial	2092	ICSI and IVF cycles from autologous oocytes	Embryoscope	Cc2, s2, t5, s3, tSB, MN, irregular division
Petersen et al. (2016) [63]	Retrospective evaluation (multicentre)	3275	ICSI and IVF cycles	Embryoscope	t3 – tPNf, (t5 – t3/t5 – t2), cells 66 hrs <8
Zhan et al. (2016) [40]	Retrospective observational study	3189	ICSI/IVF cycles	Embryoscope	DUC
Desai et al. (2016) [64]	Retrospective study	669	Vitrified-warmed blastocyst	Embryoscope	MN, delayed blastulation
Carrasco et al. (2017) [65]	Retrospective study	800	ICSI Cycles	EmbryoScope	t4, t7, s3
Mizobe et al. (2017) [66]	Retrospective study	299	IVF/ICSI cycles	Embryoscope	t2, t4, tC, tB
Ebner et al. (2017) [67]	Retrospective study	144	Devitrification cycle	Miri	tRE, tCRE, tCRE–tRE
Kovacic et al. (2018) [68]	Retrospective observational study	143	Devitrification cycle	PrimoVision	tCRE–tRE
Gonzalez et al. (2018) [69]	Retrospective study	234	Devitrification cycle	EmbryoScope	Blastocyst contraction
Bartolacci et al. (2021) [70]	Retrospective study	1801	ICSI cycle	Embryoscope	tPNf, tPNa, t2, t3, t4, t8, t2–tPNf

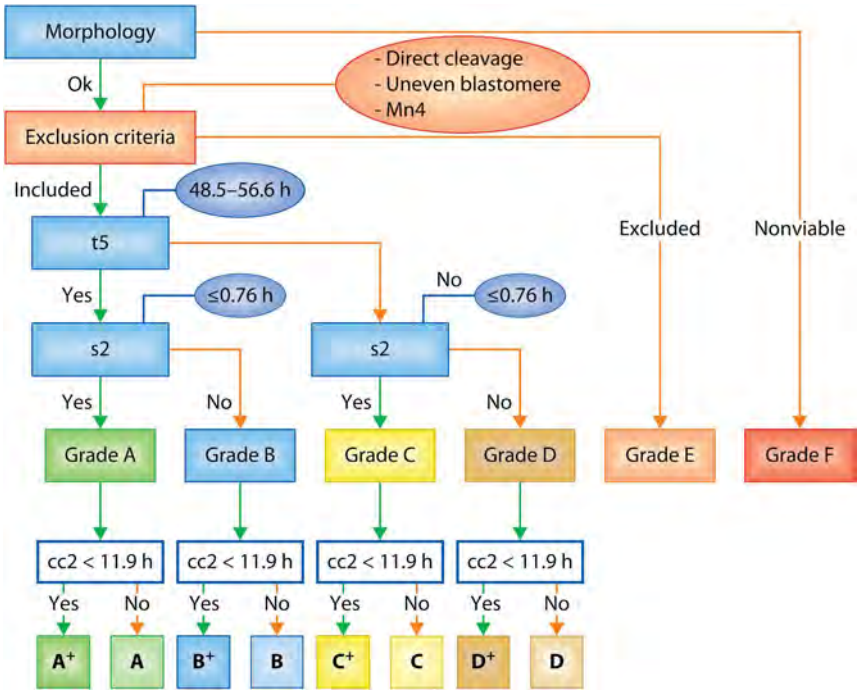

FIGURE 17.7 Original embryo categorization algorithm. (From [1], reproduced with permission.)

following the first cleavage were faster in embryos that implanted versus those that did not, and that nuclei appearance in the first two blastomeres was significantly more synchronous ($p < 0.05$).

Three years later, Meseguer et al. [1] published a study where several parameters were correlated with embryo implantation and suggested a hierarchical algorithm known as the "Meseguer" algorithm or "Meseguer" model. The study was based on 247 known implantation data (KID) embryos that subdivided embryos into six categories from A to F. Four of these categories (A–D) were further subdivided into two subcategories: (+) or (–) (Figure 17.7). The Meseguer model was extensively studied and validated externally by a few studies [25, 48, 49].

In 2012, Azzarello et al. [50] performed a prospective study transferring 159 embryos and proposed the variable "time of pronuclear breakdown" as a predictor of pregnancy. In this study, the pronuclear breakdown of embryos resulting in live births occurred significantly later than those that did not. In fact, the authors proposed the limit of 20 hours and 45 minutes and recommended to avoid transferring embryos presenting pronuclear breakdowns at earlier times.

In the same year, Hlinka et al. [27] proposed a novel method to predict implantation. The model relied on cleavage ratings of the embryos; more specifically, time patterning of cleavage clusters and interphases were used to select the highest-quality embryos. The diagnostic relation between blastocyst implantation and cleavage success was 100% specific for all the embryos analysed (n = 180) and all the pregnancies resulted from timely cleaved embryos.

Direct cleavage is another parameter that has been correlated with implantation. Meseguer et al. [1] initially observed this phenomenon based on 247 KID embryos. Later, these findings were confirmed by a multicentre retrospective study performed by Rubio et al. [51]. In this case, the number of embryos analysed was much higher (n = 5225) and embryo implantation for embryos presenting direct cleavage from two to three cells (DC2–3 <5 hours) was statistically lower than for those with a normal cleavage pattern. Only 1 out of 109 embryos with DC2–3 resulted in clinical pregnancy.

The impact of extrinsic factors on embryo kinetics and their relationship with implantation has been studied as well. In 2013, Freour et al. [52] focused on women who smoked, and the authors observed that embryo divisions occurred later in smokers than in non-smokers, resulting in worse outcomes for the first group. The authors analysed 191 embryos and indicated t4 and s3 as the most relevant kinetic parameters with respect to implantation. According to the distributions of these two variables, implantation was significantly higher in the first two quartiles. Embryos were graded as A or B depending on the optimal range defined for t4 (A = inside the range and B = outside the range). In addition, embryos were given a "+" or "–" value according to the optimal range of s3 ("+" = inside the range and "–" = outside the range). The authors validated this classification model in a database including all transferred embryos, observing implantation rates of 38.7%, 33.3%, 30.7%, and 15.3% for A+, A–, B+, and B– categories, respectively. The proportions of A+ and A– embryos were higher in non-smoker patients.

Chamayou et al. [24] retrospectively compared morphokinetic parameters of 72 implanted and 106 nonimplanted embryos. No differences were found for PN appearance, PN disappearance, t1, t2, t4, t7, t8, tC–tF, and s3 parameters. The authors concluded that these markers were not predictors of implantation, but that they could predict embryo development to the blastocyst stage. In this study, the only predictor marker of implantation and production of a viable pregnancy was cc3.

As opposed to many authors, Kirkegaard et al. [71] showed no differences in the timings of cellular division or embryonic

stage between implanted and non-implanted embryos. The study was based on the observation of 84 SETs. The author identified the duration of first cytokinesis, duration of the 3-cell stage, and direct cleavage to three cells as predictors of high-quality embryo development but not of implantation or pregnancy. Therefore, this group concluded that a universal algorithm for optimal timing might not be feasible.

In 2014, Aguilar et al. [53] studied the human's first cell cycle and its impact on implantation based on morphokinetics. To this aim, the authors conducted a retrospective analysis of 1448 transferred embryos and compared the timings of second polar extrusion, first and second pronuclear appearance, pronuclear abuttal, pronuclear fading, and length of S-phase between implanted and nonimplanted embryos. The time ranges successfully linked to implantation were 3.3–10.6 hours for second polar body extrusion, 22.2–25.9 hours for pronuclear fading, and 5.7–13.8 hours for the length of S-phase.

In 2015, Basile et al. [54] continued the study by Meseguer et al. [1] and published an improved version of the algorithm by studying a larger data set of embryos from four different IVF clinics and included new kinetic parameters (Figure 17.8).

VerMileya et al. [56] extended the EEVA I algorithm to EEVA II and established the relationship between implantation and three embryo categories derived from a computer-automated TLT. The system classified embryos into the categories high EEVA, medium EEVA, or low EEVA based on the variables P2 and P3. According to this multicentre study (205 patients), implantation rates were significantly linked to the three categories; more specifically: 37%, 35%, and 15% for high, medium, and low, respectively. In addition, the clinical pregnancy rate for patients that had one or more "high" transferred embryos was significantly higher (51% vs. 34%; $p = 0.02$) (Figure 17.9). The EEVA algorithm was validated externally by many studies.

Adamson et al., in 2016 [60], tested the same technology in a prospective way. The aim of the study was to prove if an automatic time-lapse test (TL test) combined with traditional morphology improves day-3 implantation rates compared with morphology alone. Two concurrently collected groups of patients were compared: those who received a day-3 transfer with the use of the TL test (EEVA test) together with morphology (test group), and those who received a day-3 transfer with the use of morphology alone (control group). Analysis of the study's primary end point—implantation rate—showed a significantly higher implantation rate for day-3 transfer among the test group (30.2%, 58/192) than the control group (19.0%, 84/442; $p = 0.003$).

In the same year, Goodman et al. [62] published a new scoring algorithm based on kinetic event t5, s2, s3, tSB, and cc2. The presence of multi-nucleation and irregular division was also included as deselection criteria in the algorithm. The algorithm provides a score between –2 and 4 (Figure 17.10).

Liu et al. [39] validated the "Meseguer" algorithm and derived a new hierarchical model with high AUC in 2016, and this algorithm is known as the "Liu" algorithm, which is based on kinetic parameter s2, t5, and tPNf. Abnormal cleavage and cells at 68 hours less than 8 were described as deselection criteria (Figure 17.11).

Later in the year 2016, Petersen et al. [63] provide a hierarchical algorithm based on KID known as KIDScore D3 algorithm, which is based on t3, tPNf, t2, and t5. This algorithm also considers the counts of cells should be greater or equal to 8 at 66 hpi (Figure 17.12).

Limitations on the external performance and the universal use of published algorithms have been addressed [11]. In a study published by Freour et al. [52], an external validation of Meseguer et al.'s algorithm [1] was performed in an unselected patient population. The model was applied showing a heterogeneous distribution of implantation rates in the resultant categories. In addition, correlation coefficients were significantly lower than the ones in the original study. However, a simplified version of the model (in which only the two main morphokinetic

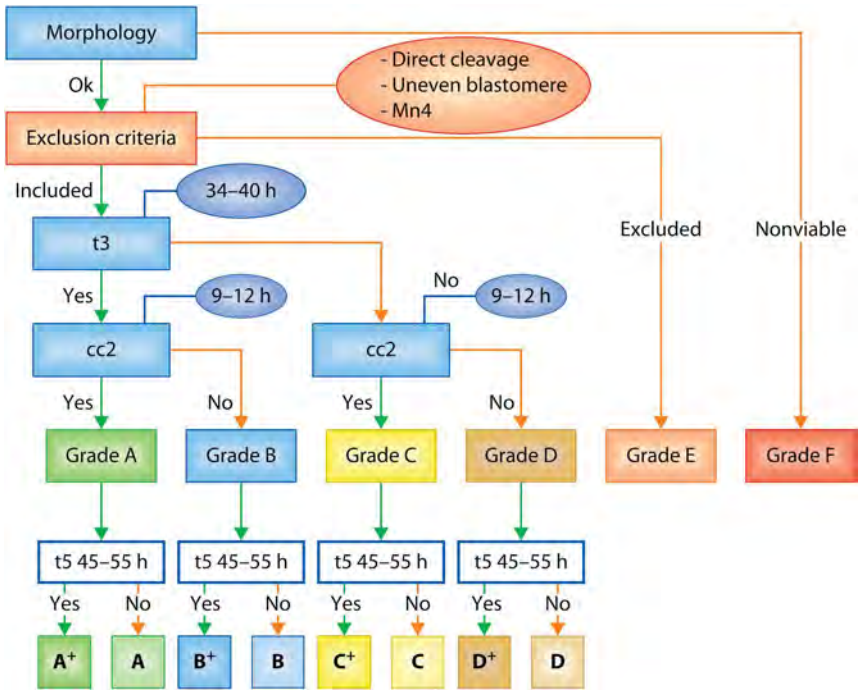

FIGURE 17.8 Revised embryo categorization algorithm. (From [16], reproduced with permission.)

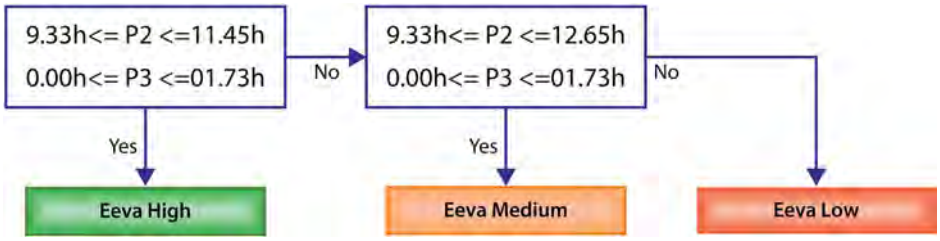

FIGURE 17.9 Modified embryo categorization algorithm known as EEVA II. (Based on data from [56].)

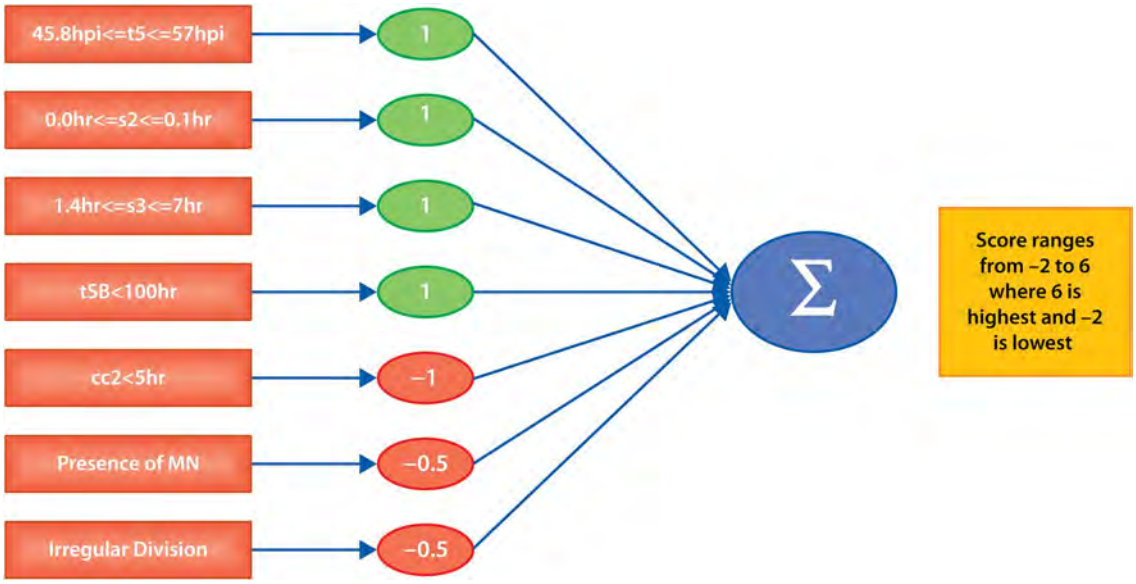

FIGURE 17.10 Goodman algorithm for embryo implantation (described by [62]).

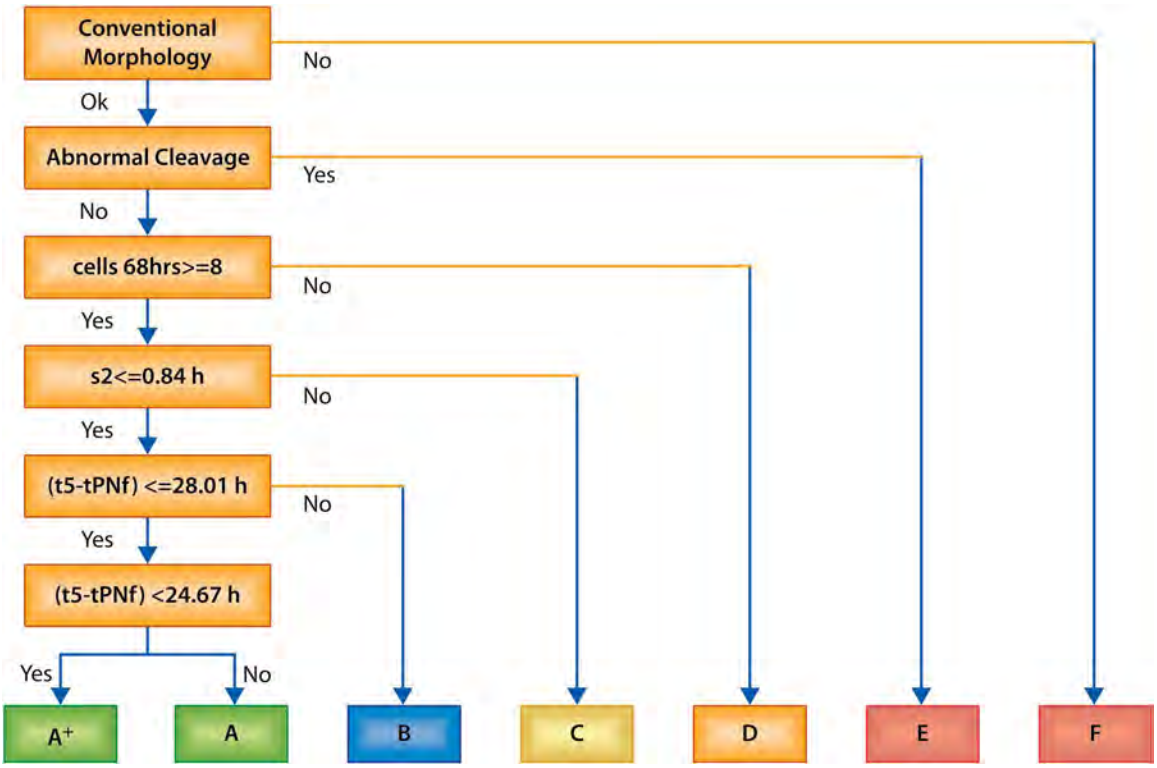

FIGURE 17.11 Liu algorithm for embryo implantation (described by [39]).

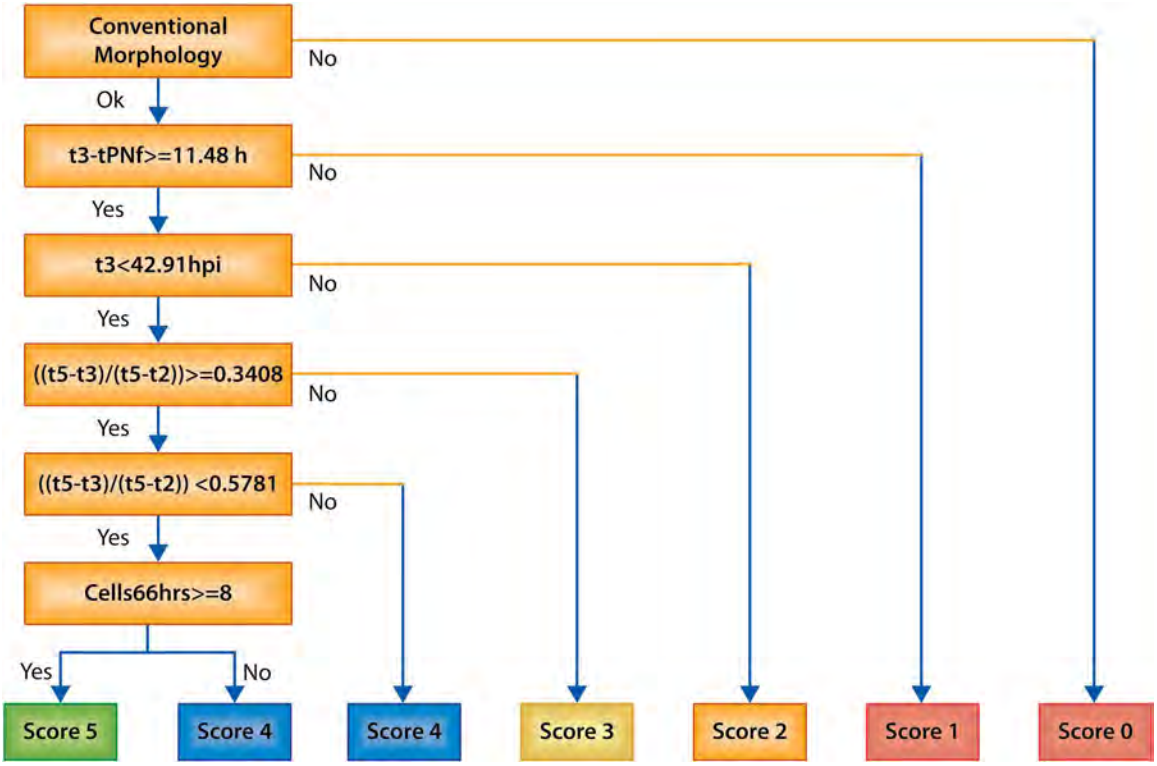

FIGURE 17.12 Petersen algorithm for embryo implantation known as KIDScore D3 algorithm (described by [63]).

variables—t5 and s2—were considered and not cc2) performed acceptably. The authors explained that the differences could be the result of variations in oxygen culture conditions, oocyte source (donor cycles vs. autologous cycles), restrictions in the studied population, and/or the stimulation protocols used. The conclusion was that a hierarchical prediction model should not be used universally in an unselected population; it should be centre specific.

The combination of technologies may be the key to improving results in the future. Dominguez et al. [59] combined proteomics and time-lapse analysis of implanted (n = 16) and non-implanted (n = 12) embryos. After logistic regression analysis, the model identified the presence or absence of protein interleukin-6 and the duration of cc2 as the most relevant embryo features. Based on these results, the authors developed a hierarchical model (Figure 17.13) based on these two variables, classifying embryos

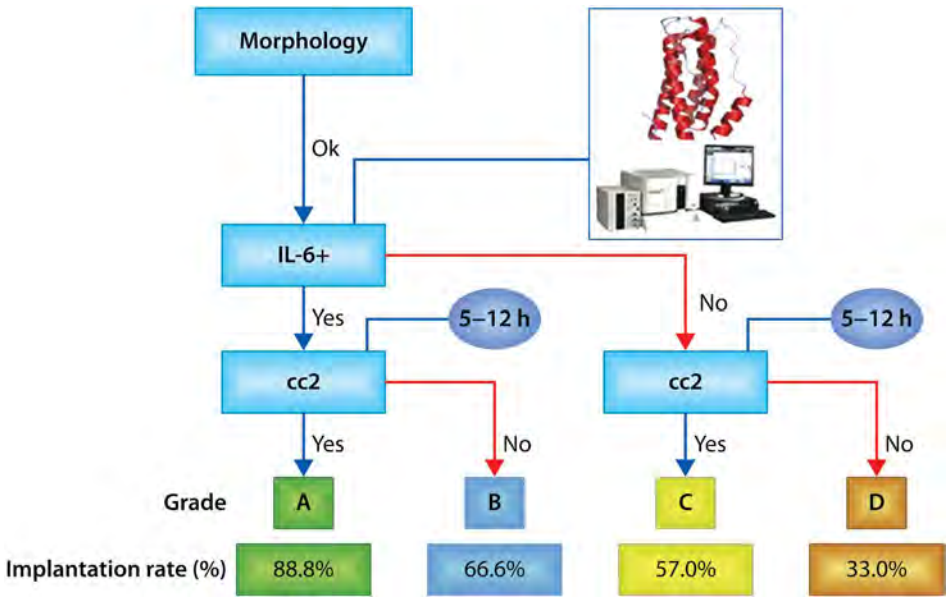

FIGURE 17.13 Combined embryo categorization algorithm. (From [59], reproduced with permission.)

TABLE 17.6 Known Implantation Algorithms that Are Widely Used

Known Model	Score Ranges	Kinetic Parameter	Type of Model
Meseguer Model	A+ – F (10)	t5, s2, cc2, UN 2 cell, MN 4 cell, DC 1–3 cells	Hierarchical
Conaghan Model (EEVA I)	EEVA low, EEVA high	P2, P3	Selection/deselection
VerMileya Model (EEVA II)	EEVA low, EEVA medium, EEVA high	P2, P3	Selection/deselection
EEVA Xtend	1 (highest) – 5 (lowest)	Egg age, cell count, and post P3 analysis	Selection/deselection/hierarchical
Basile Model	A+ – F (10)	cc2, t3, t5, UN 2 cell, MN 4 cell, DC 1–3 cells	Hierarchical
Liu Model	A+ – F	t5, tPNf, s2, cells 68 h, abnormal cleavage	Hierarchical
Motato Model	A–D	T5, t8, tEB	Hierarchical
Goodman Model	–2–4	cc2, MN, IRRD, t5, s2, s3, tSB	Hierarchical
Milewski Model	-		Based on quartile
Petersen	0–5	Not2PN, t2, t3, t5, cells 66hr	Hierarchical
KIDScore D3 (different version)	0–5	Not2PN, tPNf, t2, t3, t4, t5, t8, cells 66hr	Hierarchical
KIDScore D5 V2	0–10	Not2PN, t2, t3, t4, t5, tB, tE	Hierarchical (algorithm not shared)
KIDScore D5 V3 (Different version)	0–10	Not2PN, t2, t3, t4, t5, tB, TCM, TE	Hierarchical (algorithm not shared)
iDA Score	0–10		ANN

into four categories of A–D. Implantation rates are expected to decrease as we move on from A to D as observed in this study (A = 88.8%, B = 66.6%, C = 57%, and D = 33%) [59].

In studies from Kovacic et al. and Ebner et al. observed during warmed blastocyst transfer, kinetic parameter related to blastocyst expansion has been linked to implantation potential [67, 68].

The main algorithms that are widely used are described in Table 17.6.

Aneuploidy studies and where are we

Logically, transferring only euploid embryos should increase live birth rates through increased implantation rates and/or decreased miscarriage rates. However, so far this has been difficult to demonstrate in practice. The correlation between euploidy and embryo kinetics has been studied as well (Table 17.6).

In 2010, Wong et al. [19] collected single embryos for gene expression analysis and revealed that embryos with P1, P2, and P3 outside of the optimal ranges exhibited abnormal RNA patterns for embryo cytokinesis, microRNA biogenesis, and maternal mRNA reserve, suggesting that embryo fate may be predetermined and inherited very early in development (by the 4-cell stage).

Chavez et al. [72] subsequently observed that euploid embryos clustered tightly in the P1, P2, or P3 window, which was predictive of blastocyst formation according to Wong et al.'s study [19]. Performing further molecular analysis, the authors discovered that fragmentation dynamics, together with P1, P2, and P3, could potentially distinguish euploid from aneuploid embryos at the 4-cell stage, considering that the fragments contained nuclear DNA, kinetochore proteins, and whole chromosomes as detected by fluorescence *in situ* hybridization.

In 2013, Campbell et al. [73] elaborated an aneuploidy risk model based on the differences of tSB and tB between euploid and aneuploid embryos that had undergone TE biopsy. The model included three categories: low risk, tB <122.9 hpi and tSB <96.2 hpi; medium risk, tB <122.9 hpi and tSB 96.2 hpi; and high risk, tB 122.9 hpi [52]. The same group in a different study [74] applied this model to evaluate its effectiveness and potential clinical impact for unselected IVF patients without undergoing pre-implantation genetic screening after analysing KID embryos. The study revealed significant differences in fetal heart rate (72.7, 25.5, and 0 beats per minute) and live birth rate (61.1%, 19.2%, and 0%) between the three categories low, medium, and high, respectively. This demonstrates that time-lapse imaging using defined morphokinetic data classifies human pre-implantation embryos according to their risk of aneuploidy without performing a biopsy and pre-implantation genetic screening, and that this correlates well with clinical outcomes (Figure 17.14).

In the following year, Basile et al. [75] also correlated morphokinetics with embryo aneuploidy based on 77 patients undergoing genetic screening due to recurrent miscarriage or implantation failure. In this case, embryo biopsy was performed on day 3 of development and the total number of embryos analysed was 504. A logistic regression analysis was used to select and organize which observed timing events (expressed as binary variables inside or outside the optimal range) were most relevant to selecting embryos with higher probabilities of being chromosomally normal. The model identified t5 – t2 (odds ratio [OR] = 2.853, 95% CI = 1.763–4.616) followed by cc3 (OR = 2.095, 95% CI = 1.356–3.238) as the most relevant variables related to normal chromosomal content. An algorithm for embryo selection based on these two variables classified embryos from A to D (Figure 17.15) with significant differences in the percentages of normal embryos as we move on from A to D. More specifically, A = 35.9%, B = 26.4%, C = 12.1%, and D = 9.8% ($p < 0.001$).

As opposed to the previous studies, Rienzi et al. [78] reported no correlation at all between 16 commonly detected morphokinetic parameters and embryo ploidy. This was a longitudinal cohort study conducted using 455 blastocysts from 138 patients at increased risk of aneuploidy because of advance maternal age, history of unsuccessful IVF treatments, or both. The analysed parameters included t2, t3, t4, t5, t8, cc1, cc2, s2, s3, cc3, cc3/cc2,

TABLE 17.7 Studies Correlating Euploidy and Embryo Kinetics

Author	Study Design	N	TL System	Biopsy Day	PDG Technology	Parameters with Significant Differences Found
Chavez et al. (2012) [72]	Prospective observational	75	Custom-built microscope	D3	aCGH	P1, P2, P3, and fragmentation
Campbell et al. (2013) [73]	Retrospective cohort	98	Embryoscope	D5	aCGH/SNP array	tSB and tB
Campbell et al. (2013) [74]	Retrospective cohort	88	Embryoscope	D5	aCGH/SNP array	tSB and tB
Basile et al. (2014) [75]	Retrospective cohort	504	Embryoscope	D3	aCGH	t5 – t2 and cc3
Kramer et al. (2014) [76]	Retrospective cohort	149	Embryoscope	D5	aCGH	None
Yang et al. (2014) [77]	Prospective	285	Embryoscope	D5	aCGH	None
Rienzi et al. (2015) [78]	Longitudinal cohort	455	Embryoscope	D5	CCS	None
Chawla et al. (2015) [79]	Retrospective cohort	460	Embryoscope	D3	aCGH	tPNf, t2, t5, cc2, cc3, t5 – t2
Vera-Rodriguez et al. (2015) [80]	Prospective observational	85	EEVA	D3	aCGH	Time between PN disappearance and the start of 1st cytokinesis; 3 to 4 cell
Minasi et al. (2016) [81]	Retrospective cohort	1730/928 cultured in TLT	Embryoscope	D5	aCGH	tSB, tB, tEB, tHB
Balakier et al. (2016) [82]	Retrospective cohort	2441/607 with PGS	Embryoscope	D5	aCGH	NA
Patel et al. (2016) [83]	Retrospective cohort	167	Embryoscope	D3	aCGH	None
Zhan et al. (2016) [40]	Retrospective observational study	1434	Embryoscope	D3/D5/D6	aCGH	DUC
Mumusoglu et al. (2017) [84]	Retrospective cohort	415	Embryoscope	D5	aCGH	t9, tM, tSB, tB, tEB
Del Carmen Nogales et al. (2017) [85]	Retrospective cohort	485	Embryoscope	D3	aCGH	t3, t5 – t2
Zhang et al. (2017) [86]	Retrospective study	256	Embryoscope	D5/6	aCGH	None
Desai et al. (2018) [43]	Retrospective study	767	Embryoscope	D5/6	aCGH	2+ dysmorphism (MN, RC, IDC, DUC), tEB, tSB
Huang et al. (2019) [87]	Retrospective observational study	188	Embryoscope	D5/6	-	Average blastocyst expansion rate
Pennetta et al. (2021) [45]	Retrospective cohort study	287	Embryoscope	D3	-	tPNa

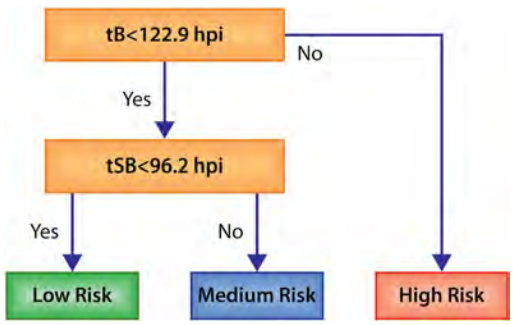

FIGURE 17.14 Campbell algorithm for ploidy detection (described by [73]).

t5–t2, syngamy, tSB, tSC, and tB. Apart from Reinzi, Patel et al., Kramer et al., Yang et al., and Zhang et al. were also not able to correlate aneuploidy detection with kinetic parameters [76, 77, 82–84].

In 2015, two studies observed correlations between embryo kinetics and euploidy. The first one, reported by Chawla et al. [79], identified tPNf, t2, t5, cc2, cc3, and t5–t2 as parameters that significantly differed between chromosomally normal and abnormal embryos. The second one, by Vera-Rodriguez et al. [80], combined chromosomal assessment and single-cell quantitative reverse transcription polymerase chain reaction (RT-qPCR) to simultaneously obtain information from all the blastomeres of human embryos until approximately the 8-cell stage (n = 85). According to their results, the chromosomal status of aneuploid

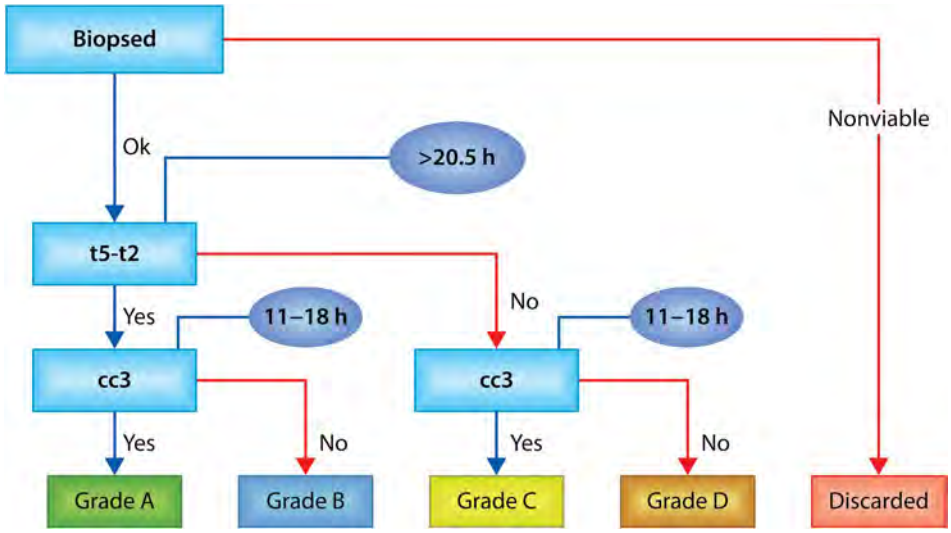

FIGURE 17.15 Embryo selection algorithm. (From [75], reproduced with permission.)

embryos (n = 26) correlates with significant differences in the duration of the first mitotic phase when compared with euploid embryos (n = 28). Moreover, gene expression profiling in this study suggested that a subset of genes is differentially expressed in aneuploid embryos during the first 30 hours of development.

In 2017, Mumusoglu et al. found statistically significant difference in the kinetic parameter (t9, tM, tSB, tB, tEB) of euploid and aneuploid blastocyst through multilevel mixed effect logistic regression analysis [84]. In same year, Del Carmen et al. developed an aneuploidy detection algorithm based on kinetic parameters [85] (Figure 17.16).

In 2018, Desai et al. found more than two dysmorphisms (MN, RC, IDC, DUC) can increase the aneuploidy [43]. In a recent study from Pennetta et al. [86] observed tPNa is linked to the ploidy detection. In the last decade, many studies have tried to correlate different kinetic markers and events to ploidy detection but still PGT-A is standard for ploidy detection. As of now, we stand at a point where TLT can only help to select a better embryo for implantation if the patient is not at a risk for aneuploidy; but for the patient at risk, TLT technology cannot be trusted from the point of aneuploidy detection, PGT-A needs to be done. In recent years, AI seems to be promising to help the ploidy detection, but a lot of studies, specifically RCT, need to be done.

Time-lapse technology and review

In the last decade, TLT system went through many changes from the point of embryo assessment. The continuous monitoring of embryos provided enough data to embryologist and technology providers to analyse this data. Artificial intelligence and many other statistical approaches have been used to understand the data to improve the IVF process, from selection of embryo to ploidy detection. The development of TLT from manual annotation to fully automatic annotation is a remarkable achievement. Different scoring methods and algorithms have been developed in the last decade using machine learning approaches based on

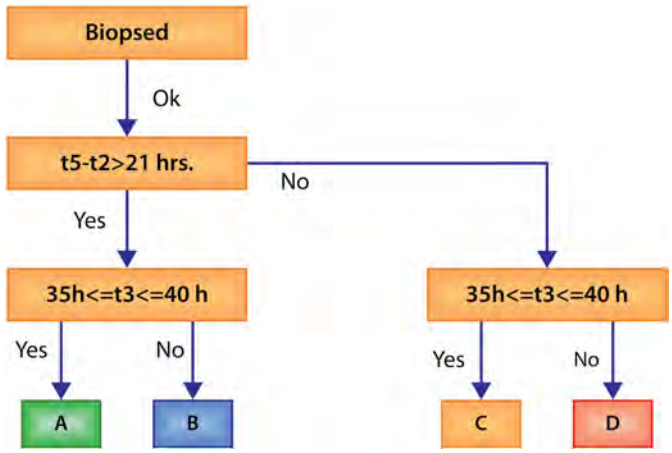

FIGURE 17.16 Del Carmen algorithm for ploidy detection (described by [85]).

TABLE 17.8 Review Studies on Morphokinetics.

Review	Title
Kirkegaard et al. 2012 [71]	Time-Lapse Monitoring as a Tool for Clinical Embryo Assessment
Chen et al. 2013 [89]	Biomarkers Identified with Time-Lapse Imaging: Discovery, Validation, and Practical Application
Kaser et al. 2014 [10]	Clinical Outcomes Following Selection of Human Preimplantation Embryos with Time-Lapse Monitoring: A Systematic Review
Kirkegaard et al. 2014 [11]	Choosing the Best Embryo by Time Lapse versus Standard Morphology
Polanski et al. 2014 [90]	Time-Lapse Embryo Imaging for Improving Reproductive Outcomes: Systematic Review and Meta-Analysis
Gardner et al. 2015 [4]	Diagnosis of Human Preimplantation Embryo Viability
Lundin et al. 2015 [91]	Quality Control and Standardization of Embryo Morphology Scoring and Viability Markers
Racowsky et al. 2015 [92]	A Critical Appraisal of Time-Lapse Imaging for Embryo Selection: Where Are We and Where Do We Need to Go?
Chen et al. 2017 [93]	Does Time-Lapse Imaging Have Favourable Results for Embryo Incubation and Selection Compared with Conventional Methods in Clinical In Vitro Fertilization? A Meta-Analysis and Systematic Review of Randomized Controlled Trials
Milewski et al. 2017 [94]	Time-Lapse Imaging of Cleavage Divisions in Embryo Quality Assessment
Pribenszky et al. 2017 [95]	Time-Lapse Culture with Morphokinetic Embryo Selection Improves Pregnancy and Live Birth Chances and Reduces Early Pregnancy Loss: A Meta-Analysis
Zaninovic et al. 2017 [96]	Assessment of Embryo Morphology and Developmental Dynamics by Time-Lapse Microscopy: Is There a Relation to Implantation and Ploidy?
Adolfsson et al. 2018 [97]	Morphology vs Morphokinetics: A Retrospective Comparison of Interobserver and Intra-Observer Agreement between Embryologists on Blastocysts with Known Implantation Outcome
Reignier et al. 2018 [98]	Can Time-Lapse Parameters Predict Embryo Ploidy? A Systematic Review
Armstrong et al. 2019 [99]	Time-Lapse Systems for Embryo Incubation and Assessment in Assisted Reproduction
Gallego et al. 2019 [100]	Time-Lapse Imaging: The State of the Art
Apter et al. 2020 [17]	Good Practice Recommendations for the Use of Time-Lapse Technology
Fernandez et al. 2020 [101]	Artificial Intelligence in the IVF Laboratory: Overview through the Application of Different Types of Algorithms for the Classification of Reproductive Data
Liu et al. 2020 [102]	Between-Laboratory Reproducibility of Time-Lapse Embryo Selection Using Qualitative and Quantitative Parameters: A Systematic Review and Meta-Analysis
Lundin et al. 2020 [103]	Time-Lapse Technology for Embryo Culture and Selection
Minasi et al. 2020 [104]	The Clinical Use of Time-Lapse in Human-Assisted Reproduction
Sciorio et al. 2021 [105]	Focus on Time-Lapse Analysis: Blastocyst Collapse and Morphometric Assessment as New Features of Embryo Viability
Kragh et al. 2021 [106]	Embryo Selection with Artificial Intelligence: How to Evaluate and Compare Methods?
Dimitriadis et al. 2022 [107]	Artificial Intelligence in the Embryology Laboratory: A Review

blastocyst formation, implantation (KID score), and fetal heartbeat (iDA score).

A lot of the reviews, opinions, and future directions regarding time-lapse that have been published in last decade are mentioned in Table 17.8.

Conclusion

Static observations obtained from standard microscopes have contributed significantly to our knowledge of embryo development; however, it is becoming more challenging to identify embryos with the highest implantation potential due to the static and notoriously subjective character of this type of morphological evaluation. The study of embryo kinetics through time-lapse technology has given rise to new markers for embryo selection, representing a new and excitingly powerful tool for viewing cellular activity and embryogenesis in a coherent and uninterrupted manner that is otherwise not available through standard microscopy. The current chapter presents an overview of the most recent studies that describe the use of this new technology in the IVF laboratory. Currently, TLT has been introduced into the IVF laboratory as a routine procedure, but many laboratories are still using standard incubators as a routine procedure because of cost and other factors. TLT provides the safest and most stable environment for the embryo culture and continuous embryo monitoring, which allowed us to identify many more undetected and unknown parameters of embryonic development. TLT provides a great hope for the future for non-invasive markers in the detection of aneuploidy, embryo abnormality, embryo selectivity, etc., with a combination of AI, metabolomics, proteomics, and secretomics.

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18

ARTIFICIAL INTELLIGENCE (AI)

Daniella Gilboa

Artificial intelligence (AI) is one of the greatest promises of the near future. By most accounts, it is going to be pertinent to the field of medicine, very significantly and very soon. As a recent editorial in the *Lancet* stated "[a] scenario in which medical information, gathered at the point of care, is analyzed using sophisticated machine algorithms to provide real-time actionable analytics seems to be within touching distance" [1].

A myriad of emerging technologies seems to be flooding hospitals, primary care practices, and the like, but to a large degree human-to-human interactions still drive the healthcare industry. AI is poised to change that—complementing human decision-making by working alongside physicians. At long last, the expert-only approach to medicine is beginning to change, thanks to the massive growth of medical data and the power of AI.

The healthcare industry is digitizing at a rapid rate: health records, medical images, and even discussions about treatment options are all being recorded digitally. In 1950, it took 50 years for the total volume of medical knowledge to double—by 2020, that doubling time is just about 73 days. Deep learning is considered a form of AI, as it bears similarities to human-based learning. The growing wide availability of digitalized data allows deep learning, which can recognize patterns in complex data sets, to revolutionize the way we practice medicine.

The main aspects of human intelligence are quite similar to AI. In the same way that humans gather information, process it, and determine an output, machines can do this as well. Because machines do not have physical senses like people do, the way they gather input differs. AI gathers information through things like speech recognition, visual recognition, and other data sources.

The processing piece of the formula also mimics how human intelligence works. Like the way people acquire memories and build knowledge, machines can create representations of knowledge and databases where information is stored. And, in the same manner that people draw inferences and make decisions, machines can predict, optimize, and determine what the best "next steps" should be in order to accomplish a particular goal.

Just as humans learn, machines can also be "taught." For instance, supervised machine learning means learning by example: the computer is provided with a data set containing labels that act as answers. Over time the machine can essentially "learn" to differentiate between those labels to produce the correct outcome.

Unsupervised machine learning is like learning by observation. The computer recognizes and identifies certain patterns and subsequently learns how to distinguish groups and patterns on its own [2]. To understand the concept of deep neural networks (DNNs), we should define the basic unit—a neuron. A neuron is a mathematical function which represents a learning unit.

A neural network is a network of functions, meaning all functions (or learning units) and all their inputs and outputs are intertwined and feed each other in order to learn the problem out of a set of examples (Figure 18.1). A neural network can learn relationships between the features that other algorithms cannot easily discover [3].

While the roots of AI date back more than 80 years from concepts laid out by Alan Turing [5, 6], Warren McCulloch, and Walter Pitts [7], it was not until 2012 that the subtype of deep learning was widely accepted as a viable form of AI. A deep learning neural network consists of digitized inputs, such as an image or speech, which proceed through multiple layers of connected "neurons" that progressively detect features, and ultimately provide an output. The basic DNN architecture is like a club sandwich turned on its side, with an input layer, several hidden layers ranging from 5 to 1000, each responding to different features of the image (like shape or edges), and an output layer. The layers are "neurons," comprising a neural network. A key differentiating feature of deep learning compared with other subtypes of AI is its autodidactic quality; the neural network is not designed by humans, but rather the number of layers is determined by the data itself. There are many types of DNNs and learning, including convolutional, recurrent, generative adversarial, reinforcement, representation, and transfer [8, 9].

Deep learning algorithms have been the backbone of computer performance. They exceed human ability in multiple games and are largely responsible for the exceptional progress in autonomous cars (Figure 18.2). Notably, except in the cases of games and self-driving cars, a major limitation to interpretation of claims reporting superhuman performance of these algorithms is that analytics are performed on previously generated data rather than prospectively in real-world clinical conditions. Furthermore, the lack of large data sets of carefully annotated images has been limiting across various disciplines in medicine.

Recent advances in generative AI (GenAI) have opened a new perspective for AI.

GenAI is a type of AI model that can create a wide variety of content such as text, images, videos, audios, and 3D models. It does so by using large language models (LLMs) to train on very large amounts of data, and then uses this knowledge to generate new and unique outputs. GenAI primarily differs from previous forms of AI or analytics because it can generate new content, often in "unstructured" forms.

LLMs are recent advances in deep learning models that work on human languages. An LLM is a trained deep-learning model that understands and generates text in a human-like fashion. Behind the scenes, it is a large transformer model that does all the magic. A transformer model is a neural network that learns context and meaning by tracking relationships in sequential data, like the words in a sentence.

Transformer models apply an evolving set of mathematical techniques called the attention mechanism, which allows us to see the entire sentence (or even the paragraph) at once rather than one word at a time. This allows the transformer model to understand the context of a word better [10].

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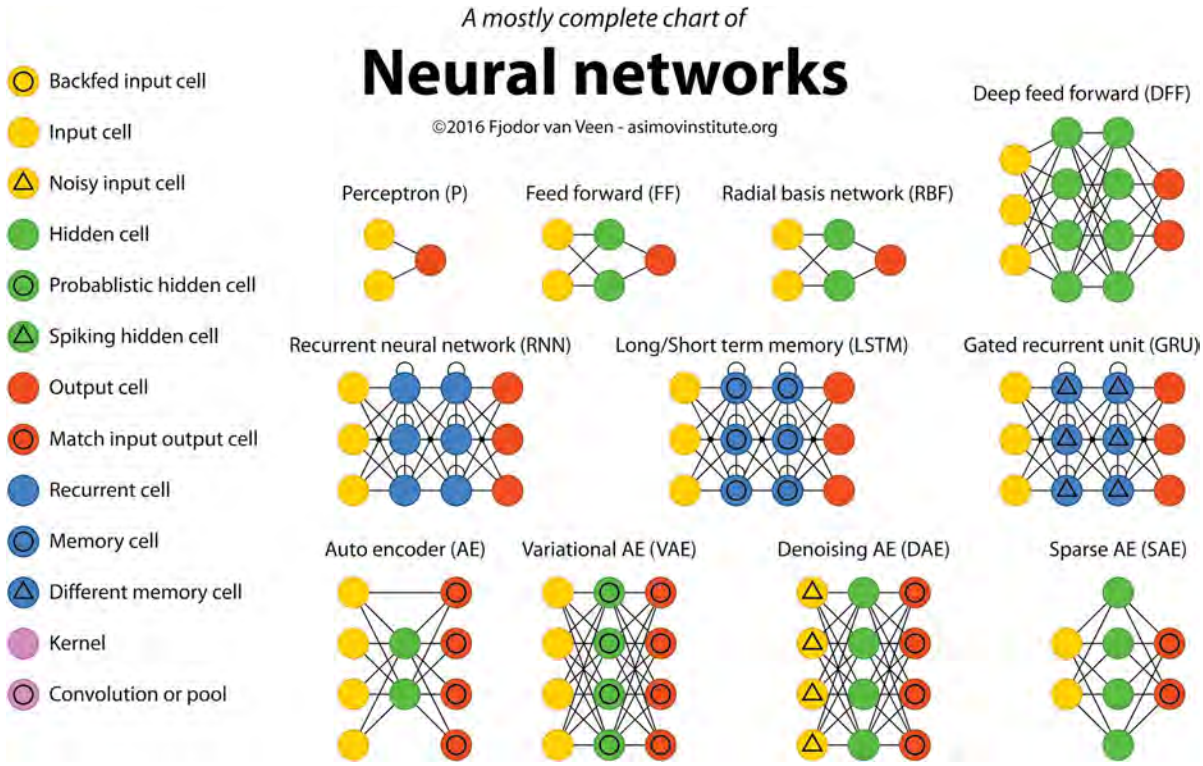

FIGURE 18.1 Examples of neural networks. (From [4], with permission.)

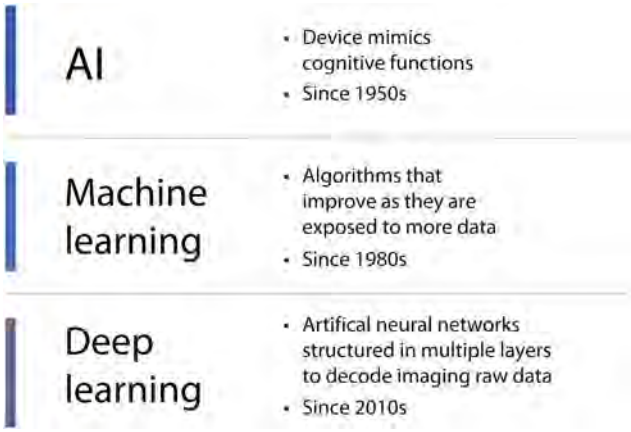

FIGURE 18.2 Summary of AI terminology.

Transformers are among the newest and one of the most powerful classes of AI models invented to date and are regarded as "foundation models" [11].

To grasp what lies ahead requires an understanding of the breakthroughs that have enabled the rise of GenAI, which was in the making for decades. Deep learning has powered many of the recent advances in AI, but the foundation models powering GenAI applications are a step-change evolution within deep learning. Unlike previous deep learning models, they can process extremely large and varied sets of unstructured data and perform more than one task.

The speed at which GenAI technology is developing is amazing. ChatGPT was released in November 2022. Four months later, OpenAI released a new LLM called GPT-4 with markedly improved capabilities. In May 2023, Google announced several new features powered by GenAI, including Search Generative Experience and a new LLM called PaLM 2 that will power its Bard chatbot, among other Google products.

These recent advances in GenAI have opened a new perspective for AI in healthcare, which include simulation of medical data for model training, synthetic medical data, drug discovery, drug development, education and automation of medical notetaking, among many more applications.

AI in healthcare

The fruitful collaborative research of clinicians and AI scientists is currently leading to a growing surge in groundbreaking publications. For instance, successful use of DNNs was reported for the analysis of skin cancer images with greater accuracy than a dermatologist [8]. A deep learning system was shown effective for the diagnosis of diabetic retinopathy and related eye diseases from retinal images [9]. DNNs have also been successfully implemented in classification of ovarian cancer types [12] and cervical cancer [13] from cytological images. "Nature" have launched a new journal, *Nature Machine Intelligence*, which will provides the research community with a forum for these themes and explores a broad spectrum of topics that connect various scientific disciplines with machine intelligence.

The revolution does not stop in academic research. The US Food and Drug Administration (FDA) has fast-tracked certain categories of AI services, opening "commercial pathways" for more than 100 AI imaging and diagnostics companies. It was recently claimed the "next big thing" in radiology may not be a new scanner technology but rather new discoveries in the way AI can be utilized for imaging.

The need for AI and the enormous potential that it holds are now clear. On one hand, the limitations of classic medicine, carried out by human caregivers, is becoming apparent—cost and human limitations are making such services more and more difficult to provide at a good standard across the entire population; on the other hand, vast improvements in computational powers and technical abilities make the incorporation of computers and machines in medical decision-making and treatment a very viable option.

In a recent paper published in The JAMA [14], the researchers evaluated the ability of ChatGPT (specifically the version that was released in November 2022) to provide quality and empathetic responses to patient questions. They used a public database of questions from a public social media forum (Reddit's r/AskDocs) to randomly draw 195 exchanges, where a verified physician responded to a public question. Chatbot responses were generated by entering the original question into a fresh session. The original question along with anonymized and randomly ordered physician and chatbot responses were evaluated by a team of licensed health care professionals. Evaluators chose "which response was better" and judged both "the quality of information provided" and "the empathy or bedside manner provided."

The results were in favor of the chatbot. Evaluators preferred chatbot responses to physician responses in 78.6% of the cases. Chatbot responses were of significantly higher quality than physician responses. In a post-COVID era when rapid expansion of virtual health care has caused a surge in patient messages concomitant with more work and burnout among health care professionals, this is just one example of an AI assistant that could aid in creating answers to patient questions by drafting responses that could be reviewed by clinicians.

AI for reproductive care

The idea of applying AI to infertility has been around for two decades. Early products presenting a solution which uses time-lapse imaging microscopy to collect data over the length of the embryo's culture period, and an algorithm to predict which embryo has the best chance of progressing, are now in the market.

AI systems for IVF are already showing promising results in clinical practice. In one study, the system used human embryos to identify the ones most likely to survive. Overall, the AI system had a 67% accuracy rating [15]. This demonstrates that a fully automated model can perform better than the models based on morphokinetic parameters, and this is obtained without the need for assessment/annotation by the embryologist.

Identifying viable embryos is only the first hurdle in IVF. A true "end-to-end" AI solution for infertility care will have to integrate complex (and diverse) data sets that are currently managed in multiple, incompatible systems—patient demographics and medical histories; drug treatment regimens; pre-implantation genetic screening; and clinical pregnancy outcome data. AI-based systems are helping physicians to choose among several treatment options that have the highest success rates, and accept new information based on the patient's responses to treatments.

In clinical embryology, we don't yet know the feature or set of features that is most predictive of IVF success. It is possible that the most important variable for a successful IVF cycle could still be unknown to science, but, in principle, may be uncovered by AI systems. This may be referred to as computational embryology.

A particular problem with using AI for IVF is the huge, multidimensional solution space that an AI system would have to cope with. Unlike in some other medical disciplines, there isn't a tumour to find in a scan, an aneurysm to detect on an image, or any single feature or cluster of features that directly link to treatment success or failure. In time-lapse imaging, success or failure may be hiding in any of hundreds of images and in their relationship over time—for example, in the timing of mitosis events.

The problem is made yet more difficult by the nature of time-lapse imaging of embryos. A time-lapse video suffers from all the challenges of unsupervised photography: inconsistent lights, bad focus in part or in whole, and artefacts such as bubbles interfering with some or all frames. The embryo itself is sometimes only partly visible because it could be at the edge of the dish from the camera's point of view. AI systems are overcoming all of these challenges and are becoming clinically useful (Figure 18.3a–d).

Biological constraints mean that only a tiny portion of this solution space represents real embryos. In AI-speak, this is a non-Euclidean problem [16] as it does not occupy a typical Euclidean space where all points are possible (Figure 18.4a). Non-Euclidean problems pose a particular challenge for neural networks, as the networks struggle to learn the relevant part of the solution space. (Figure 18.4b–d shows examples of different non-Euclidean spaces.)

Newly emerging experimental approaches in AI for non-Euclidean problems are showing great promise and it is expected that these will soon contribute to improvements in the accuracy of AI systems. In parallel, an approach to use computer vision and AI to explicitly find known features and incorporate these into the neural net is helping to mitigate the non-Euclidean nature of the problem by reducing the solution space.

AI technologies have tremendous potential to help the field of infertility medicine to transcend its current narrow focus on individual embryos and uncover new patterns hidden in the patient data for the treatment of stubborn infertility [17].

The excitement that lies ahead, albeit much further along than many have forecasted, is for software that will ingest and meaningfully process massive sets of data quickly, accurately, and inexpensively. Moreover, AI machines are predicted to see and do things that are not humanly possible. This capability will ultimately lay the foundation for high-performance medicine which is truly data-driven, negating our reliance on human resources, and eventually taking us well beyond the sum of the parts of human and machine intelligence. Reproductive medicine is likely to be one of the fields to effectively adopt the AI revolution, greatly advancing our ability to accurately prescribe personalized care for our infertility patients along with improving the success rates in the embryology lab.

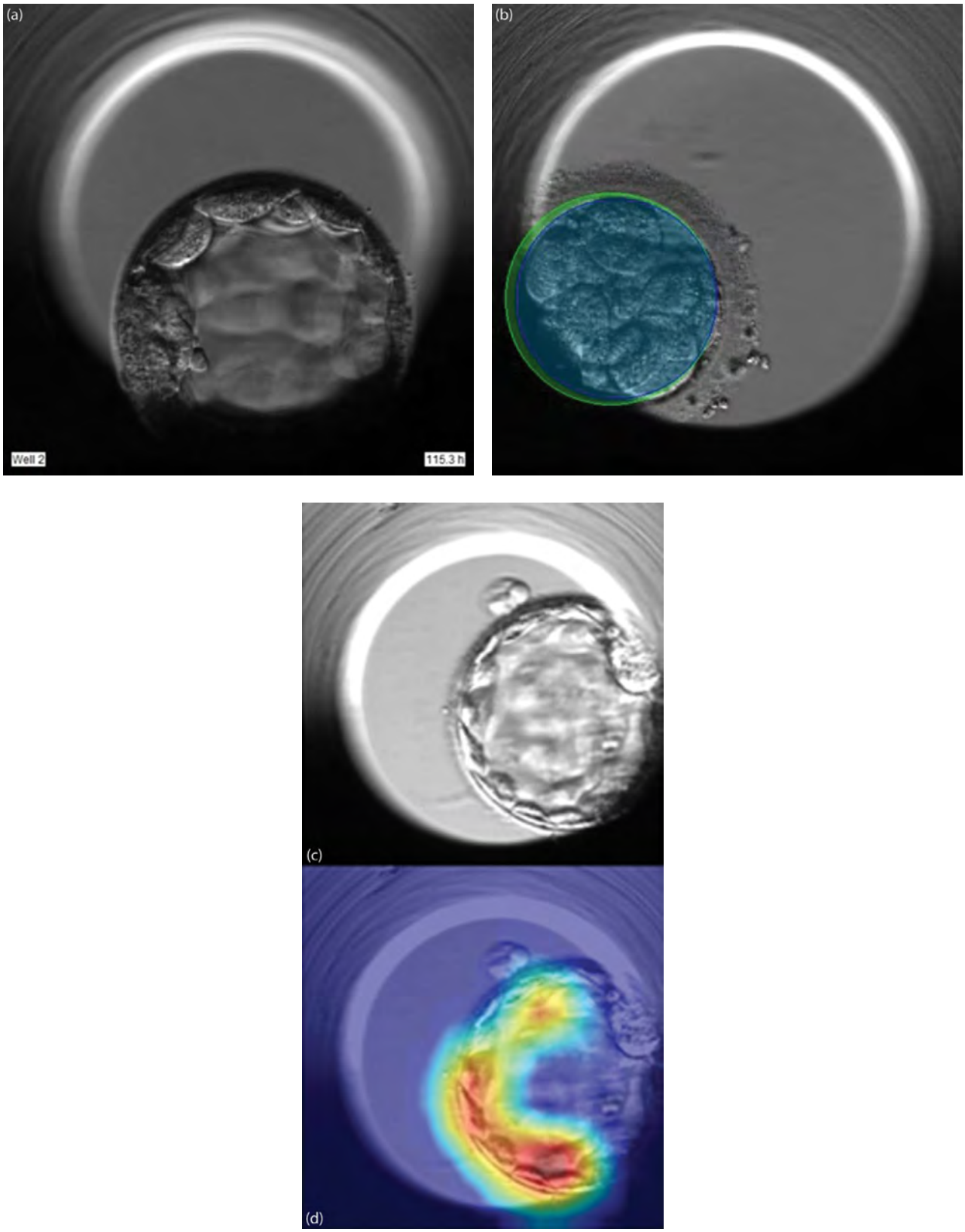

FIGURE 18.3 (a) The human brain intuitively identifies this displaced embryo at the bottom of the round dish. For scale, the dish is 200 microns in diameter. (b) Human segmentation of another displaced embryo. (c–d) Displaced embryo and the accurate detection of the model after training.

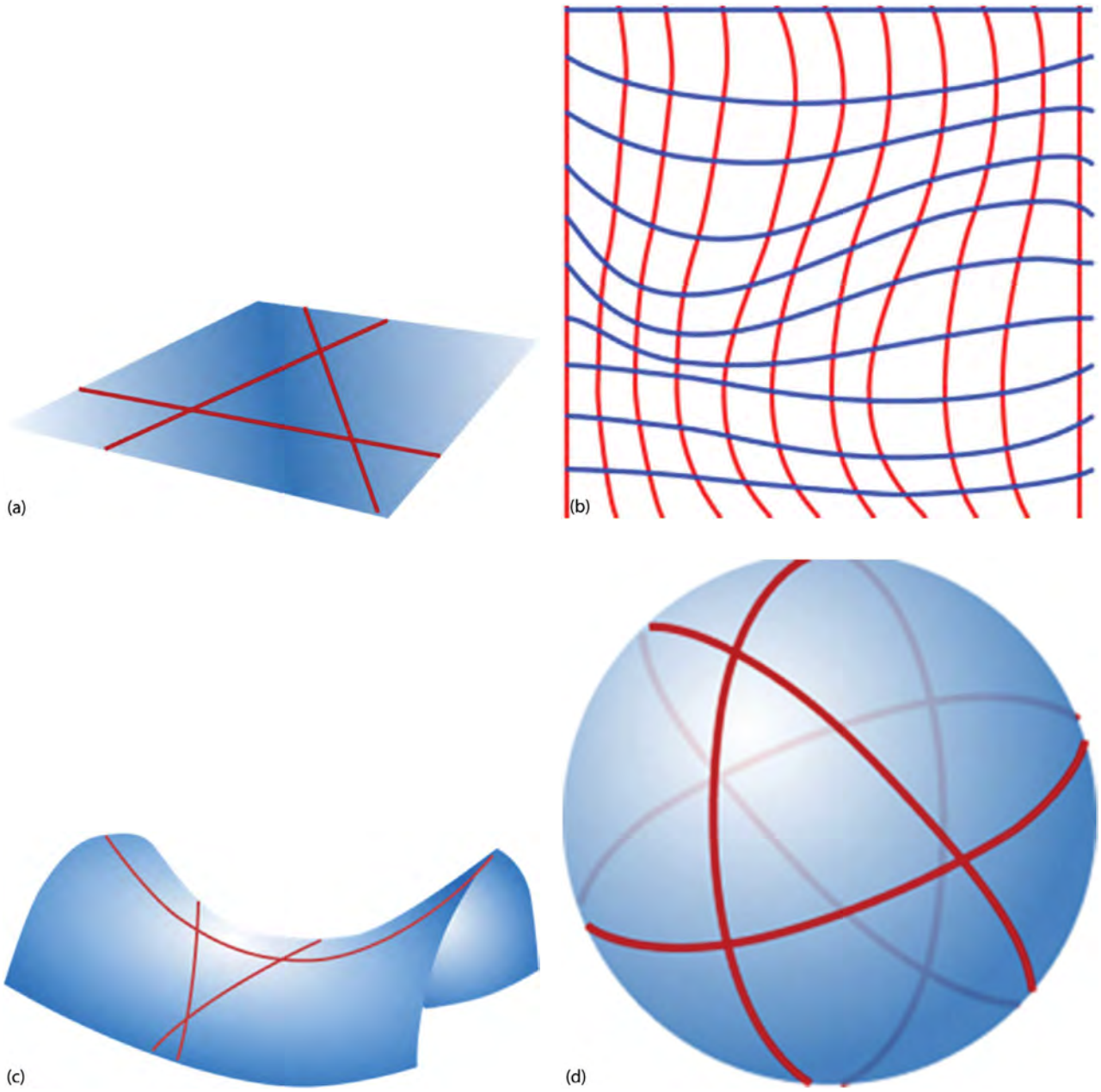

FIGURE 18.4 (a) A Euclidean space such as the physical world, where geometric rules are aligned with human intuition. (b) A two-dimension illustration of a non-Euclidean space. Coordinate lines are not parallel to each other, and each grid square does not have the same area. Mathematical problems are often represented by non-Euclidean spaces, but to our intuition these spaces seem "wrong." (c) Non-Euclidean space-hyperbolic. The angles of a triangle add up to more than 180 degrees, as opposed to a triangle in a Euclidean space. (d) Non-Euclidean space-spherical. The angles of a triangle add up to less than 180 degrees.

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19

ARTIFICIAL INTELLIGENCE (AI) IN GAMETE AND EMBRYO SELECTION

Chloe He, Neringa Karpaviciute, Cristina Hickman, and Nikica Zaninovic

Over the past few decades, computers have revolutionized the field of medicine. Today, computers and computer-based technologies have become an indispensable tool for clinicians, researchers, and patients, supporting their efforts to monitor conditions, process data, and access healthcare services. But the field of computing itself is also witnessing its own revolution. Since the early 2010s, the collection of large data sets and increases in computing power have enabled numerous successes in the field of artificial intelligence (AI). Many of these successes have hinged on machine learning (ML), a data-driven subfield of AI that focuses on the automatic discovery of patterns in data.

The plethora of techniques coming out of AI research have the potential to resolve many of the challenges experienced within the field of reproductive medicine, including prediction, classification, and operational efficiency. If incorporated effectively into clinical processes, the automation associated with AI could enhance consistency, efficiency, and efficacy as well as reduce risk and human errors. The promise of AI has led to an increase in research towards the development of AI-based tools to assist in gamete and embryo selection. In this chapter, we will review the current landscape of AI in gamete and embryo selection in the *in vitro* fertilization (IVF) laboratory.

AI in gamete selection

Ensuring gamete quality is a crucial step towards assisted reproductive technology (ART) cycle success. Manual gamete selection is a labour-intensive, time-consuming, and highly subjective process. The application of AI to oocyte and sperm selection greatly benefits gamete donation and cryopreservation, and it reduces embryo waste, particularly in countries with restrictive ART laws. AI-based oocyte selection has the potential to be able to identify oocytes with the highest developmental potential that are "worth" fertilizing. This could increase the efficiency of the IVF process and reduce the creation of incompetent, nonviable, or chromosomally abnormal embryos that will never be used in clinical treatment. It could also reduce storage requirements for cryopreserved oocytes and embryos. The following sections explore gamete selection methods driven by ML. A summary of the studies discussed can be found in Table 19.1.

Oocyte selection

The ability to noninvasively assess oocyte quality without damaging the oocyte itself is key to the success of ART cycles. Such assessment typically involves visual inspection of the cumulus–oocyte complex, cytoplasm, zona pellucida, perivitelline space, and polar bodies. Because the appearance of these structures has been associated with successful fertilization, ML algorithms have been aimed at analysing them [1].

Early attempts used classical image analysis methods to predict oocyte capacity to result in live birth [2, 3]. Manna et al. [4] used multiple artificial neural networks to predict pregnancy from oocyte images using textural descriptors of the oocytes (derived local binary patterns). The textural descriptors were obtained using classical image analysis techniques and were fed into each network. A classification was arrived at through a majority-voting procedure between the networks. More recently, Baručić et al. [5] proposed a system for the automatic detection of oocytes with high developmental potential. Unlike Manna et al. [4], Baručić et al. [5] considered physical measurements. Individual parts of the oocyte were segmented (that is, identified and highlighted) using a convolutional neural network (CNN) that took the raw oocyte images as input and returned segmentation maps. Measurements derived from the segmentation maps were then fed into a support vector machine model that predicted the oocyte's viability. The model achieved performance comparable to that of a human embryologist.

Several studies have also solely focused on the automatic identification and segmentation of key oocyte structures such as vacuoles and polar bodies. The systems developed in these studies have several applications, including downstream image analysis [5] and robotic cell manipulation [6]. Firuzinia et al. [7] developed a deep learning system for segmenting low-resolution images of metaphase II (MII) oocytes. Targosz et al. [8], carried out a benchmark study of popular CNN architectures for the segmentation of cytoplasm, vacuoles, polar bodies, zona pellucida, cumulus cells, and other structures.

Semen analysis and sperm selection

Although a limited number of oocytes are retrieved for an IVF cycle, tens of thousands of sperm are placed around an oocyte during IVF. Sperm sorting must take place in the embryology laboratory to select sperm with high fertilization potential. Research into the application of AI systems to sperm evaluation, sorting, and selection has generally been more advanced than that for oocytes, with many major laboratories making use of computer-assisted sperm assessment systems. Sperm morphology, motility, and concentration are important parameters evaluated in semen analyses by embryologists and andrologists. Moreover, unlike oocytes, visually identifiable parameters such as sperm motility and morphology have been shown to reflect DNA integrity [9]. As a result, AI systems have been developed to automate this evaluation to save time and avoid the subjectivity and variability inherent in manual assessment.

Several studies have proposed automated systems for sperm morphology analysis. Among the earliest of these systems is FERTECH, which classified sperm according to World Health Organization (WHO) criteria [10]. More recent studies have tackled the problem using modern data-driven image analysis techniques [11–21]. While most of these works analysed the spermatozoon as a whole, Javadi et al. [12] developed a CNN-based system that explicitly separated the evaluation of the head, vacuole, and acrosome of the sperm.

The assessment of sperm motility has also been a target for automation [22–27]. Goodson et al. [24] developed a support vector machine model that reported motility characteristics

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TABLE 19.1 AI Gamete Selection Literature

Gamete	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
Oocyte	Image Segmentation	Basile et al. (2010)	[2]	A texture-based image processing approach for the description of human oocyte cytoplasm	Identified and segmented the cytoplasm in oocyte images. Created clusterings of cytoplasm textures.	No
		Targosz et al. (2021)	[8]	Semantic segmentation of human oocyte images using deep neural networks	Benchmarked different convolutional neural network (CNN) architectures for segmentation of key structures in images of oocytes.	No
		Firuzinia et al. (2021)	[7]	A robust deep learning-based multiclass segmentation method for analyzing human metaphase II oocyte images	Proposed a CNN model for key structures in human metaphase II oocytes.	No
	Developmental Potential Prediction	Manna et al. (2013)	[4]	Artificial Intelligence (AI) techniques for embryo and oocyte classification	Predicted live birth from textural descriptors of oocytes using an ensemble of Levenberg-Marquard neural networks.	No
		Baručić et al. (2021)	[5]	Automatic evaluation of human oocyte developmental potential from microscopy images	Extracted measurements from CNN-generated oocyte segmentation maps and used them to predict developmental potential.	Yes
Sperm	Morphological Analysis	Kruger et al. (1993)	[10]	A new computerized method of reading sperm morphology (strict criteria) is as efficient as technician reading.	Proposed an image analysis program for the classification of sperm morphology according to strict criteria.	Yes
		Shaker et al. (2017)	[15]	A dictionary learning approach for human sperm heads classification	Used an adaptive patch-based dictionary learning method to classify sperm heads. Publicly released the widely used HuSHeM data set of sperm heads images.	No
		Riordon et al. (2019)	[14]	Deep learning for the classification of human sperm	Fine-tuned an existing VGG16 CNN to classify sperm head morphology from images.	No
		Javadi and Mirroshandel (2019)	[12]	A novel deep learning method for automatic assessment of human sperm images	Proposed a CNN with low computational cost for the assessment of morphological deformities in head, acrosome, neck, tail, and vacuole.	No
		Ilhan et al. (2020)	[17]	A fully automated hybrid human sperm detection and classification system based on mobile-net and the performance comparison with conventional methods	Proposed a system for the segmentation and classification of sperm images.	No
		Yüzkat et al. (2021)	[16]	Multi-model CNN fusion for sperm morphology analysis	Trained six CNNs on different data sets to assess sperm morphology. At inference time, predictions from all models are combined via a voting procedure to arrive at a final assessment.	No
		Abbasi et al. (2021)	[18]	Effect of deep transfer and multi-task learning on sperm abnormality detection	Adapted an existing VGG19 CNN to detect head, acrosome, and vacuole abnormalities in sperm images.	No
		Sato et al. (2022)	[26]	A new deep-learning model using YOLOv3 to support sperm selection during intracytoplasmic sperm injection procedure	Trained a YOLOv3 CNN to simultaneously track and morphologically assess sperm in real time.	No
		Chandra et al. (2022)	[19]	Prolificacy assessment of spermatozoan via state-of-the-art deep learning frameworks	Benchmarked different CNN architectures for the detection of head, acrosome, and vacuole abnormalities.	Yes

(Continued)

TABLE 19.1 AI Gamete Selection Literature *(Continued)*

Gamete	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
	Motility Analysis	Goodson et al. (2017)	[24]	CASAnova: A multiclass support vector machine model for the classification of human sperm motility patterns	Analysed kinematic parameters of sperm obtained through sperm tracking software using a support vector machine to assess motility.	No
		Dimitriadis et al. (2019)	[31]	Automated smartphone-based system for measuring sperm viability, DNA fragmentation, and hyaluronic binding assay score	Proposed a smartphone-based video analysis system for the assessment of sperm viability using 3D-printed hardware.	No
		Agarwal et al. (2019)	[27]	Automation of human semen analysis using a novel AI optical microscopic technology	Comparison between a proprietary automated semen analysis device and human experts on assessment of sperm concentration and motility.	Yes
		Hicks et al. (2019)	[28]	Machine learning (ML)-based analysis of sperm videos and participant data for male fertility prediction	Trained a CNN to predict sperm motility from multimodal video data and clinical data.	No
		Somasundaram et al. (2021)	[29]	Faster region CNN and semen tracking algorithm for sperm analysis	Combined the outputs of a faster region CNN network with other algorithms for the assessment of sperm morphology and motility from video data.	No
		Alameri et al. (2021)	[23]	Multistage optimization using a modified Gaussian mixture model in sperm motility tracking	Presented a system for the evaluation of motility from videos based on a modified Gaussian mixture model.	No
		Valiuškaitė et al. (2021)	[22]	Deep learning based evaluation of spermatozoid motility for artificial insemination	Trained a faster region CNN network for the assessment of sperm morphology and motility from videos.	No
	Image Segmentation	Chang et al. (2014)	[11]	Gold-standard and improved framework for sperm head segmentation	Used classical image analysis techniques to perform segmentation of the sperm head, nucleus, and acrosome.	No
		Movahed et al. (2019)	[20]	Automatic segmentation of sperm's parts in microscopic images of human semen smears using concatenated learning approaches	Proposed a system proposed of a variety of ML algorithms (including CNNs, k-means clustering, and support vector machines) for the segmentation of the head, acrosome, nucleus, axial filament, mid-piece, and tail.	No
		Kandel et al. (2020)	[13]	Reproductive outcomes predicted by phase imaging with computational specificity of spermatozoon ultrastructure	Proposed a U-Net CNN for the segmentation of the head, mid-piece, and tail from high-sensitive phase imaging data. These segmentations were used to calculate dry-mass ratios between the different parts of the sperm.	No
		Marín et al. (2021)	[21]	Impact of transfer learning for human sperm segmentation using deep learning	Demonstrated that a CNN pre-trained on larger sperm data set for segmentation can be adapted to new sperm data sets via transfer learning leading to performance gains over training from scratch.	No

(Continued)

TABLE 19.1 AI Gamete Selection Literature ***(Continued)***

Gamete	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
	DNA Integrity Prediction	McCallum et al. (2019)	[32]	Deep learning-based selection of human sperm with high DNA integrity	Fine-tuned an existing VGG16 CNN to predict DNA integrity from brightfield images.	No
		Zhang et al. (2021)	[9]	Quantitative selection of single human sperm with high DNA integrity for intracytoplasmic sperm injection	Created a program to predict DNA fragmentation from morphological and motility assessments derived from computer vision algorithms.	Yes
	Sperm Ranking	Mendizabal-Ruiz et al. (2022)	[25]	Computer software (SiD) assisted real-time single sperm selection correlates with fertilization and blastocyst formation	Evaluated a proprietary sperm scoring system with respect to whether human embryologists selected sperm for ICSI, fertilization, and blastocyst formation.	No
	Clinical Outcome Prediction	Gunderson et al. (2021)	[30]	Machine-learning algorithm incorporating capacitated sperm intracellular pH predicts conventional in vitro fertilization success in normospermic patients	Predicted fertilization after conventional IVF using gradient-boosted ML algorithm trained on clinical data, sperm pH, and membrane potential.	No

with 89.9% accuracy. The model was also able to identify subpopulations of sperm cells [24]. More recently, deep learning methods have been used to improve the accuracy achieved by computer-assisted motility analysis [22, 28, 29]. Mendizabal-Ruiz et al. [25] used a proprietary computer vision system to select individual spermatozoon for intracytoplasmic sperm injection (ICSI) based on kinematic data (straight-line velocity, linearity of the curvilinear path, and head movement pattern). They found statistically significant differences between spermatozoa that resulted in IVF success and those that did not. Furthermore, Sato et al. [26] developed a CNN-based system to simultaneously perform morphological assessment and tracking of sperm in real time to assist in sperm selection for ICSI. The model was able to distinguish between normal and abnormal sperm with high sensitivity and precision (79.4% and 88.1%, respectively).

Other sperm selection techniques include measuring intracellular sperm pH, which was found to correlate with IVF success [30]. Furthermore, smartphone-based semen analysis systems are becoming more popular, which enable at-home testing [31]. A study by McCallum et al. [32] was the first to demonstrate that it is possible to predict the DNA integrity of an individual spermatozoon solely from imaging data by training a CNN with sperm images labelled with different DNA fragmentation indices.

AI in embryo selection

The application of AI to embryo selection in ART cycles has also been an area of considerable research interest. Most of these approaches are based on the analysis of both static and time-lapse embryo imaging data. As a result, many state-of-the-art systems have been based on deep learning, a subset of AI approaches that have proved especially successful at image analysis tasks. In this section, we will take a high-level look at the landscape for AI systems for embryo selection. A summary of the studies discussed can be found in Table 19.2.

What is the ground truth?

To date, almost all work in AI for embryo selection has involved the use of supervised learning to predict a clinically relevant outcome from input data. While the input data is usually time-lapse or static imagery, there is a wide range of target variables in the literature that include blastocyst formation [33–35], blastocyst grade [36–39], ploidy [40–44], implantation [33, 40, 45–50], fetal heartbeat pregnancy [47, 48, 51–57], and live birth [52, 57–61].

This heterogeneity arises from several factors. Clearly, the different outcomes carry different biological and clinical significance. For instance, it may be argued that automated embryo selection systems should be trained using live birth data since a live birth is the end goal of the ART cycle. It may, however, also be argued that the occurrence of live birth greatly depends on factors other than the embryo, and thus the prediction of blastocyst grade or implantation may be more appropriate. Another factor is data availability and volume; as a rule of thumb, the more developmentally advanced an embryo must be to measure a target variable, the scarcer the data available on the target variable will be by virtue of fewer embryos reaching that stage. A more comprehensive treatment of the advantages and disadvantages of each prediction target can be found in Table 19.3.

Static image analysis

Many early studies examining the potential for the use of AI in embryo selection focused on single static images. Khosravi et al. [37] fine-tuned an InceptionV3 CNN model to classify Hoffman modulation contrast (HMC) microscopy images of D5 blastocysts as "good" or "poor." The ground-truth classifications were based on the majority vote of a panel of four embryologists. The model outperformed each individual embryologist, predicting the majority vote with 96.9% accuracy. Ver Milyea et al. [55] also made use of a CNN. In this study, it was to predict fetal heartbeat from D5 blastocyst images captured on a standard light microscope. The system achieved 64.3% accuracy with 70.1% sensitivity and 60.5% specificity.

TABLE 19.2 AI Embryo Selection Literature

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
Static Image	Embryo Grading	Filho et al. (2012)	[36]	A method for semi-automatic grading of human blastocyst microscope images	Used measurements derived from segmentation maps of blastocysts to train a support vector machine to predict blastocyst grade.	No
		Khosravi et al. (2019)	[37]	Deep learning enables robust assessment and selection of human blastocysts after in vitro fertilization	Fine-tuned an existing InceptionV3 CNN to predict blastocyst grade. The system outperformed individual embryologists at embryo selection.	Yes
		Wu et al. (2020)	[62]	A classification system of day 3 human embryos using deep learning	Proposed an ensemble of CNN models for the prediction of gradings for D3 embryos.	Yes
		Thirumalaraju et al. (2021)	[39]	Evaluation of deep CNNs in classifying human embryo images based on their morphological quality	Benchmarked several CNN architectures for the prediction of developmental stage and, where applicable, blastocyst grade of D5 embryos. Also generated heatmaps to help identify parts of the image deemed to be important by the models.	No
	Image Enhancement	Raudonis et al. (2021)	[73]	Fast multi-focus fusion based on deep learning for early-stage embryo image enhancement	Proposed a CNN-based system to fuse multiple focal planes into a single image without the loss of useful information.	No
	Image Segmentation	Singh et al. (2015)	[68]	Automatic segmentation of trophectoderm in microscopic images of human blastocysts	Used a level-set algorithm to segment the trophectoderm in blastocyst images.	No
		Saeedi et al. (2017)	[70]	Automatic identification of human blastocyst components via texture	Used a pipeline of classical computer vision techniques to segment the trophectoderm and inner cell mass.	No
		Rad et al. (2019)	[67]	BLAST-NET: Semantic segmentation of human blastocyst components via cascaded atrous pyramid and dense progressive upsampling	Proposed the first CNN architecture for the segmentation of the zona pellucida, trophectoderm, inner cell mass, and blastocoel.	No
		Rad et al. (2020)	[66]	Trophectoderm segmentation in human embryo images via inceptioned U-Net	Proposed a novel U-Net CNN architecture for the segmentation of the zona pellucida, trophectoderm, and inner cell mass.	No
		Arsalan et al. (2022)	[71]	Detecting blastocyst components by AI for human embryological analysis to improve success rate of in vitro fertilization	Proposed a novel CNN architecture using sprint convolutional blocks for the segmentation of the zona pellucida, trophectoderm, inner cell mass, and blastocoel.	No
	Live Birth Prediction	Manna et al. (2013)	[4]	AI techniques for embryo and oocyte classification	Predicted live birth from textural descriptors of embryos using an ensemble of Levenberg-Marquard neural networks.	No

(Continued)

TABLE 19.2 AI Embryo Selection Literature ***(Continued)***

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
		Geller et al. (2021)	[48]	An AI-based algorithm for predicting pregnancy success using static images captured by optical light microscopy during intracytoplasmic sperm injection	Finetuned an existing InceptionV1 CNN to predict pregnancy and live birth from pictures of D5 embryos.	No
		B. Huang et al. (2022)	[52]	Using deep learning to predict the outcome of live birth from more than 10,000 embryo data	Proposed a CNN model for the prediction of live birth outcome from a single blastocyst transfer using imaging data.	No
		Enatsu et al. (2022)	[47]	A novel system based on AI for predicting blastocyst viability and visualizing the explanation	Proposed a CNN model for the prediction of pregnancy and live birth from images of D5 embryos. In addition, explanations for the CNN's decisions were provided using heatmaps. The model outperformed embryologist evaluation using the Gardner scale.	Yes
	Pregnancy Prediction	Bormann et al. (2020)	[45]	Performance of a deep learning based neural network in the selection of human blastocysts for implantation	Evaluated a system for the automatic prediction of implantation against fifteen trained embryologists. The system was based on a CNN combined with genetic algorithms. The system outperformed the embryologists.	Yes
		Chavez-Badiola (2020)	[46]	Predicting pregnancy test results after embryo transfer by image feature extraction and analysis using ML	Compared several algorithms for the prediction of biochemical pregnancy from parameters derived from dimensionality reduction and image analysis of D5 and D6 blastocysts.	No
		ver Milyea et al. (2020)	[55]	Development of an AI-based assessment model for prediction of embryo viability using static images captured by optical light microscopy during IVF	Presented a CNN model for the prediction of pregnancy from images of D5 blastocysts. The model significantly outperformed embryologists.	Yes
		Fitz et al. (2021)	[49]	Should there be an "AI" in TEAM? Embryologists' selection of high implantation potential embryos improves with the aid of an AI algorithm	Demonstrated improvements in the ability of embryologists to select embryos with high implantation potential when aided by a CNN-based system.	Yes
		Loewke et al. (2022)	[56]	Characterization of an AI model for ranking static images of blastocyst stage embryos	Proposed a CNN model for the prediction of pregnancy from images of blastocysts. Explanations for the CNN's decisions were provided using heatmaps. Inspection of the heatmaps revealed the features learned by the model overlapped with the features considered by manual grading systems.	Yes

(Continued)

TABLE 19.2 AI Embryo Selection Literature *(Continued)*

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
	Ploidy Prediction	Chavez-Badiola (2020)	[40]	embryo ranking intelligent classification algorithm (ERICA): AI clinical assistant predicting embryo ploidy and implantation	Trained a deep neural network to predict ploidy and implantation from parameters derived from the analysis of blastocyst images. The network outperformed two human embryologists.	Yes
	Image Generation	Dirvanauskas et al. (2019)	[87]	HEMIGEN: Human embryo image generator based on generative adversarial networks	Trained a generative adversarial network to generate synthetic images of cleavage stage embryos.	No
	Pronuclei Detection	Fukunaga et al. (2020)	[75]	Development of an automated two pronuclei detection system on time-lapse embryo images using deep learning techniques	Presented a CNN model for the detection of pronuclei in fertilized oocytes.	No
Time-lapse	Embryo Grading	Kragh et al. (2019)	[38]	Automatic grading of human blastocysts from time-lapse imaging	Presented a recurrent CNN-based system for the prediction of inner cell mass and trophectoderm grades from time-lapses. The model performed on par with embryologists.	Yes
	Pregnancy Prediction	Tran et al. (2019)	[54]	Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer	Introduced a deep learning model for the prediction of fetal heart beat pregnancy from time-lapse videos.	No
		Alegre et al. (2021)	[50]	Assessment of embryo implantation potential with a cloud-based automatic software	Predicted implantation potential using morphokinetic parameters obtained by a CNN model.	No
		Kan-Tor et al. (2021)	[33]	Automated evaluation of human embryo blastulation and implantation potential using deep-learning	Predicted blastocyst formation and implantation using a system based on deep neural networks. To gain some insight into the features the system deemed most important, the trained models were analysed using Shapley additive explanations.	No
		Berntsen et al. (2022)	[51]	Robust and generalizable embryo selection based on AI and time-lapse image sequences	Proposed a deep learning model for the prediction of fetal heart beat pregnancy from time-lapses. The model consisted of a two-stream inflated 3D CNN augmented with bidirectional long short-term memory modules.	No
		Kragh et al. (2022)	[53]	Predicting embryo viability based on self-supervised alignment of time-lapse videos	Proposed a self-supervised training set-up that enabled the use of unlabelled data in the training of deep learning models. Models trained to predict pregnancy using the proposed method outperformed models trained with supervised learning alone.	No

(Continued)

TABLE 19.2 AI Embryo Selection Literature ***(Continued)***

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
		Erlich et al. (2022)	[82]	Pseudo contrastive labeling for predicting IVF embryo developmental potential	Proposed a pseudo-contrastive labelling scheme that assigned similar labels to embryos with similar developmental patterns. This allowed a CNN-based system to be trained to predict implantation despite ambiguity arising from multiple embryo transfers and missing labels. The model outperformed human embryologists.	Yes
	Live Birth Prediction	Sawada et al. (2021)	[61]	Evaluation of AI using time-lapse images of IVF embryos to predict live birth	Trained an attention branch network to predict live birth from time-lapse videos.	No
		Nagaya et al. (2022)	[60]	Embryo grading with unreliable labels due to chromosome abnormalities by regularized PU learning with ranking	Presented a new training scheme for deep learning models for the prediction of live birth in the presence of confounders such as chromosome abnormalities.	No
	Ploidy Prediction	B. Huang et al. (2021)	[43]	An AI model (euploid prediction algorithm) can predict embryo ploidy status based on time-lapse data	Presented a pipeline of deep learning models trained to predict ploidy from time-lapse videos.	No
		Lee et al. (2021)	[44]	End-to-end deep learning for recognition of ploidy status using time-lapse videos	Trained a two-stream inflated 3D CNN for the prediction of ploidy from time-lapse videos.	No
	Image Segmentation	Zhao et al. (2021)	[69]	Application of CNN on early human embryo segmentation during in vitro fertilization	Proposed a CNN model for the segmentation of the cytoplasm, pronuclei, and zona pellucida in D1 embryo time-lapses.	No
		T. T. F. Huang et al. (2021)	[80]	Deep learning neural network analysis of human blastocyst expansion from time-lapse image files	Trained a U-Net CNN model to segment blastocysts. The model was applied to time-lapses and blastocyst expansion curves were generated.	No
	Blastocyst Formation Prediction	Coticchio et al. (2021)	[81]	Cytoplasmic movements of the early human embryo: imaging and AI to predict blastocyst development	Evaluated several ML models for the prediction of blastocyst formation from the movement of cytoplasmic particles. Movement of the particles was measured using a particle image velocimetry algorithm.	No
		Liao et al. (2021)	[34]	Development of deep learning algorithms for predicting blastocyst formation and quality by time-lapse monitoring	Trained a long short-term memory neural network to predict blastocyst formation and quality from morphokinetic parameters derived from a CNN model.	Yes
	Developmental Stage Prediction	Lau et al. (2019)	[76]	Embryo staging with weakly-supervised region selection and dynamically-decoded predictions	Predicted embryo developmental stage using a CNN model augmented with monotonicity constraints. Images were automatically cropped by a reinforcement learning agent as a pre-processing step.	No

(Continued)

TABLE 19.2 AI Embryo Selection Literature *(Continued)*

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
		Leahy et al. (2020)	[65]	Automated Measurements of Key Morphological Features of Human Embryos for IVF	Predicted embryo developmental stage and cell segmentation masks using a CNN-based system.	No
		Malmsten et al. (2020)	[79]	Automated cell division classification in early mouse and human embryos using CNNs	Trained a CNN to detect and classify cell divisions up to the 8-cell stage.	No
		Lockhart et al. (2021)	[78]	Automating embryo development stage detection in time-lapse imaging with synergic loss and temporal learning	Proposed a novel CNN model and loss function for the detection of developmental stages in time-lapses.	No
		Lukyanenko et al. (2021)	[77]	Developmental stage classification of embryos using two-stream neural network with linear-chain conditional random field	Proposed a novel system for the detection of developmental stages in time-lapses. The system consisted of two CNNs (one for the prediction of developmental stage and the other for the detection of transitions between stages) and a linear-chain conditional random field.	No
Morphokinetic Annotations	Pregnancy Prediction	Petersen et al. (2016)	[74]	Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on day 3	Predicted pregnancy from manually provided morphokinetic annotations using a decision tree approach.	No
	Live Birth Prediction	Bodri et al. (2018)	[59]	Predicting live birth by combining cleavage and blastocyst-stage time-lapse variables using a hierarchical and a data mining-based statistical model	Trained two models for the prediction of live birth from morphokinetic parameters. The first was a hierarchical model. The second was a logistic regression model using features derived from principal component analysis.	No
		D'Estaing et al. (2021)	[35]	An ML system with reinforcement capacity for predicting the fate of an ART embryo	Predicted blastocyst formation and live birth using a scoring system based on logistic regression over morphokinetic parameters.	No
	Ploidy Prediction	de Gheselle et al. (2022)	[42]	ML for prediction of euploidy in human embryos: In search of the best-performing model and predictive features	Benchmarked several ML models for the prediction of euploidy from clinical and morphokinetic data.	No
Clinical Data	Live Birth Prediction	Amini et al. (2021)	[58]	Factors associated with in vitro fertilization live birth outcome: A comparison of different classification methods	Benchmarked several ML models for the prediction of live birth from demographic, clinical, and treatment parameters.	No

(Continued)

TABLE 19.2 AI Embryo Selection Literature *(Continued)*

Input Modality	Task	Authors (Year)	Reference	Title	Summary	Comparison with Human Experts?
	Pregnancy Prediction	Liu et al. (2021)	[57]	Multifactor prediction of embryo transfer outcomes based on a ML algorithm	Benchmarked several ML models for the prediction of pregnancy following frozen embryo transfer from demographic, clinical and treatment parameters.	No
	Optimal Transfer Policy Prediction	Xi et al. (2021)	[63]	Individualized embryo selection strategy developed by stacking ML model for better in vitro fertilization outcomes: An application study	Combined patient factors with embryologist gradings of D3 embryos to propose a personalized embryo transfer strategy that maximized chances of pregnancy within a given risk of twin pregnancy.	No
Proteomic Profile	Live Birth Prediction	Bori et al. (2021)	[64]	An AI model based on the proteomic profile of euploid embryos and blastocyst morphology: A preliminary study	Trained a multilayer perceptron for the prediction of live birth based on blastocyst morphology and the protein profiles of spent culture media.	No
Chromosome Sequencing	Pregnancy Prediction	Chen et al. (2022)	[41]	Non-invasive embryo selection strategy for clinical IVF to avoid wastage of potentially competent embryos	Predicted blastocyst ploidy from chromosome sequencing of the culture medium using a range of ML models. The best model (a random forest) was validated in a blinded prospective observational study.	No

TABLE 19.3 Comparison of Common Prediction Targets for Embryo Selection AI

Target	For	Against
Blastocyst Formation	• Lots of data available • Objective	• Not a strong predictor of pregnancy or live birth
Blastocyst Grade	• Lots of data available • Correlation with pregnancy and live birth	• Requires embryologist annotation • Subjective, with variation between annotators
Ploidy	• Correlation with early pregnancy loss	• Requires biopsy • Relatively little data available
Implantation (Biochemical Pregnancy)	• Strong correlation with live birth	• Little data available • Multiple embryo transfers can make it difficult to attribute implantation to a single embryo
Fetal Heartbeat Pregnancy	• Strong correlation with live birth	• Little data available • Maternal factors can have a confounding effect • Multiple embryo transfers can make it difficult to attribute the pregnancy to a single embryo
Live Birth	• Corresponds to the ultimate goal of the ART cycle	• Little data available • Maternal and environmental factors can have a confounding effect • Multiple embryo transfers can make it difficult to attribute the birth to a single embryo

Automated static image analysis has also been applied by Wu et al. [62] to classify D3 cleavage-stage embryos. Their classification scale consisted of four categories and captured the equality in blastomere size as well as the severity of fragmentation. The CNN-based system achieved 74.1% accuracy and an area under the curve (AUC) of 0.935. Other recent work has looked at combining insights from static image analysis with other data. For instance, Xi et al. [63] combined patient factors with embryologist gradings of D3 embryos to propose a personalized embryo transfer strategy (single/double embryo transfer along with specific embryos to transfer) that maximized chances of pregnancy within a given risk of twin pregnancy. The system used the XGBoost algorithm and achieved an AUC of 0.72 for the prediction of twin pregnancy. In a preliminary study, Bori et al. [64] used a multilayer perceptron to analyse how protein profiles of spent culture media and blastocyst morphology contribute to live birth. The system was able to predict live birth with 72.7% accuracy.

Not all research in static image analysis, however, has focused on classifying embryo images according to clinical outcomes. Several studies have proposed systems for image segmentation [65–71]. One such system proposed by Rad et al. [67] used a CNN to identify the zona pellucida, trophectoderm, inner cell mass, and blastocoel in images of blastocysts. Once segmented, each structure can be analysed separately in downstream tasks such as taking measurements or assigning quality grades. Other work has focused on pre-processing and enhancing images before they are used as input into models [72, 73].

Time-lapse analysis

The majority of recent studies on AI for embryo selection focus on the analysis of time-lapse videos captured on incubators such as the EmbryoScope, Geri, and MIRI. An advantage of using these incubation systems is that they allow a high degree of standardization in imaging set-ups between clinics. Early systems utilizing time-lapse analysis relied on the manual annotation of morphokinetic events by embryologists. An example of these systems (that is, both commercially available and FDA-approved) is the Known implantation data on day 3 (KIDScoreD3) [74], a decision tree algorithm trained on 3275 embryos with known outcome data that predicted blastocyst formation and blastocyst quality from morphokinetic markers during the cleavage stage.

Since around 2020, the focus has shifted towards the use of fully automated image analysis. A clear advantage of taking such an approach is that it avoids intra- and inter-operator variation in embryologist evaluation and saves time from manual annotation. As with static image analysis, most of these systems are based on CNNs (albeit slightly modified using techniques such as long short-term memory modules to handle the temporal aspect of the data).

Broadly speaking, most research into automated time-lapse analysis tackles the problem from one of two angles. The first involves the creation of algorithms that identify markers such as pronuclei formation and fading [65, 75, 76], cell divisions [65, 76–79], blastocyst formation and development [65, 76–78, 80], and cytoplasm movement [81]. These markers may then be used downstream by either embryologists or another algorithm to make inferences about the embryo. The second involves the creation of algorithms that directly predict clinical outcomes from time-lapses in an end-to-end fashion [34, 43, 44, 51, 53, 54, 82]. This latter group of methods has the advantage that algorithms are free to pick up markers (or interactions between markers) unaccounted for in standard morphokinetic evaluation. As a result, several such systems have been reported to outperform professional embryologists in the evaluation of time-lapses [34, 82].

AI in gamete and embryo selection: Challenges and opportunities

Data availability

A major challenge experienced by many researchers and practitioners building data-driven AI systems for healthcare is a lack of data [83]. This is no less true for the field of ART. Due to data protection, privacy, or commercial considerations, the data landscape in ART is, at the time of this writing, fragmented and siloed [84]. Moreover, because of the great amount of effort required by embryologists to annotate data sets, the labelled data sets that do exist are often quite small or contain missing labels. This poses a problem for the development of modern data-driven AI systems, which require large, diverse data sets in order to accurately capture variation across the whole patient population. As a result, AI systems that build upon data sets from single clinics may only perform well for a certain clinic's patient demographics. Moreover, the current state of the ART data landscape makes it difficult to verify and reproduce study results.

Nonetheless, in recent years, the field of ART has made its first steps towards a more open data landscape. Several publicly available sperm imaging data sets exist [11, 15, 85]. Gomez et al. [86] released an open data set for benchmarking morphokinetic parameter prediction models. Dirvanauskas et al. [87] tackled the data availability problem from a more technical angle, proposing a generative adversarial network model capable of generating artificial images of embryos that could be used as training data. Progress has also been made towards making effective use of unlabelled data. Kragh et al. [53] used self-supervised learning techniques on unlabelled data to augment the performance of a supervised model. Such an approach allowed the model to be trained on a data set that was only 16% labelled.

The fields of AI and ML have also recently seen several innovations, such as federated learning. Federated learning allows AI models to be trained across multiple devices without transferring each device's data set to a centralized server. As such, federated learning may be used to enable multi-clinic collaborations in which a model is securely trained across data sets held at each clinic, without the need for individual clinics to send each other their own data sets [84]. Another such innovation is differentially private learning, a collection of techniques that allows models to be trained so that they satisfy strong mathematical privacy guarantees [88]. The adoption of such techniques may pave the way towards greater data availability in ART.

Technical challenges

The application of AI to gamete and embryo selection also faces technical hurdles. One such hurdle is the problem of noisy labels. As supervised learning remains the prevailing paradigm for building AI systems for ART, the quality of annotated labels is of great importance. Although these labels are typically provided by expert embryologists, the annotation process is nevertheless noisy. For instance, Khosravi et al. [37] found that a panel of five embryologists grading embryos into three categories ("good," "fair," and "poor") using the Gardner system only agreed on the grades of 89 out of 394 embryos. There are also instances in which noise and uncertainty are inherent in labels obtained from biological processes. Consider, for example, the problem of

attributing a single pregnancy after a multiple embryo transfer to a specific transferred embryo.

Recent studies have taken different approaches to dealing with noisy labels. Khosravi et al. [37] used the majority vote of a panel of embryologists as "gold standard" labels for model training. Erlich et al. [82] addressed the problem of ambiguity in implantation data due to the impact of maternal factors by creating pseudo-labels. These pseudo-labels were generated by using a CNN so that embryos with similar developmental patterns were given the same label. This allowed viable embryos to be identified even if they failed to implant due to maternal factors. Moreover, the method is also applicable to training with unlabelled data.

The ability of AI systems to remain robust across different clinics (known as domain adaptation) presents another hurdle. This is especially problematic for deep learning image analysis models that can be thrown off by variations in imaging set-ups (this can range from different lighting conditions to the use of different imaging apparatus). Common methods used to help improve robustness include data normalization, data augmentation (training models using slightly modified copies of images in the original data set), and data collection from multiple clinics (which has its own challenges, as previously discussed). More recent work has made use of techniques such as adversarial learning, in which an AI system is taught to ignore artefacts specific to a particular imaging set-up. Such an approach enabled Kanakasabapathy et al. [72] to adapt a CNN trained on one image modality (e.g. HMC microscopy) to another (e.g. a smartphone-based imaging system).

Reporting, accountability, and ethical challenges

Various frameworks have been proposed to create unified reporting standards for AI systems in healthcare and other high-impact application domains [89–92]. Among the most popular of these are the guidelines by Collins et al. [92] for the transparent reporting of multivariate prediction models for individual prognosis or diagnosis (TRIPOD). The guidelines are targeted at healthcare publications (to date, no ART-specific reporting guidelines exist) and take the form of a checklist of 22 items to report, including details on the participant population, model development, performance evaluation, and study limitations. However, many AI publications in the ART field do not currently adhere to these TRIPOD guidelines and thus fail to report sufficient information to allow for suitable scrutiny as to the validity of their claims.

Prior to incorporation into clinical practice, it is critical to assess how well AI systems generalize in terms of different patient populations, clinical practices, and rare biological events. For instance, models derived from data sets consisting predominantly of Caucasian populations may see a decrease in performance when used on patients of other ethnicities. Such a model would be a cause for ethical concern because it would lead to inequality in the application of research findings and thus varied success rates with different ethnicities. Moreover, a data-driven AI system may learn to reproduce harmful unconscious biases present in the clinical decisions made while collecting the data set. Thus, there is a need for large, diverse, international testing data sets to allow for AI systems to be suitably validated for generalization and harmful biases.

It is important to highlight that, at this stage, there is no evidence that AI can replace fertility practitioners in clinical decision-making. Instead, the goal of the vast majority of AI systems is to support and simplify clinical decision-making. As such, fertility practitioners continue to be fully accountable for the advice received from such tools. This presents a particular challenge when the AI system underlying a decision support tool is a so-called "black box": a system that provides very little visibility or explanation as to how decisions are made. An example of a black box system is a deep neural network that conducts millions of mathematical operations to arrive at a decision. This can lead to concerns about the trustworthiness of AI tools. For instance, when a blastocyst ranking tool proposes that a poor-quality blastocyst be prioritized over a good-quality blastocyst, the embryologist may struggle to follow the AI tool blindly without explanation.

It is therefore imperative that AI tools used in clinical practice provide biologically sound explanations that can be readily understood by clinicians and embryologists. This would help ensure frictionless integration into clinical decision-making processes. For instance, returning to the previous example of a poor-quality blastocyst being prioritized over a good-quality blastocyst, the recommendation of an explainable AI decision support tool might be accompanied by an explanation that despite the first blastocyst's poor morphology, the second blastocyst had certain morphokinetics outside the normal range and a direct cell division from one to three cells. These parameters would have otherwise been missed by the embryologist had they not been using an AI tool to support embryo selection. Therefore, the understanding, transparency, and explainability of AI decision support tools is essential for their incorporation into clinical practice, especially because the fertility practitioner continues to be accountable for decisions.

Nonetheless, the creation of AI systems that are explainable without compromising predictive power remains an open research problem; very few studies have investigated the use of existing explainability techniques in the context of AI for embryo and gamete selection [39, 47, 56]. Among these is a study by Enatsu et al. [47] who have proposed a CNN-based system in which pregnancy predictions from blastocyst images are accompanied by heatmaps over the image that indicate where the model was "looking."

Regulatory bodies, particularly in the United States and Europe, are adapting to the introduction of AI into medical devices, and there are a range of guidelines and standards that are currently being introduced into the regulatory pathway. This will help to resolve the risks and challenges associated with AI, and to ensure the safe introduction of this new technology into clinical practice.

The future of AI for gamete and embryo selection

In recent years, the field of ART has made great strides in AI-supported gamete and embryo selection tools. We anticipate that AI will provide a baseline for further technological advances, shifting the way that embryology is practiced away from technical, hands-on processes and towards intellectual processes. This will necessitate a different skill set for the next generation of embryologists. The use of AI may also dramatically enhance the operational efficiencies in the IVF laboratory, increasing embryologists' capacity for cycles. This will in turn reduce the operational costs of IVF, making it more accessible to the patients who need it.

From a technical standpoint, the landscape of research into AI systems for gamete and embryo selection remains relatively immature when compared to other medical domains, particularly those involving imaging. Recently, the field has seen a trend towards the analysis of videos in lieu of single images,

allowing embryos and gametes to be evaluated over longer time periods and in more detail than currently feasible. Several studies have also begun to analyse or even combine multiple data modalities such as protein profiles and imaging data. It is quite possible that the development of such systems, together with explainability techniques, may prove instrumental in the discovery of new parameters for embryo and gamete selection. Combined with large data sets due to a progressively more open data landscape and technologies such as federated learning, AI may provide insights at a speed and scale that more traditional methods such as randomized control trials (RCTs) have failed to achieve.

Moreover, up until now, studies applying AI to ART have largely been based on supervised learning, which requires large quantities of labelled data. But there are signs of the beginning of a shift away from the paradigm of supervised learning towards the use of semi-supervised, self-supervised, and unsupervised learning techniques. In this way, future studies will likely work with much larger data sets of which only a subset is expertly labelled. The field may also see the use of AI, mathematical modelling, and computer graphics to generate synthetic data and simulations that can reduce the cost of data acquisition and thereby the cost of developing not only AI but also non-AI–based technologies.

The future of AI for gamete and embryo selection is very promising for the field of reproductive medicine.

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20

DEMYSTIFYING VITRIFICATION

Debra A. Gook and Kelly Lewis

Introduction

The theory and results from the application of vitrification in assisted reproductive technology (ART) have been extensively covered elsewhere [1–8]. The aim of this chapter is not to repeat this body of work, but to discuss the technical issues that arise while vitrifying biological material in ART. It will also put these issues into context by providing evidence relating to their role as critical parameters and provide practical information to assist in achieving optimal vitrification outcomes.

By combining more than 30 years' experience in cryopreservation (including teaching and watching many scientists performing vitrification in the laboratory) with theoretical knowledge, this chapter will provide the basis for understanding why some small variations in methodology can have an impact on the outcomes of vitrification. The information provided is applicable to the vitrification of both human oocytes and embryos, although much of the experimental data has been generated using discarded human oocytes (metaphase I [MI] and germinal vesicle [GV] stage oocytes matured overnight to the metaphase II [MII] stage) and aims to assist in eliminating user variation and achieving reproducibly high outcomes with vitrification.

Cryopreservation, regardless of whether using a controlled rate of cooling (often referred to a slow cooling) or vitrification, requires the expulsion of intracellular water prior to cooling and replacing it with a permeable cryoprotectant. The aim of both approaches is to have no free water and a minimal amount of bound water which could form ice during cooling or warming. Most commercial vitrification kits on the market are composed of three solutions: (i) a buffer solution without cryoprotectant; (ii) a solution containing approximately a 3 M concentration of two permeating cryoprotectants, often dimethyl sulfoxide (DMSO) and ethylene glycol (EG), which will be referred throughout as the equilibration solution (ES); and (iii) a solution containing double the concentration of permeating cryoprotectants in the ES with the addition of a non-permeating molecule, generally a sugar such as sucrose or trehalose, which will be referred to as the vitrification solution (VS) throughout. To reduce individual cryoprotectant toxicity at high concentrations, a combination of two cryoprotectants has the benefit of achieving the required dehydration while reducing toxicity [9]. Regardless of the developmental stage, i.e. oocytes, cleavage stage embryos, or blastocysts, most kits are recommended for universal application and only vary in the recommended time spent in the solutions in each case. This relates to the size of the cell and therefore the water content within that cell.

Oocyte vitrification

The impetus for many clinics to attempt to vitrify human oocytes came from the high survival rates achieved with donor oocytes reported by Cobo 2010 using the Kitazato kit [10]. The method originally reported by Cobo 2008 [11] is described in Figures 20.1 and 20.2. This will be referred to as the standard procedure when comparing parameters throughout this chapter. However, when this approach was applied to oocytes from infertile women, survival rates were more variable [12–14]. As with much of ART, the likely explanation was thought to be female age, but comparison of survival rates relative to female age showed no significant effect other than a trend towards slightly lower survival with advanced age [15]. Survival rates for oocytes from young (<38-year-old) and older (>38-year-old) infertile women were also not significantly different in our vitrification system [16]. In contrast, others have shown age to a be significant factor [17]. Oocyte quality is also more variable in infertile women, potentially impacting on survival, but this was also reported to not be responsible for reduced survival [18].

Dehydration

What is variable across a cohort of oocytes is the rate at which water moves, referred to as the hydraulic permeability coefficient [19], which is temperature-dependent. This can vary within a cohort by as much as eightfold between individual oocytes from the same stimulation cycle [20], implying that there will be variability in the level of dehydration achieved in the ES when applied for a set time. The time at which an oocyte re-expands in the ES solution to 80% of its initial volume is an indication of the hydraulic permeability coefficient for that oocyte. We have measured this for a large number of oocytes (Table 20.1) and confirmed this observation of variability between individual oocytes from a single cohort/patient cycle. However, no relationship was observed between the re-expansion time and maturation stage or re-expansion time and patient age. In the standard method (Figure 20.1) oocytes were moved out of the ES when re-expansion was achieved, thereby allowing for this variation, but commercial methods recommend a set time in the ES solution. In our experiments, although survival rates for those transferred to VS once re-expanded in ES compared to those following a set time of 10 minutes in ES were not significantly different (re-expansion 92.4% [73/79]; set 10 minutes 84.0% [79/94]), there is a suggestion that fewer oocytes have survived with 10 minutes in ES, and that a proportion may require slightly longer to remove all of the water.

In the case of blastocysts, although inner cell mass (ICM) cells are more similar in size and therefore water content, there will be a concentration gradient of water and cryoprotectant across the ICM. This implies that the position of individual ICM cells relative to the zona pellucida and cavity will dictate the time required for each cell to dehydrate, indicating again, that a set time in ES may not be appropriate for all cells within blastocysts and that any recommended time in ES at a particular temperature is likely to be a compromise. With respect to the blastocyst, this will be explored further in the "to collapse or not" section later in the chapter.

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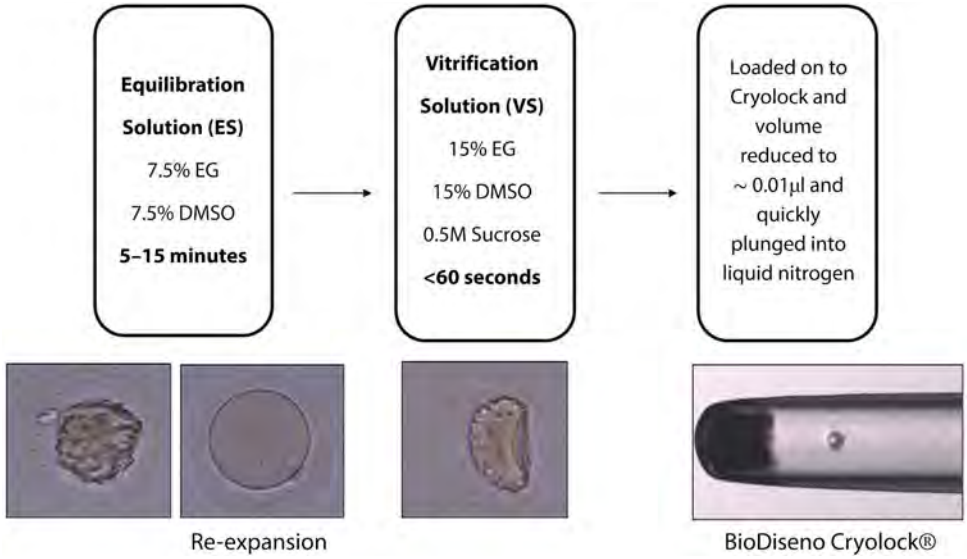

FIGURE 20.1 Oocyte vitrification method. All procedures performed at room temperature. (Kuwayama M, Vajta G, Kato O, Leibo SP, Highly efficient vitrification method for cryopreservation of human oocytes. Reprod Biomed Online. 2005 Sep;11(3):300–8. doi: 10.1016/s1472-6483(10)60837-1; modified by [11]).

It is also worth noting that increased variation in methodology for the ES exposure, such as the drop merging system or floating on the surface of the ES, will increase variability in the extent of dehydration.

In the VS the concentration gradient results in further dehydration. How quickly this is achieved is dependent on the amount of intracellular water remaining after the ES exposure. The technical ability of the embryologist is also an important factor and in some cases concern regarding the high concentration of cryoprotectant in VS has prompted a tendency to only expose oocytes to VS for as short a time as possible. Again, for a large cell such as the oocyte this is counterintuitive, since the oocyte will still contain water after exposure to the ES and will, therefore, require additional time in the VS solution. Analysis of the impact of duration in the VS has shown that a longer time of 90 seconds in VS results in similar survival to the standard method (Figure 20.1) (100% [20/20]). In contrast, a longer time of 80 seconds in the VS followed by holding the cryolock in air for 10 seconds before plunging has a significantly negative impact on survival (71% [15/21]). This scenario is designed to mimic the situation when too much solution is pipetted onto the cryolock and there is a delay before plunging while removing the excess VS surrounding the oocyte. Knowledge that this has an impact prompts the next question, as to whether the smallest volume is required on the cryolock to achieve the cooling rate required to successfully vitrify oocytes.

Cooling rate

The volume of VS surrounding the oocyte or blastocyst on the cryolock has been reported to be the most critical parameter

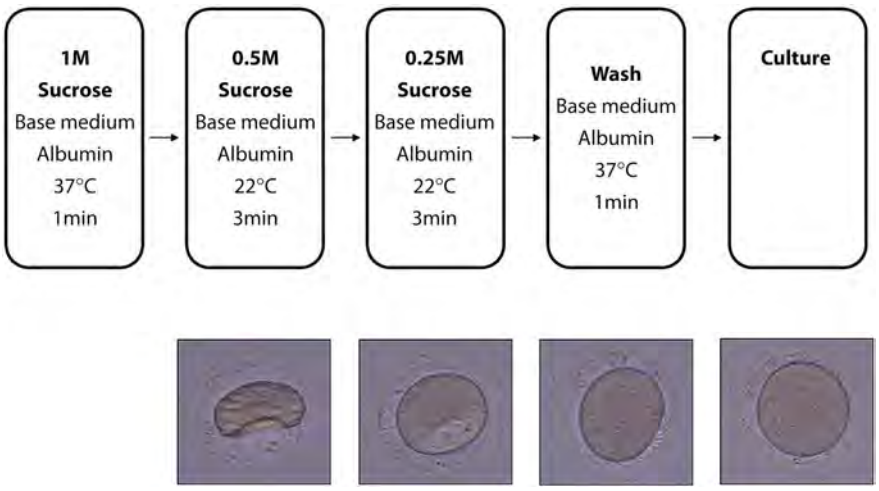

FIGURE 20.2 Oocyte warming.

TABLE 20.1 Re-expansion Time for Oocytes

Oocyte	Mean (Minutes)	Range (Minutes)
GV → MII	6.45	5.0–10.15
MI → MII	7.1	5.2–9.25
MII (<37 year) (n = 2293)	6.7	4.0–12.0
MII (≥37 year) (n = 1317)	6.5	3.5–11.0

in achieving the ultra-high cooling rate required for successful vitrification [21]. The risk of excess solution increases when vitrifying multiple oocytes on the one tool; therefore, to minimize this the excess solution is usually aspirated from around the oocyte before plunging. To assess the effect of a lower cooling rate, an excess of 0.5 µL of VS around the oocyte was tested and no impact on survival (95.2% [20/21]) was observed. This indicates that a slower cooling rate, at least as associated with this amount of excess volume, was not the critical parameter it was previously thought to be, and that exposure to air when removing excess VS had a greater impact on survival.

In fact, a slower cooling rate of –1220°C/minute achieved with a closed vitrification system (Rapid-I), which vitrifies using super-cooled air, can also achieve high survival rates with oocytes [16] that are not significantly different to those achieved with the standard open system (Figure 20.1). The oocyte is loaded in a hole on the Rapid-I that holds 30 nl of VS. This tiny volume of fluid facilitates in achieving the cooling rate reported with super-cooled air. Outside the hole, the plastic is reasonably thick and placing an oocyte on this area in a relatively large volume of 0.5 µL of VS had an impact on survival (85.7% [30/35]), suggesting that a slower cooling rate than –1220°C/minute is less favourable for oocyte vitrification.

Warming rate

Seki and Mazur [22] clearly showed with mouse oocytes that an extremely rapid warming rate—not the cooling rate—is the critical parameter. They showed, regardless of the cryoprotectant concentration and slower cooling rates (100 and 1000°C/min), that high survival can be achieved when the speed of warming is 117,500°C/minute (Figure 20.3). To achieve the extremely rapid warming rate, the tool is plunged directly into the first warm solution at 37°C and this is fundamental to achieving high survival rates. Therefore, the first warming solution should be warmed to 37°C for sufficient time to establish even temperature throughout the solution and verified in-house with dishes used for warming.

There is a risk of transient warming at any time after vitrification when tools have not been maintained under liquid nitrogen. Due to the low thermal mass of the vitrified tools, and the process required to load and unload transport tanks, there is a high-risk of temperature fluctuations during the transport process, which has been shown to impact on survival [23]. Comparison of exposure to a mock process involved with transporting and receiving vitrified oocytes (Figure 20.3), to vitrified oocytes not exposed to the transport system (i.e. remaining in storage tank until warming), showed similar initial survival rates, but with time in culture (24 hours) the mock transport oocytes' survival deteriorated and an increase in spindle abnormalities was observed within these oocytes. In contrast, survival was similar for those kept in storage

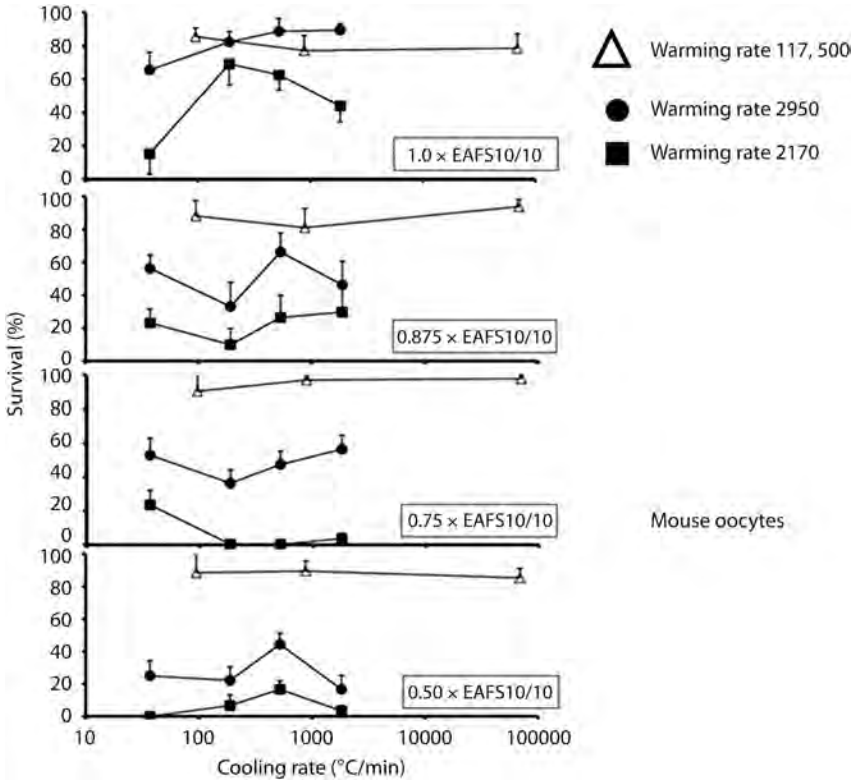

FIGURE 20.3 The rate of warming may be more critical to successful vitrification than cooling.

and the mock transport when liquid nitrogen was decanted into the transport tank before removing the goblets.

To assess the impact of temperature, goblets containing vitrified oocytes were exposed to different temperatures in a transport tank. The temperature within a transport tank will start to increase over time (Figure 20.4), providing a sub-zero temperature range that the goblet contents can be exposed to but remain cryopreserved, after which the goblet is quickly returned to liquid nitrogen for warming at a later time. Using this system, the survival of oocytes exposed to a range of sub-zero temperatures can be compared (Figure 20.5). A significant ($p < 0.001$) reduction in survival was observed with a brief exposure to −63.8°C and −53.8°C suggesting that, at these temperatures, there has been a transient devitrification and, on returning to storage under liquid nitrogen, a revitrification. Whether this damage is a consequence of devitrification or revitrification is unknown.

Rehydration

There are two general methods employed to rehydrate vitrified ART cells, i.e. by (i) exposure to a high concentration of non-permeating cryoprotectant only and (ii) exposure to reduced

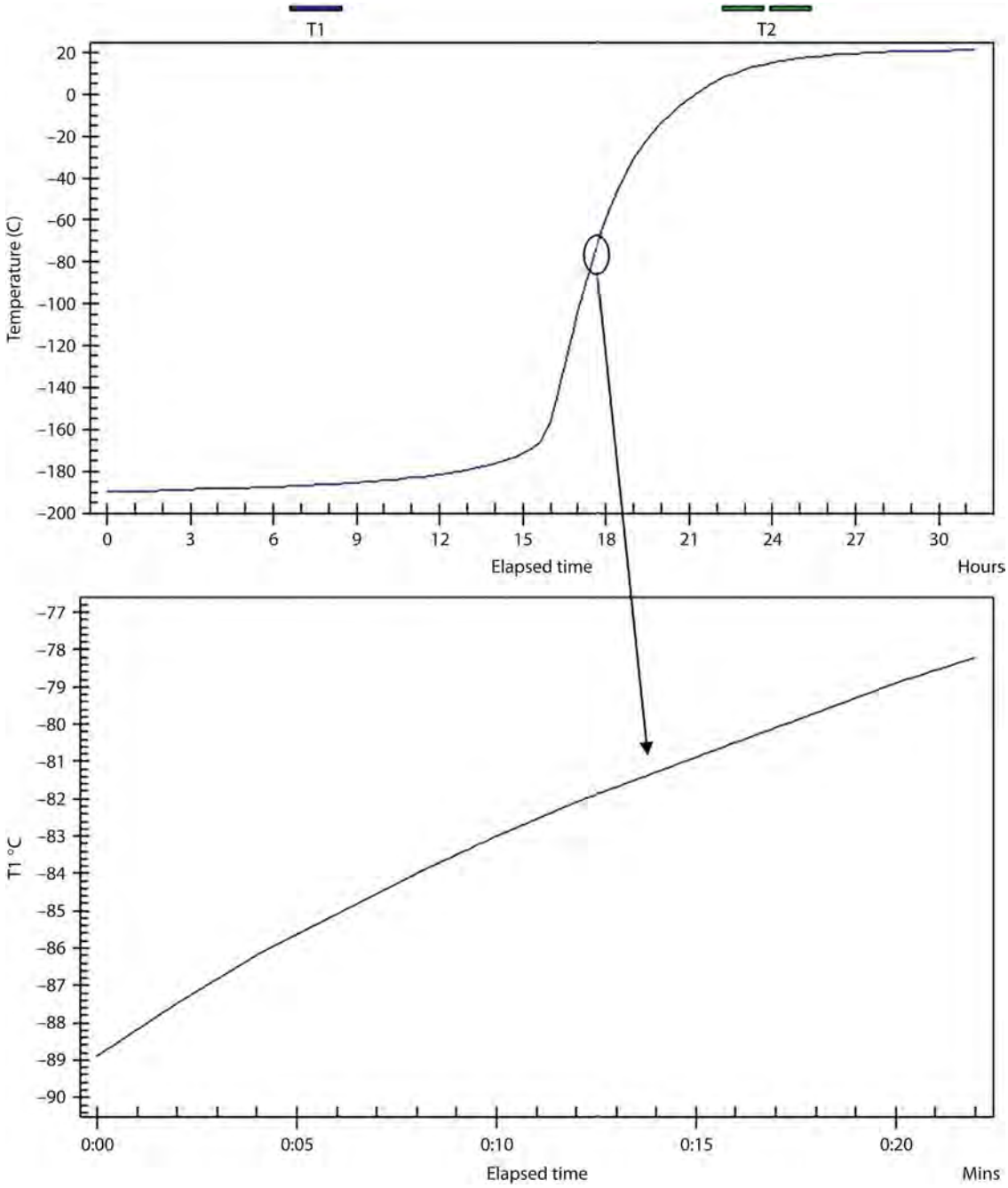

FIGURE 20.4 Temperature during dry shipper during warmer phase. Temperature range −88.9°C to −78.2°C; time 22 minutes; mean temperature −83.1°C.

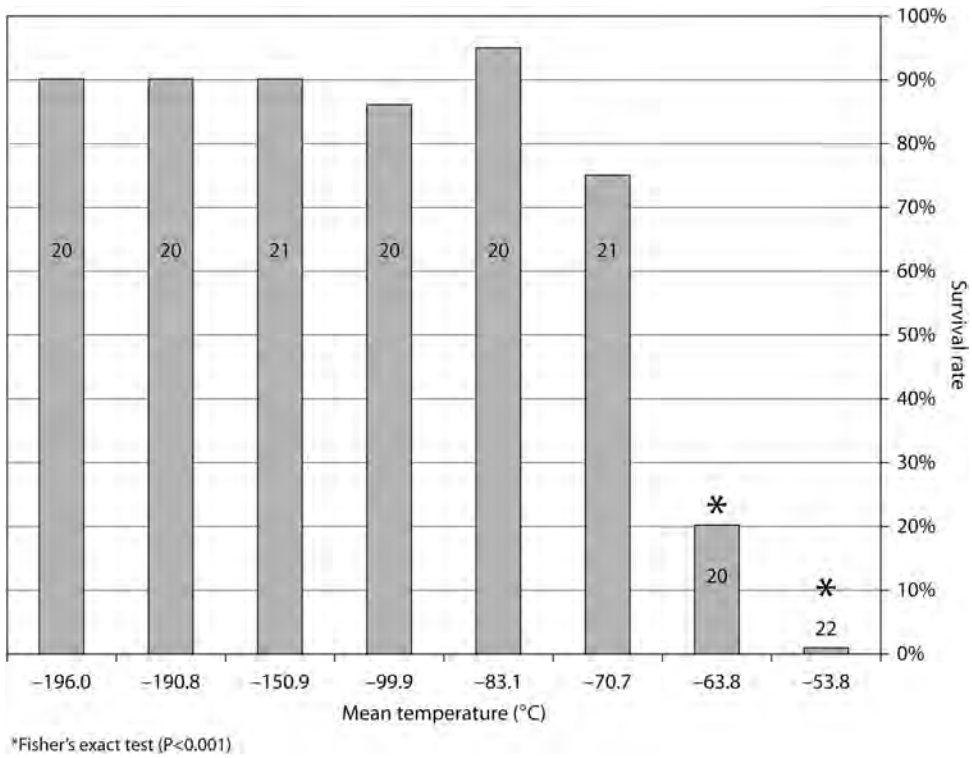

FIGURE 20.5 Oocyte survival rates after temperature exposure.

concentrations of permeating cryoprotectants with a higher concentration of non-permeating cryoprotectant, both followed by transfer to basal medium. The first rehydration method, used by Cobo 2008 (Figure 20.2), is the principle used in most commercial warming kits and prescribes 60 seconds in the high non-permeating (generally 1M sucrose) solution. How critical the time is in this solution was examined and a reduction in survival was observed when oocytes were exposed for only 30 seconds (64.0%) and also for a longer time (90 seconds; 48.8%). Both were significantly lower than when exposure was for 60 seconds ($p < 0.01$ for both compared to 60 seconds; 92.4%).

In the Cobo 2008 procedure, and most commercial kits, the subsequent solution is half the concentration of non-permeating cryoprotectant (generally sucrose) but for this solution the exposure is now at room temperature. It is difficult to understand the rationale for this change in temperature, apart from the fact that the hydraulic permeability coefficient is temperature-dependent and, therefore, the rate of permeating cryoprotectant moving out of the oocyte at room temperature is slower. However, at this high non-permeating concentration (0.5 M) there is probably very little water and permeating cryoprotectant movement. Changing the temperature of this solution to 37°C had no impact on survival when exposure time was reduced to 1 minute in our experimental procedure (100% [12/12]) and with clinical vitrified oocytes donated to research (100% [30/30]). This approach has the added benefit that all solutions in the warming process are at 37°C physiological temperature. This has been confirmed for oocytes showing high survival, fertilization, and implantation rates when all warming steps are at 37°C [24].

A frequently asked question is whether a universal warming protocol is appropriate to warm oocytes and blastocysts vitrified with a variety of kits (Table 20.2). A crossover study of

TABLE 20.2 Broad Composition of Vitrification Kits

Vitrification Kit	EG	DMSO	PROH	Sucrose	Trehalose	Other
Kitazato[a]	X	X			X	Hydroxypropyl cellulose
SAGE	X	X		X		
Irvine	X	X		X		
COOK	X	X			X	
Medicult	X		X	X		
VITROLIFE Cleave and OMNI	X		X	X		Ficoll Hyaluronan

Note:

[a] Initial composition of the Kitazato kit was sucrose and synthetic serum substitute; since 2016 these have been replaced with trehalose and hydroxypropyl cellulose.

Kitazato and SAGE blastocyst vitrification kits and in-house warming solutions of 1.0 M sucrose, 0.5 M sucrose, showed no difference in blastocyst survival and implantation rates, which were similar to fresh [25]. Both vitrification kits consist of the same permeating cryoprotectants, and their concentrations are the same, but vary in the base buffer and the non-permeating cryoprotectant (see Table 20.2). Considering that the dilution from medium on the vitrification tool is minuscule, and that the non-permeating cryoprotectants are only outside the blastocyst, it is not surprising that the outcomes are the same. This has been repeated in another clinical study [26] with similar composition kits and no difference was observed. However, there are major differences in composition with other kits; the COOK kit has a different non-permeating cryoprotectant (trehalose 0.68 M) and slightly higher concentrations of permeating cryoprotectants (both EG and DMSO at 16%), and the Vitrolife vitrification kits (blastocyst, cleavage, and OMNI) have propanediol and EG (both 16%) as the permeating cryoprotectants and 0.65 M sucrose, Ficoll, and hyaluronan, which all act as non-permeating cryoprotectants (Table 20.2). A comparison of mouse blastocysts vitrified with Sage (same composition as Figure 20.1), COOK, or Vitrolife kits and warmed with the same kit warming solution or the universal warming kit (sucrose) showed no difference in survival in the paired comparison for both Cook and Vitrolife initially and 10 hours post warming (unpublished data Moazzam 2021). However, in contrast to the Parmegiani 2018 study, SAGE vitrification with SAGE warming was significantly better than with the universal warming ($p < 0.05$). This is surprising considering both have 1.0 M sucrose followed by 0.5 M sucrose as their warm solutions with the same timing and temperature (Table 20.3). Time to full re-expansion was similar across all groups (three to four hours) with the exception of those vitrified with the Vitrolife kit and warmed with the universal warm solutions, which took significantly ($p < 0.05$) longer to re-expand (7.5 hours). A similar study (unpublished data Aarshiya 2018) with mouse oocytes vitrified with SAGE, Vitrolife (OMNI) kits and an in-house trehalose vitrification kit, all with matched warming solutions or the universal solution showed no difference. There was a suggestion of reduced survival with the Vitrolife vitrification solutions regardless of warming solutions but numbers were insufficient to show a statistical difference. Subsequent fertilization and blastocyst development were similar across all groups. This concept requires further assessment but may indicate the potential of a universal warming method.

To collapse or not

In the early days of vitrification, there were a number of publications describing various methods for collapsing the blastocoel cavity prior to dehydration [27–33] and reports on the value of artificial shrinkage have continued to appear in the literature [34–37]. Regardless of how the cavity size was reduced—needle, laser, or micropipette [29]—post-vitrification survival was improved following the artificial collapsing of the cavity compared to no intervention [27, 28]. In many groups, breeching the zona and trophectoderm continues to be performed and is reported to be superior to vitrifying expanded blastocysts [38]. The process of reducing the fluid cavity artificially will reduce the water and permit faster dehydration of the cells on the inner side of the ICM. However, the cavity also contains other components [39] that will also leak out due to the rupture and these will not be replaced during warming. This approach may be adopted to increase the rate of dehydration or to promote subsequent implantation (i.e. assisted hatching), in the latter case based on an unsubstantiated belief that zona hardening occurs as a result of vitrification [40]. Irrespective of the aim, some of the contents of the cavity will be lost. As stated earlier in the dehydration section, the aim is to remove all free water prior to vitrification and, therefore, it is vital to reduce the size of the cavity so that cells will not be ruptured by ice during vitrification and/or warming. Many cavities will collapse by themselves in the ES solution without assistance, but those cannot be predicted on the basis of blastocyst morphology. Therefore, collapsing the cavity by the aforementioned methods or pipetting [41, 42] during the time in ES will facilitate movement of water and dehydration prior to moving to the VS solution in which dehydration is completed. Similarly, vitrifying biopsied blastocysts shortly after biopsy while collapsing has been shown to be beneficial [38].

Conclusion

As we increase our knowledge of the critical factors affecting vitrification, our ability to achieve high survival levels should also increase. It then becomes a matter of the quality of training of staff in what is required to achieve high survival. This should be complemented by promoting and developing an understanding of the principles underlying the kit composition, the method, and the critical parameters. Regular refresher courses will also be important to maintain KPIs and ensure that individual drift in methodology has not occurred.

TABLE 20.3 Broad Composition of Warming Kits

Warming Kit	EG	DMSO	PROH	Sucrose[a]	Trehalose	Other
Kitazato[b]					X	Hydroxypropyl cellulose
SAGE				X		
Medicult				X		
COOK					X	
VITROLIFE Blast, Cleave and OMNI				X		Hyaluronan

Notes:

[a] Sucrose concentration is 1.0 M except for VITROLIFE Blastocyst and Cleave Kits (<1.0 M).

[b] Initial composition of the Kitazato kit was sucrose and synthetic serum substitute; since 2016 these have been replaced with trehalose and hydroxypropyl cellulose.

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21

VITRIFICATION OF THE HUMAN OOCYTE

Masashige Kuwayama

Introduction

The past 50 years have yielded impressive breakthroughs in cryopreservation as applied to the discipline of reproductive biology. Techniques were usually derived in experimental and domestic animals, and subsequently applied to humans. The first success in freezing cells was achieved in spermatozoa [1], followed by successful cryopreservation of pre-implantation embryos at different stages of development [2–4]. Since the first report in 1972 of cryopreservation of mammalian embryos resulting in the birth of live mice offspring [2], attempts to cryopreserve human oocytes, similar to the results with oocytes of domestic animals, mostly failed for many years. However, the development of an ultra-rapid vitrification method now means that oocytes can be cryopreserved without significant loss of their viability, and such oocytes may be used clinically [5].

The reasons to cryopreserve human oocytes are widely known and were summarized recently [6, 7]. Common indications for this procedure include diseases and their treatments, i.e. to preserve the reproductive competence of unmarried young women with cancer who need irradiation of the pelvic region or chemotherapy, or who require surgical intervention before or during their reproductive age that may involve removal of ovaries. Another reason for cryopreservation is when patients have problems resulting from ovarian malfunction, including premature menopause, ovarian hyperstimulation syndrome, or poor response to ovarian stimulation. There are also legal, ethical, social, and practical problems that may also require oocyte cryopreservation: some countries restrict or prohibit embryo cryopreservation which only leaves the option to preserve oocytes; women may wish to delay motherhood for various reasons, such as career priorities; and there may cases where there is no semen available after a successful oocyte retrieval, to mention a few examples.

However, as discussed in detail recently [6–9], in broader terms, oocyte cryopreservation is also needed to compensate for the unique situation of women in regards to reproduction. As in most mammalian species, women suffer more and sacrifice more for their offspring both physically and emotionally. Yet, a woman's reproductive capability is restricted in terms of quantity and duration. Males produce hundreds of millions of sperm in a single ejaculation, while females ovulate normally only one oocytes every 28 days. From the time that he reaches puberty, a man's reproductive capability is almost unlimited, while that of a woman (without considering special treatments) is limited to a period of just 15 to 20 years. Assisted reproductive techniques did not eliminate this difference. In fact, with the introduction of the procedure of intracytoplasmic sperm injection (ICSI) and successful cryopreservation of sperm, the gap has widened considerably. Apart from the practical goals, our moral duty is to help develop an efficient and safe oocyte cryopreservation method to enhance the reproductive capability of women.

Unfortunately, the task is rather demanding. Although the first pregnancy from a cryopreserved oocyte was reported about 40 years ago [10], advances until recently were very slow. Generally, inefficiency and lack of consistency were the two main problems [11]. Oocytes are unique cells; their large size, spherical shape, low membrane permeability, and general fragility explain many of the difficulties that occur during cryopreservation.

Oocytes are often described as the largest cells of the mammalian body, and this represents a real challenge in cryopreservation. Cell volume is known to be a crucial parameter that determines the likelihood of success when a cell is cryopreserved. Viruses and bacteria, which have a very tiny volume, may survive deep freezing without any special treatment, such as use of cryoprotectants or controlled rate cooling. Freezing of fibroblasts or epidermal cells is usually an easy and efficient routine task in tissue culture laboratories, and does not need any special instruments. Sperm cryopreservation can be efficiently performed with the use of a controlled-rate freezer. Early cleavage-stage embryos with individual blastomeres having 50% to as little as 10% of the original size of oocytes survive traditional slow-rate freezing very well, and their developmental competence is usually well preserved. Preantral and primary follicles can also be frozen successfully, in contrast to the large, fully developed, metaphase II-stage (MII) oocytes.

The near-spherical shape of the oocyte does not confer an advantage from the point of view of cryopreservation. During equilibration and dilution before and after cooling and warming, permeable cryo-protectants must be distributed rapidly and uniformly throughout the ooplasm. A large spherical object, such as an oocyte, has the lowest surface area/volume ratio of any geometric shape. An irregular object, such as a fibroblast or lymphocyte, has a much larger surface area/volume ratio and will equilibrate osmotically much faster than an oocyte.

The one cell-stage of an oocyte also severely limits options, as there is no margin for error. The single cell survives *or* it does not. Multicellular embryos may survive and develop even if more than 50% of their cells are damaged. This fact is clearly demonstrated by successful births resulting from bisected embryos of domestic animals.

However, apart from the size, shape, and cell number, other factors may also play an important role in limiting successful oocyte cryopreservation. Germinal vesicle-stage (GV) oocytes and fertilized zygotes have almost exactly the same characteristics. However, zygotes are considerably more resistant to cryoinjuries while GV-stage oocytes are even more sensitive than MII-stage oocytes. Factors that are known to influence their sensitivity include chilling-injury, serious deformation of shape during exposure to and/or removal of cryo-protectants, and hardening of the zona pellucida.

Chilling-injury is probably one of the least understood types of injuries during cryopreservation, involving damage of lipid droplets, lipid-rich membranes, and microtubules. The temperature zone at which such injury occurs is rather high, between +15° (in some biological objects +20°C) and −5°C [12]. The damage to lipids is irreversible and causes death of the oocytes. Compared

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to other species, the lipid content of human oocytes is relatively low. Yet, their sensitivity to chilling is still considerable, caused probably by membrane damage and depolymerization of microtubules, with all of the subsequent consequences, including misalignment of chromosomes and aneuploidy [13–17]; however, the latter effect may be less detrimental than earlier supposed [17]. Chilling damage of membranes in human mature oocytes seems to be much more serious than at later developmental stages, e.g. zygotes, a possible cause for the well-known stage-dependent sensitivity [18].

As a result of osmotic effects, serious deformation of the shape may occur when oocytes are exposed to cryo-protectant solutions. However, in spite of the somewhat peculiar morphology that oocytes may exhibit during exposure to cryo-protectants, they do seem to tolerate these deformations rather well. Careful addition of cryo-protectants may minimize deleterious effects of such morphological alterations. An alternative strategy, such as addition of cytoskeleton relaxants used with porcine embryos [19], may not be required in humans. On the other hand, during removal of the cryo-protectant, the spherical shape of the oocyte may allow only a minimal expansion; accordingly, the in-rushing water may disrupt the cell membrane.

Vitrification versus slow freezing

During the past five decades, two major strategies for cryopreservation of oocytes and embryos in mammalian species have been developed [20]. Traditional slow-rate freezing establishes a delicate balance between various sources of injuries, while the principal goal of vitrification is to eliminate ice crystal formation entirely in the whole solution containing the embryos and oocytes. To achieve this ice-free glass-like solidification of solutions, which may also be defined as an extremely increased viscosity, high cryo-protectant concentrations and/or very high cooling rates are required. To decrease the potential osmotic and toxic damage caused by cryo-protectants, recent vitrification methods have focused on increasing the cooling and warming rates [21–24]. Most successful vitrification methods are based on use of extremely small volumes of solution containing the specimens and direct contact between this solution and liquid nitrogen.

One of these approaches, the minimum drop size (MSD) method, was first applied by Arav [25], and further modified by Hamawaki et al. [26]. Based on these earlier results, a novel method, called the ultra-rapid vitrification, was developed for cryopreservation of oocytes and embryos [27]. Ultra-rapid vitrification has been used successfully to cryopreserve oocytes and embryos from a wide variety of mammalian species, and has resulted in a considerable increase in the overall efficiency of cryopreservation of human oocytes and embryos [28–31]. And more recently, based on these huge results and experiences using the ultra-rapid vitrification method for more than two million clinical cases for two decades all over the world, a non-invasive survival vitrification method was established for the standard clinical protocol for human oocytes and embryos (the Cryotec method [9, 32]).

The danger of liquid nitrogen mediated disease transmission

Safety issues regarding open methods of vitrification have been discussed recently in detail [5, 20, 33]. Liquid nitrogen may become contaminated with pathogenic agents and can transmit these agents to other samples stored in the same tank of liquid nitrogen. Under experimental conditions, transmission has also been demonstrated between embryological samples [34, 35]. Although no disease transmission related to liquid nitrogen-mediated contamination and embryo transfer has been reported for humans or for animals during the past 40 years, a theoretical danger exists and should be minimized with rational measures. According to most observations, hermetical isolation of samples from liquid nitrogen or medium during cooling and thawing considerably decreases cooling and warming rates and, as a consequence, also reduces survival of oocytes. One reasonable solution to this problem is to separate cooling and thawing of oocytes from their storage. Cooling can be performed in liquid nitrogen that is directly provided from the factory, hasn't been in contact with any other biological samples, and has been filtered before use [36, 37]. For storage, samples may be sealed into a pre-cooled, hermetically isolated container, for example 1-mL-diameter cryo-bio-straw (CBS) (IMV, L'Aigle, France). An analogue of the system has been used for OPS vitrification [35] and the required instrument is commercially available (VitSet, Minitüb, Landshut, Germany; [33]). At warming, the end of the 1-mL straw may be cut with sterile scissors while the rest of the straw is still submerged in liquid nitrogen, and the Cryotec can be quickly removed with narrow forceps for immersion into the proper medium. However, high post-warm survival rates of oocytes have not been obtained in these partially closed or fully closed systems, possibly because of the lower cooling and warming rate than those in ultra-rapid vitrification. The fact is that no viral transmission problems have occurred after more than four million cases of clinical applications of the Cryotop and Cryotec method for 20 years in 76 countries. This provides the best practical evidence to indicate the safety of this method with respect to possible liquid nitrogen-mediated disease transmission.

Recent outcomes using ultra-rapid vitrification protocols

The first baby born after human oocyte vitrification was reported by Kuleshova using the OPS method [22, 38] in 1999. However, the survival rate was not high and no replicate results have been reported. This is similar to the first success of human oocyte slow freezing in 1986 [10]. The volume of vitrification solution (VS) is much larger in this method and resulted in a lower cooling/warming rate. Nevertheless, this technique does work well for mammalian embryos, even if less efficiently for oocytes.

In the same year, Kuwayama also obtained the first success of human oocytes vitrification using the ultra-rapid method with an acceptable high survival rate [27]. The protocol of this vitrification has been improved to be simpler and more efficient for everyone's use. The protocol has gradually become used around the world, being adapted for various clinical needs in each country [7, 28, 29].

In Japan, using this method, 91% post-thaw survival rate, 90% fertilization, and 50% blastocysts formation rate after ICSI and *in vitro* culture were first reported [29]. After embryo transfer, a pregnancy rate of 41% was achieved. The ultimate birth rate of those embryos that implanted was 83%. A total of 20 healthy babies were delivered in the clinical trial. This ultra-rapid vitrification method was used to establish the first oocyte bank for unmarried cancer patients in 2001. More than 600 oocytes from 112 patients have been cryopreserved for their future IVF treatment use, and the first delivery was obtained in malignant patients in 2013. Two oocytes of a 16-year-old unmarried malignant student

were vitrified in 2001. They were warmed and ICSI-ET was performed in 2013, and a healthy baby was born in 2014.

In the United States, Katayama et al. [28] repeatedly used ultra-rapid vitrification and achieved a post-warm survival rate for oocytes of 97%, and they obtained the first live baby from a vitrified oocyte in the United States in 2003. They also established the first oocyte bank for unmarried cancer patients and also for healthy women for social reasons in USA in 2002.

In Spain, Cobo et al. [37] reported that the survival of 231 oocytes that were warmed after vitrification was 97%; the respective fertilization, cleavage, and blastocyst rates were 76%, 94%, and 49%. Embryo transfer performed on 23 patients resulted in a 65% pregnancy rate, although with a miscarriage rate of 20%. This Spanish team has used oocyte vitrification for an egg donation program [39]. More than 1000 healthy babies have been born from oocytes that were vitrified by this team alone.

In these two decades, based on huge clinical experiences of ultra-rapid vitrification all over the world, difficulties in the protocol for the embryologists and reasons for lethal damage of the oocytes during the vitrification process have become clear.

And to minimize oocyte loss due to human error for embryologists, improved instrumentation for easier handling has been developed in vitrification and warming-plates. This is because it is a more universal and reliable clinical technique for patients. Regarding VS, there was still lower viscosity of the solutions, difficulty judging completion of the oocyte in VS equilibration, difficulty loading oocytes on vitrification container sheets within a limited time, and stickiness of oocytes to the sheet at warming in thawing solution (TS). But these problems in the method have been solved by the improvement of composition of the solution by the addition of thickening agents, like hydroxy propyl cellulose and xanthan gum [7].

Details of the latest protocol for ultra-rapid vitrification follow.

Latest ultra-rapid vitrification protocol to cryopreserve human oocytes

Timing of vitrification, and ICSI after warming

Oocytes can be vitrified between one and six hours after ovum pick up, and immediately after denudation (cumulus cell removal). ICSI can be performed within two to four hours in culture after the vitrified oocytes have been warmed. This short time of culture is required to allow the oocytes to be recognized as survival and also recover the plasticity of their membranes during the puncture by the ICSI needle.

Vitrification media and container

The media is composed of minimal essential media (MEM), ethylene glycol, and dimethyl sulfoxide as permeable cryo-protectants and Trehalose as non-permeable cryo-protectant. All solutions are serum and protein free.

To obtain the best ultra-rapid cooling and warming rates of the VS containing oocytes, the vitrification container, Cryotec, consists of a 1.0-mm wide, 1.4-mm long, 0.1-mm thick flexible filmstrip attached to a rigid plastic handle. To protect the filmstrip and the vitrified oocytes on it, a 65-mm long transparent plastic cap is also provided to cover this part during storage in liquid nitrogen (Figure 21.1). The device is sterilized, and should be handled under aseptic conditions and only for one cycle of vitrification.

Working environment and preparation steps

The vitrification procedure has to be performed in a well-ventilated laboratory at room temperature of 25°C to 27°C. Because all equilibration and dilution parameters described here were adjusted for this temperature, it is very important to warm media that have been stored in the refrigerator to 25°C to 27°C. This is easily achieved by placing all the solutions and vials on a clean bench for more than one hour, preferably inside a laminar-flow hood. The only exception is the TS, which should be warmed to 37°C to obtain the highest warming rate of the vitrified oocytes. Note that the basic solution contains Hepes buffer along with bicarbonate buffer, and has been adjusted to maintain the appropriate pH even when exposed to air. Therefore, a carbon dioxide incubator is not required for warming of solutions in closed vials.

Additional tools

Vitrification has to be performed in 300-µL volume three-well plate (Vitri-Plate, REPROLIFE, Japan; Figure 21.2). To obtain the optimum gradual change of osmolality of the extracellular solutions for the best post-warm survival of the oocytes, it is very important that precise proportions of the volume of each solution and transferred solution be used. For practical reasons, a relatively small, thick-walled Styrofoam box (approximately 250 × 150 × 200 cm for length, width, and height) with a minimum of 3-cm thick walls and bottom is suggested, preferably with an appropriate Styrofoam cover. The box should be placed on a stable surface within easy reach but with little risk of accidentally spilling it or pouring off the liquid nitrogen. All safety instructions related to work with liquid nitrogen should be strictly followed. Points for selection of optimal sources and possible pre-treatment of liquid nitrogen will be discussed later. The Styrofoam box should also contain plastic racks for temporary storage of the device.

Ultra-rapid vitrification requires adept handling of oocytes and embryos. For vitrification and warming, a relatively simple stereomicroscope equipped with a zoom lens and capable of providing sharp, contrast images is appropriate. Except for special purposes, there is no need for an upright or inverted compound microscope or for fluorescent equipment. There is no need to restrict illumination if light sources are filtered for UV lights. Use microscope lights only when required.

Equilibration and cooling

Gently mix vials of pre-warmed equilibration solution (ES) and VS (one vial of each). Pour 300 µL of ES into well 1, and 300 µL of VS into wells 2 and 3 in the proper Vitri-Plate (Figure 21.2).

FIGURE 21.1 Cryotec vitrification container, with and without cover cap.

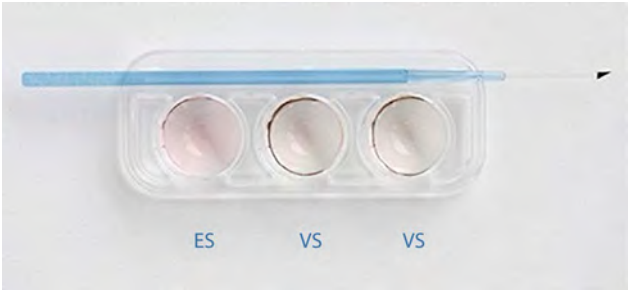

FIGURE 21.2 Vitrification solutions in vitrification plate. *Abbreviations*: ES, equilibration solution; VS, vitrification solution.

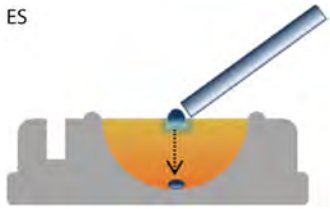

FIGURE 21.3 Equilibration of oocytes in ES. *Abbreviation*: ES, equilibration solution.

Before starting a vitrification procedure, check the quality and perivitelline space of the oocytes, compare it to the thickness of the zona pellucida, and record any characteristics that might affect oocyte survival. The equilibration and vitrification procedure consist of the following steps.

1. Place the oocytes in the centre of the surface of ES. The oocytes will begin to contract osmotically and they will sink by their own density to the bottom of the well (Figure 21.3).
2. Contraction of the oocytes should occur at the latest within 90 seconds after placing them into the ES. Wait for 12 minutes and observe the recovery of the oocytes. If full re-expansion of oocytes occurs (the perivitelline space should be the same as before equilibration), oocytes should be picked up for the next step. If the volumetric recovery of the oocytes is incomplete, continue the equilibration until 15 minutes all together. The recovery period can be used to prepare the liquid nitrogen container and to label the cryo-containers.
3. Pick up oocytes with the pipette, and expel the oocytes at the middle depth of VS1 with ES. Oocytes will immediately float to the surface of VS1. Expel and wash the inside of the pipette with fresh VS, and pick up the oocytes and expel them again at the bottom of VS. The oocytes will then very slowly float to the middle and stop. When oocytes stop, it is the end of equilibration step, as the weight has become the same inside and outside of the oocytes. Aspirate the oocytes at the tip of pipette to move to VS2 (Figure 21.4).
4. Expel the oocytes into the middle depth of VS2. Expel and aspirate fresh VS from the edge of surface, and expel it outside of the well. Aspirate fresh VS2 again from the surface. Blow out VS from the pipette and mix the solution around the oocyte to watch the oocytes to be shrunk from 3D. (Figure 21.5). Expel and wash the inside of the pipette with fresh VS, and aspirate the oocytes at the tip of the pipette to put the oocytes onto the cryo-container set on the slit of the Vitri-Plate.
5. Pick up the oocytes with the pipette in the smallest possible amount of VS and place them on the strip of the cryo-container on the Vitri-Plate near the black triangle mark (Figure 21.6).
6. Immerse the cryo-container directly into the liquid nitrogen in the Styrofoam box and rapidly stir the cryo-container in the liquid nitrogen to obtain the maximum cooling rate (23,000°C/minute). While keeping the cryo-container submerged in liquid nitrogen, cover the strip of the container with the plastic cap using tweezers and then the fingers to ensure it is tightly closed (Figure 21.7).

Warming and dilution of CPAs

An unopened vial of TS and a warming-plate (Figure 21.8) should be pre-warmed to 37°C in an incubator for at least one hour. All other solutions should be kept at room temperature, i.e. 25°C to 27°C.

Gently mix pre-warmed DS and TS vials with an up-and-down movement. Pour 300 µL of DS into well 2, and 1.8 mL of 37°C TS into square well 1 of warming-plate.

The warming and dilution procedures consist of the following steps (dilution is also shown on Figure 21.8):

1. Using tweezers, remove the plastic cap of the cryo-container while it is still submerged in liquid nitrogen. This manipulation can be performed easily if the Styrofoam box is filled almost entirely with liquid nitrogen. The container should be positioned close to the microscope to avoid delay when transferring the cryo-container. The microscope

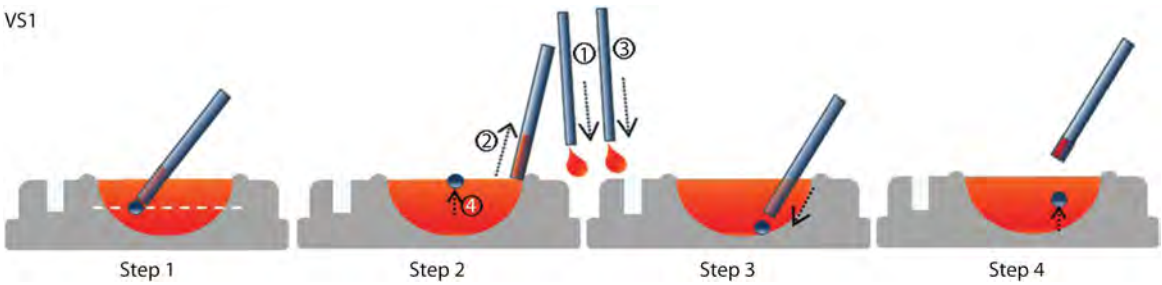

FIGURE 21.4 Equilibration of oocytes in VS1. *Abbreviation*: VS1, vitrification solution 1.

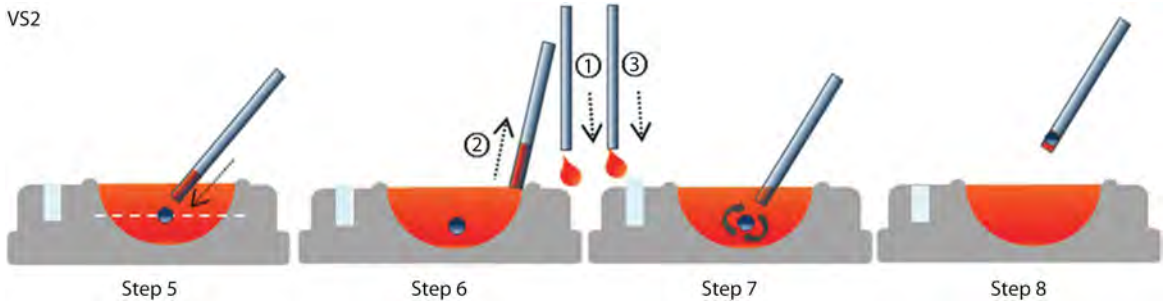

FIGURE 21.5 Confirmation of oocyte shrinkage in VS2. *Abbreviation*: VS2, vitrification solution 2.

FIGURE 21.6 Easy loading of oocytes to cryo-container using Vitri-Plate.

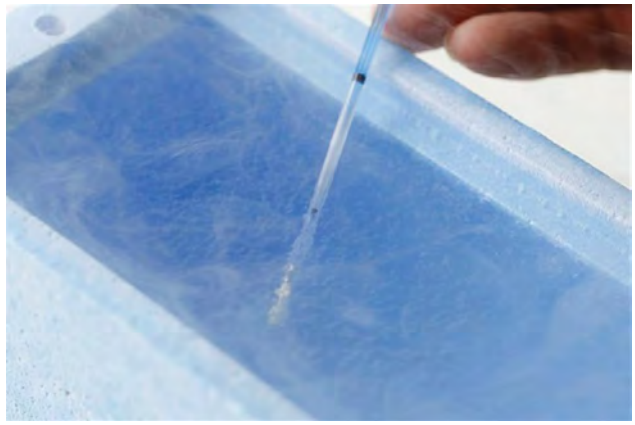

FIGURE 21.7 Vitrified oocytes (▲mark) on cryo-container in LN2 with tightly closed cover cap.

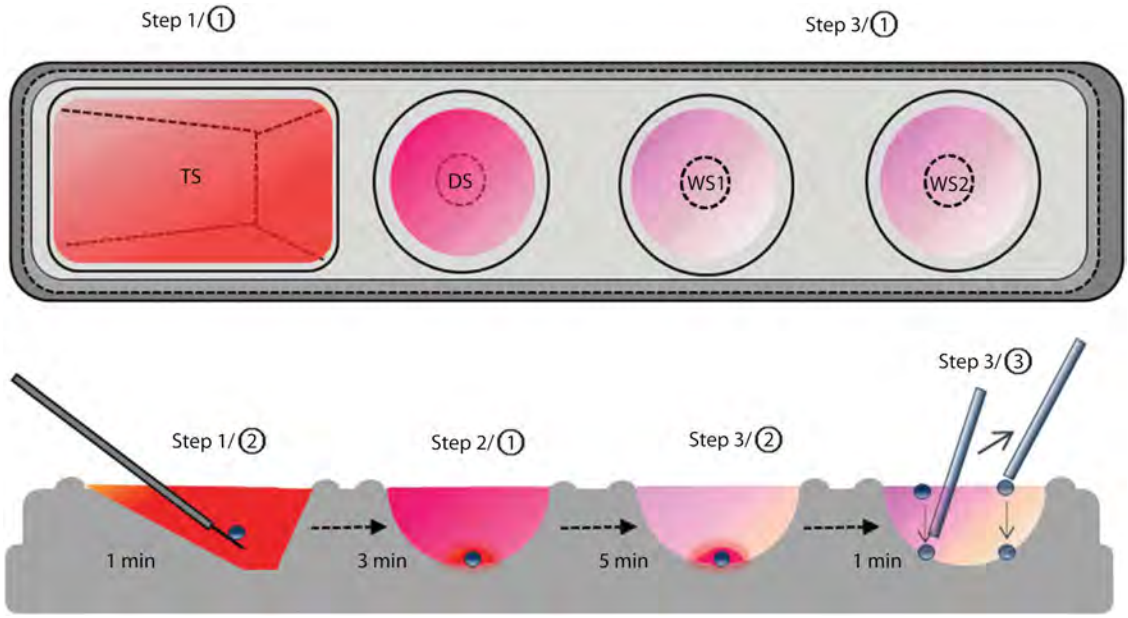

FIGURE 21.8 Warming solutions in warming plate and warming procedure. *Abbreviations*: DS, dilution solution; TS, thawing solution; WS1 washing solution 1; WS2, washing solution 2.

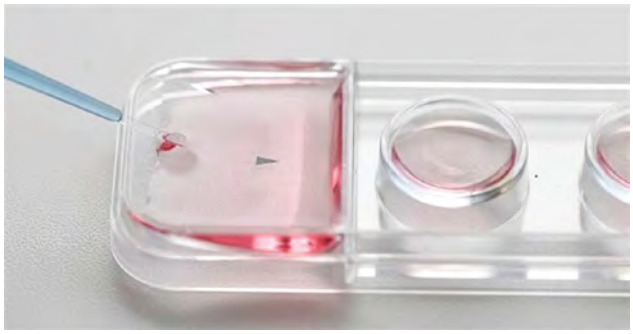

FIGURE 21.9 Warming: oocyte on cryo-container sheet in thawing solution in the warming-plate.

should be focused at the centre of the TS of the warming-plate with low magnification.

2. Hold the cryo-container and look for the black mark while maintaining the tip submerged in liquid nitrogen. Remove the cryo-container with a rapid movement from the liquid nitrogen and place the tip immediately into the middle of the square TS well of the warming plate (Figure 21.9).
3. Find the oocytes by adjusting the focus on the cryo-container sheet. One minute after immersing into TS, while keeping the sheet in the middle of TS, oocytes will separate themselves from the sheet and will begin to float. Follow all movements of the oocytes continuously, as they become transparent at this phase of the procedure and it is easy to lose them. Later, they will regain their normal appearance.
4. Gently pick up the oocytes into the pipette and aspirate an additional 3-mm-long TS column to the tip of the pipette. Transfer the pipette to the bottom of DS well and expel the contents gently to the centre of the bottom (deepest place): first the TS media, allowing it to form a small "mountain" of fluid, then the oocytes to the bottom of this mountain. Just do nothing. Wait for three minutes (Figure 21.10).
5. Subsequently, the same method of transfer should be applied but with different solutions: oocytes will be placed to the bottom of the mountain formed from DS medium in the WS1 dish for five minutes, without any stirring or mixing of the media.
6. Place oocytes onto the surface of WS2 and wait for one minute.
7. Finally, oocytes are transferred into the culture dish and their morphology is examined under the stereomicroscope. ICSI can be performed after a recovery period of at least one hour.

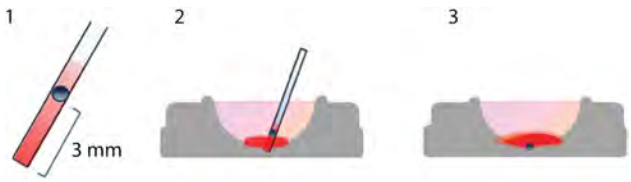

FIGURE 21.10 Gradual replacement of the solutions (thawing solution to dilution solution/dilution solution to washing solution).

High survival of human oocytes by ultra-rapid vitrification

Before being cryopreserved, the potential development rate of oocytes is 100%. If some oocytes undergo serious damage during cryopreservation, those oocytes die, resulting in a lower overall survival rate. A lower survival rate is the evidence of increasing damage caused by cryopreservation. Therefore, especially in clinical applications of vitrification, it is very important that the highest survival be attained not only for the efficiency of the treatment but also to ensure the likelihood of producing normal, healthy babies. Such high post-warming survival of oocytes can be obtained using an ultra-rapid vitrification method.

As a result of personal communication with colleagues in more than 76 countries, I estimate that more than 100,000 oocytes have been vitrified by the ultra-rapid method, and in most of the centres, the recent survival rate of vitrified oocytes after warming using the latest ultra-rapid vitrification protocol is almost 100%, and more than 10,000 healthy babies have been delivered thus far. The fact that such results have been reported by this many independent clinical groups in different countries with no direct or commercial connection for the past 10 years may indicate that a reliable clinical procedure to cryopreserve human oocytes has been established.

Conclusion

Cryopreservation of oocytes is regarded as one of the most demanding tasks of human-assisted reproduction. With scrupulous attention to numerous details and proper application of the latest vitrification techniques, efficiency of the procedure has been substantially improved.

The latest vitrification method has resulted in almost 100% survival rate followed by excellent fertilization, blastocysts development, pregnancy, and births after ETs, comparable to those achieved with non-vitrified control oocytes.

The technique can be useful in diverse situations where oocyte storage is required or considered.

Acknowledgements

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22

THE HUMAN EMBRYO

Vitrification

Zsolt Peter Nagy and Ching-Chien Chang

Introduction

Decades ago, most assisted reproductive technologies including *in vitro* fertilization (IVF) and cryopreservation of embryos by traditional freezing were applied to humans almost immediately after the first successes in some experimental or domestic species. However, there are some techniques, where efforts to adopt a new approach were insufficient and sporadic, consequently the practical application has been considerably delayed. Vitrification belongs to this group. Reasons for this delay may include the fact that slow-freezing/thawing of zygote-, cleavage-, and blastocyst-stage human embryos was somewhat efficient, providing a "reasonable" survival rate of embryos (somewhere between the 50% to 80% range). At the same time, vitrification seemed "scary," requiring the use of very high concentrations of cryo-protectants, and very precise handling/timing, while it also seemed "less sophisticated" because it relies fully on manual technology compared to slow-freezing where automatic traditional freezers are used. High concentrations of cryo-protectants required for vitrification discouraged some potential users initially. Finally, none of the major suppliers were eager to replace their expensive freezing machines with a much simpler system required for vitrification (though, very recently there have been some efforts to develop an instrument that would allow a "semi-automated" vitrification process) [1, 2].

Some scientists in the early 2000s started moving from domestic animal embryology to the human field to apply the technique of vitrification. However, additional years were still required to get the approach acknowledged, to develop commercially available tools and kits, and to teach both distributors and consumers about the benefit of vitrification. Eventually, the overwhelming comparative evidence made clear to almost everybody that in all developmental stages, vitrification produces significantly better survival and more competent oocytes/embryos than traditional freezing. Today, the rapidly increasing interest towards vitrification creates novel problems such as diversity of tools and media, lack of information regarding ingredients, inconsistency in teaching and application. Legal concerns on biosafety issues have also emerged, although no scientific proof exists for the magnitude or existence of any risks.

In this chapter, we summarize the basic features of vitrification, explain some special aspects of vitrification, and provide data about the efficiency of vitrification for cryopreservation of human pre-implantation stage embryos at different developmental stages. Also discussed is how the highly efficient vitrification (both embryo and oocyte) method has contributed to a paradigm shift in how assisted reproduction treatment is practiced today. The effect of vitrification is clearly demonstrated by the dramatic increase of cryopreservation cycles ("freeze all" IVF cycles) in the United States. Based on the Center for Disease Control (CDC) data, in 2007 there were only 2020 "freeze all" cycles, in 2013 there were 27,564, and in 2019 (the most recent available data) there were 121,086 freeze all cycles (Figure 22.1).

For terms and definitions, we accept and use the excellent review and suggestions of Shaw and Jones [3]. For the basic principles of cryobiology we refer to earlier reviews [4–7].

Main cryopreservation approaches

Within approximately a decade after the first successes with cryopreservation of mammalian embryos [8–12], the first human pregnancies were achieved [13, 14]. All these works were performed with traditional slow freezing. Vitrification was first applied for cryopreservation of mammalian embryos in 1985 [15] but regarded as a curiosity and experimental procedure for almost a decade, when practical application was started in domestic animal embryology and sporadic approaches in humans. Competitive vitrification strategies for human embryo and oocyte cryopreservation have only been developed 15–18 years ago (in the mid-2000s).

The strategies of the two approaches are basically different. Far the most important source of damage at cryopreservation is ice crystal formation. To minimize this injury, application of various chemicals (cryo-protectants) is required, which, unfortunately, may also induce various injuries including toxic and osmotic damage.

Just to "recap," the mostly "retired" traditional slow-rate freezing creates a delicate balance between these factors. Embryos are typically exposed to 1–2-mol/L solutions of permeable and (less concentrated, if any) non-permeable cryo-protectants, then loaded into a 0.25-mL straw, sealed and cooled to –6°C relatively rapidly, by placing the straws into a controlled-rate freezer. With the given cryo-protectant concentration, no spontaneous ice formation occurs at this temperature; however, ice nucleation can be induced by "seeding," i.e. touching the straw with a forceps that has been previously immersed into liquid nitrogen. The controlled-rate freezer is adjusted to make a very slow cooling (usually 0.3°C/minute, to around –30°C), then the straws are immersed into liquid nitrogen for a final cooling and storage. The slow rate of cooling allows solution exchange between the extracellular and intracellular fluids without serious osmotic effects and deformation of the cells (this fact is reflected in the other name of the procedure: equilibrium freezing; [16]).

The strategy of vitrification is much more radical. The main purpose (according to the cryobiological definition) is the complete elimination of ice formation in the whole solution the sample is cooled in.

Evidently, this can only be performed with the use of high cryo-protectant concentration, which may theoretically induce serious toxic and osmotic damages. A huge variety of cryo-protectants were tested in different studies along with many "technical approaches" (see Table 22.1), and as a result, most

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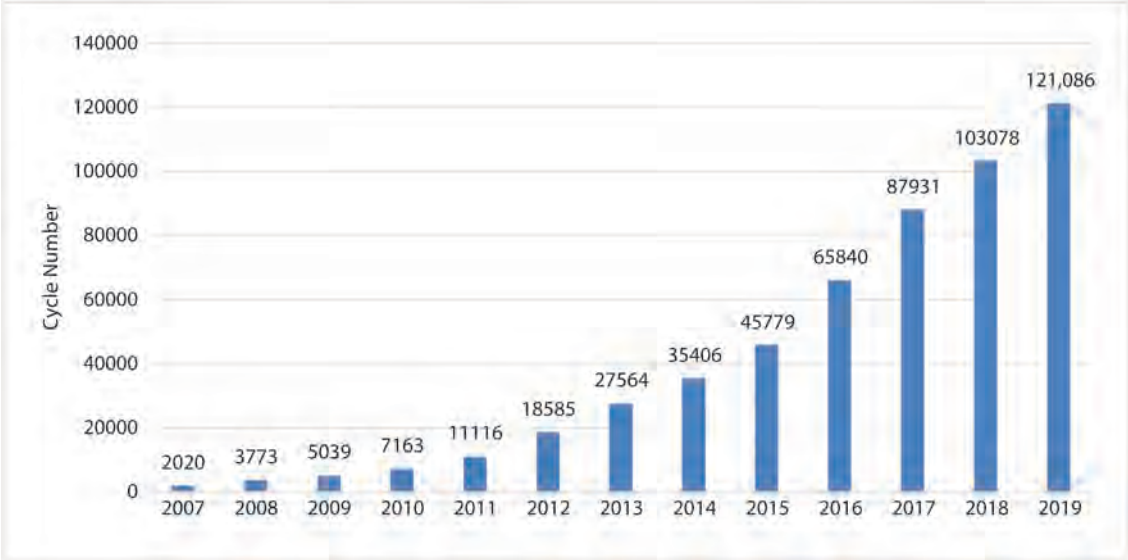

FIGURE 22.1 The impact of vitrification on the number of IVF cycles with "freeze all" in the United States, from 2007 to 2019.

TABLE 22.1 Various Vitrification Techniques in Embryology

System	Reference
Direct dropping into liquid nitrogen	Landa and Tepla [76]
Electron microscopic grids	Martino et al. [25]
Open-pulled straw (OPS)	Vajta et al. [83]
Glass micropipettes (GMP)	Kong et al. [239]
Super-finely pulled OPS (SOPS)	Isachenko et al. [240]
Gel-loading tips	Tominaga and Hamada [241]
Sterile stripper tip	Kuleshova and Lopata [125]
Flexipet denuding pipette (FDP)	Liebermann et al. [242]
Fine-diameter plastic micropipette	Cremades et al. [243]
100 µl pipetting tip	Hredzak et al. [244]
Closed-pulled straw (CPS)	Chen et al. [245]
Sealed open-pulled straws	Lopez-Bejar and Lopez-Gatius [246]
Cryotip	Kuwayama et al. [69]
Cryoloop	Lane et al. [91]
Nylon mesh	Matsumoto et al. [97]
Minimum drop size (MDS)	Arav [247]
Minimum volume cooling (MVC)	Hamawaki et al. [99]
Hemi-straw system (HSS)	Vanderzwalmen et al. [100]
Cryotop	Kuwayama et al. [68]
Vitmaster	Arav et al. [85]
Solid surface vitrification (SSV)	Dinnyes et al. [113]

Source: Reprinted from Vajta and Nagy [248] with permission from Reproductive Healthcare Ltd.

vitrification-based kits have very similar composition (similar components and concentrations) and similar techniques/handling. Cell shrinkage caused by non-permeable cryo-protectants, and the incomplete penetration of permeable components may cause a relative increase of intracellular concentration of macromolecules that is enough to hamper intracellular ice formation. Accordingly, vitrification belongs to the group of non-equilibrium cryopreservation methods.

Another possibility to minimize the chance of ice formation during vitrification is to increase the cooling and warming rates. The higher the cooling rate, the lower the required cryo-protectant concentration is, and vice versa. Eventually, even the radical approach of vitrification has to establish a delicate balance, as it requires (i) establishment of a safe system for maximal and reliable cooling (and warming) rates while avoiding consequent damage including fracture of the zona pellucida or the cells, and (ii) elimination or minimization of the toxic and osmotic effects of high cryo-protectant concentrations needed to obtain and maintain the glass-like solidification.

There is, however, a small, poorly defined group of cryopreservation techniques that shares some features with both vitrification and slow rate freezing. In this method, cryo-protectant concentrations are insufficient to establish vitrification [9, 17–19]. This approach has been established entirely empirically, and does not meet any supposed requirements of cryopreservation in embryology. Although ice is formed in the solution, under certain (and sometimes unpredictable) conditions embryos survive and develop further [20, 21]. However, the lack of control may result in inconsistent survival and developmental rates. On the other hand, some of the early experiments characterized as rapid freezing were in fact vitrifications [22, 23].

Injury and prevention during cryopreservation

Exposition to deep sub-zero temperatures is a situation mammalian cells never meet under physiological circumstances. The injury may occur at all phases of the procedure.

During cooling, different types of damage may occur when embryos pass through three overlapping temperature zones.

At relatively high temperatures, between +15 and −5°C, the chilling injury is the major factor, damaging predominantly the cytoplasmic lipid droplets and microtubules including the meiotic spindle [24–26]. While the latter damage may be reversible, the former is always irreversible and contributes to the death of cryopreserved lipid-rich oocytes and embryos of some species.

Between −5 and −80°C, extracellular or, predominantly, intracellular ice crystal formation is the main source of injury.

Temperatures between −50 and −150°C can cause fracture damage to the zona pellucida or the cytoplasm [27] are postulated to occur (although the mechanism and the actual temperature of occurrence is not entirely defined). However, it is unlikely that zona fracture could occur as a simple consequence of osmotic stress, as suggested by Smith and Silva [6].

Storage below −150°C (typically in liquid nitrogen, at −196°C) is probably the least dangerous phase of the cryopreservation procedure.

Importantly, accidental warming is probably the most frequent form of injury, which definitely puts vitrified samples at risk if not handled appropriately [28]. The effect of background irradiation seems to be less harmful than supposed, and is not a significant source for DNA injury in a realistic time interval, i.e. years, decades, or even centuries [29]. There is increasing concern regarding possible disease transmission between the stored samples mediated by the liquid nitrogen—even though there are no reported cases in literature involving embryos.

At warming, the same types of injuries may occur as at cooling, obviously in inverse order. One of the most likely reasons for injury is recrystallization during warming, which nearly always occurs. To avoid or minimize its potential damage, the addition of certain components to the cryo-solution has been investigated, as well as adjusting the speed of warming (relative to the speed of cooling) [30, 31].

Apart from these processes, there are some partially understood injuries including damage of intracellular organelles, cytoskeleton, and cell-to-cell contacts [32, 33].

All embryos subject to cryopreservation may suffer considerable damage during cooling and warming. Fortunately, they also have a remarkable, sometimes surprising ability to repair fully or partially this damage, and in the best case to continue normal development. All cryopreservation methods try to decrease the damage and facilitate the regeneration process.

Cryo-protectants are a diverse group of simple or complex, permeable or non-permeable, organic or inorganic compounds with two common features: they are water-soluble and they protect the cells from cryo-injuries. The range is wide, expanding from well-known simple organic solvents such as ethanol to the complex, partially known substances such as serum or egg yolk. Permeable cryo-protectants enter the cells and minimize ice formation with various mechanisms depending on their structure and chemical activity, whereas non-permeable cryo-protectants remain outside the cells and minimize ice formation by removing water from the cells by osmotic effect. However, there are certain overlaps between the two groups, especially in vitrification methods, where the usually applied short exposition to the concentrated, theoretically permeable components do not allow full equilibrium, therefore part of the effect of permeable cryo-protectants is dehydration, as well. Additionally, both permeable and non-permeable components may have some other specific cryo-protectant effects, for example, stabilization of cell membranes, the meiotic spindle, or other cellular structures [34]. Unfortunately, most cryo-protectants have some negative effects, including toxicity and, obviously, osmotic effect. Toxicity is usually in direct correlation with the concentration of the substance, the temperature, and the time of exposure. The osmotic effect is mostly proportional to the concentration. In case of permeable or partially permeable cryo-protectants, the osmotic effect can be minimized by slow, stepwise addition and removal during equilibration and dilution, respectively. The mechanism and reasons for damage during cryopreservation as well as the precise protective mechanisms of cryo-protectants are poorly understood at present. The effects of a given cryo-protectant may substantially differ at physiological and low temperatures; thus, the retrospective analysis of damage may result in faulty conclusions. Considering these uncertainties, it is not surprising that the vast majority of existing cryopreservation techniques were established empirically, based on rough morphological changes observed under a stereomicroscope, and have been justified by the outcome, i.e. *in vitro* and *in vivo* survival. It is more recent development that using highly sophisticated diagnostics would help to assess freezing conditions, such as using protein expression to detect gene expression [35, 36].

Vitrification

Cryo-protectants

No cryo-protectants exclusively designed or used for vitrification have been developed yet. Certain components and combinations (for example ethylene glycol, DMSO, and sucrose) are typically used for vitrification purposes, and the concentration of specific components is significantly higher at vitrification than in traditional or rapid freezing.

The most common permeable components are ethylene glycol, propylene glycol, acetamide, glycerol, raffinose, and dimethyl sulfoxide (DMSO), and these have been tested in various combinations [5, 37]. Due to low toxicity, high permeability, and excellent ice-blocking ability, ethylene glycol is an almost indispensable component of all cryo-protectant solutions. However, a common strategy to decrease the specific toxicity of any one cryo-protectant is to use the mixture of two permeable cryo-protectants, i.e. a mixture of ethylene glycol and either DMSO, propylene glycol, or, less typically other components. Eventually the mixture of ethylene glycol and DMSO appears to be used frequently [38, 39]. According to some studies, the permeability of this mixture is higher than that of the individual components [40]. It should be noted that the earlier concerns regarding the genotoxicity and cytotoxicity of DMSO have been dismissed [41, 42].

Commonly used non-permeable cryo-protectants include mono- and disaccharides, sucrose, trehalose, glucose, and galactose [43–45]. Recently, sucrose has become almost a standard component of vitrification mixtures. This is true even though nearly all comparative investigations proved the superiority of trehalose. Sucrose along with other sugars may not have any toxic effects at low temperatures, but may compromise embryo survival when applied extensively to counterbalance embryo swelling after warming [46–48], although this effect was not always demonstrated [49]. Several polymers were also suggested for the purpose, including polyvinylpyrrolidone, polyethylene glycol, Ficoll, dextran, and polyvinyl alcohol [50–55]. However, from this group the only widely used compound is Ficoll, predominantly in combination with ethylene glycol and sucrose [56]. Various forms of protein supplementation have also been used, including egg yolk, but its optically dense appearance made the microscopic manipulation rather difficult. High concentrations of sera of different origin as well as serum albumin preparations [57] are common additives. In the bovine model, recombinant albumin and hyaluronan were also effective [58]. On the other hand, the use of antifreeze proteins isolated from arctic animals [59–61] has

largely been abandoned. More recently, hydroxypropyl cellulose (HPC) was investigated as a replacement for serum-derived protein for use in cryo-protectant solutions, and results have been promising [62, 63].

Another practical feature is the stepwise addition of increasing concentrations of cryo-protectants [57, 64–66]. After several early attempts, the two-step equilibration has become the most commonly used approach, with the first solution containing approximately 50% of the final cryo-protectant concentration. Embryos and oocytes are equilibrated for a relatively long period (5–15, sometimes up to 21 minutes) in the first solution, then for a short period (approximately 1 minute) in the second one [67–69]. This approach may increase the toxic effect slightly, but provides a much better protection for the whole cell, and may be especially beneficial in the case of large substances with a low surface/volume ratios, including oocytes or early-stage embryos. On the other hand, earlier attempts to cool the concentrated solution to 4°C to decrease toxicity have been found later to be unnecessary. Because of the much higher concentrations of cryo-protectants (CPAs) used in vitrification, it was initially assumed that intracellular concentrations of CPAs are higher after vitrification than after slow-freezing—giving concerns on the toxicity of these CPAs. However, in a recent, elegant study, it was demonstrated that intracellular concentration of cryo-protectants are actually lower after vitrification than after slow freezing, despite exposure to higher concentration of cryo-protectant solutions [70]. Very recently, there are attempts to use microfluidic technique to provide a more controlled and gradual increase of cryo-protectants to the vicinity of the embryo (or oocyte) using robotic systems to improve outcomes [71].

Tools of cryopreservation used for vitrification

Plastic insemination straws or cryovials were used initially for vitrification experiments. These tools were not designed for the special purpose of vitrification, had a thick wall, and required a relatively large amount of solution for safe loading. Accordingly, the cooling and warming rates were quite limited (approximately 2500°C/minute for straws [72]; and even less for cryovials). This relatively low rate was still hazardous to perform, as direct immersion into liquid nitrogen at cooling, and transfer to a water bath at warming, induced extreme pressure changes in the closed system and frequently led to the collapse or explosion of the straws and loss of the sample. One of the other consequences of these manipulations was the decreased and inconsistent rates: the temperature of the vapour of liquid nitrogen is variable, depending on many factors, and the definition of "room temperature" laboratory air may mean 5–7°C differences, even at the same place on the same day. Consequently, a minimum 5–7-mol/L cryo-protectant concentration was required, and chilling injury could not be lowered to the level occurring at slow freezing.

Some scientists have investigated the use of an instrument, called a VitMaster, to achieve higher cooling rates. (VitMaster is able to lower the temperature of liquid nitrogen from its boiling point of –196°C to around –208°C—applying vacuum—thus the nitrogen then changes from its liquid state to a slush, which prevents an insulating pocket of gas forming around the sample, resulting in faster cooling). Although outcomes of vitrification using VitMaster tended to be somewhat better than "traditional" vitrification [73, 74], its use has not become part of the daily routine. More recently, efforts were made to develop an instrument that can offer some level of automation for vitrifying samples [1]. This "Gavi" system can automatically perform equilibration steps before closed vitrification is performed for embryos. The warming, however, has to be performed manually, and currently the system is not proven to perform equivalently for oocyte cryopreservation. Although technically challenging, there are continued efforts to create an automatic (or semi-automatic) vitrification device [75].

Increasing cooling rates with new carrier tools

Although the increased cooling and warming rate was a well-known way to keep the concentration of cryo-protectants as low as possible, and minimize the related toxic and osmotic injuries, this option has remained unexploited for a relatively long period of time. The first purpose-made tools were only produced approximately 20–25 years ago. Today, however, there is a huge variety of tools, methods, and approaches are available, and without adequate comparative studies, the selection of the best choice is a serious problem for embryologists working in a routine human IVF laboratory.

The most logical way to increase cooling and warming rates is to decrease the volume of the solution that surrounds the sample, and to establish a direct contact between the sample and the liquid nitrogen.

Seemingly the simplest way to accomplish this task is the direct dropping into liquid nitrogen [67, 76–78]. Unfortunately, to form a drop from a water-based solution requires a relatively large amount of solution (4–6 µL), and the drop does not sink immediately into the liquid nitrogen, because for the initial seconds the drop is surrounded by the vapour that is induced by the warm solution, and does not allow the sample to sink (see Table 22.1 for the different approaches investigated).

Accordingly, some carrier tools have been used to push the sample immediately below the level of liquid nitrogen, to serve as a storage device after cooling, and to facilitate quick warming as well. Electron microscopic grids used for this completely different purpose have proved the practical value of the idea first [76, 77, 79, 80]. In this system, the size of the drop surrounding the sample was extremely small, as after loading, most of it was removed by placing the grid on a filter membrane. The thermoconductive metal grid also contributed to improving the cooling and warming rates. Surprisingly, the solidified cryo-protectant solution fixed the sample safely to the grid during cooling and storage, and released it easily after warming [81]. However, the storage and handling of the tiny grid has been a demanding task.

The first purpose-made tool for vitrification was the open pulled straw (OPS), a modification of a standard 0.25-mL plastic straw, with decreased diameter and wall thickness. This modification enabled loading with the capillary effect, and the minimum volume decreased to approximately 0.5–1 µL, i.e. five to ten times smaller than that of the original straw, which results in approximately a tenfold increase in the achievable cooling and warming rates, and 30% decrease of the cryo-protectant concentration required for vitrification. The OPS has become the most widely used approach for ultra-rapid vitrification [82–90].

The cryoloop was another approach applied earlier using the small volume–direct contact principle. It consists of a small nylon loop attached to a holder and equipped with a container (Figure 22.2). It has been used for cryopreservation in crystallography and is now used widely for oocyte and embryo cryopreservation [91–94]. The solution film bridging the hole of the

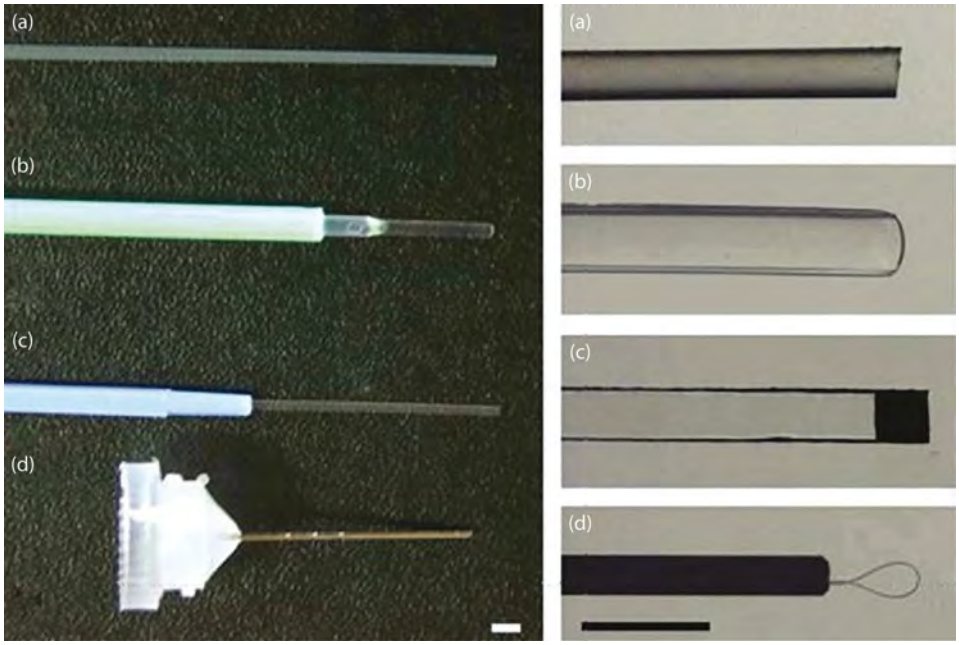

FIGURE 22.2 Examples for commercially available tools used as carriers for high-speed vitrification. (a) Open pulled straw, OPS (Minitüb, Landshut, Germany); (b) McGill Cryoleaf (MediCult, Jyllinge, Denmark); (c) Cryotop (Kitazato, Tokyo, Japan); (d) Cryoloop (Hampton Research, Aliso Viejo, California, United States). Bars represent 2 mm.

loop is strong enough to hold the oocyte or the embryo, and with this minimal solution volume, the achievable cooling rate may be extremely high, up to an estimated 700,000°C/minute [95]. Using this tool, safe cryopreservation can be achieved even in the vapour of liquid nitrogen [96, 97].

The minimum drop size (MDS) method of Arav [98] consists of a small droplet of vitrification solution containing the oocyte or embryo placed on a solid surface that is immersed into liquid nitrogen. The approach was used later with some modifications called the minimum volume cooling (MVC; [99]) or in the hemi-straw system (HSS; [100]), where the carrier tool was a cut-open straw.

Currently, the most commonly used tool for the vitrification of human oocytes and embryos is the Cryotop, an advanced version of the MVC technology [69, 99]. It consists of a flexible transparent plastic film attached to a handle, and also equipped with a protective tube to avoid damage to the film during storage in liquid nitrogen. The sample is loaded on the film, the excess solution is removed, and the film is immersed into the liquid nitrogen. At warming, the Cryotop is quickly removed from the liquid nitrogen, and the film is immersed into the warming medium. Since its first introduction, a good number of studies confirmed its value [101–103]. Yet other carriers, such as Cryolock, and Cryotec, similar in its design to Cryotop, are gaining more popularity and being used efficiently [104, 105].

Cryopette is probably the first carrier that is designed to combine the benefit of very low volume solution with the advantage of a closed system. There are other closed carriers that have been investigated and tried for the use of embryo vitrification, including the Rapid-I [106, 107] and the CBS-VIT High Security (HS) straw, demonstrating satisfactory outcomes [108, 109]. Based on published studies, it appears that closed systems are also able to provide adequate outcomes for embryo vitrification, however, open systems are more likely to provide superior results when oocytes are vitrified and to preserve the original physiological cell condition [110, 111].

The flow chart of a typical high-speed vitrification procedure is summarized in Figure 22.3.

Decreased vapour formation for increased cooling rates

One major limitation of the achievable cooling rates of the sample is the vapour that is formed around it at immersion. At –196°C, liquid nitrogen is at boiling point. Accordingly, a submerged warmer item will induce an extensive evaporation around the sample, producing a thermo-insulating coat around the sample and decreasing the achievable cooling rate, especially at the initial moments, when the chilling injury may develop.

One possibility to avoid this phenomenon is to expose liquid nitrogen to a vacuum for several minutes. Part of the liquid nitrogen will evaporate, and the rest will cool down to –203 to –207°C, where it starts to get solidified, i.e. slush is formed. As the nitrogen escapes from the fragile boiling zone, the immersed sample creates a minimal evaporation, consequently the cooling rate gets considerably higher [85, 86, 112]. While using "super-cooled" LN2 offers the aforementioned theoretical benefits, the use of VitMaster (and this process) has not gained much application in real life. Probably because it is possible to achieve excellent outcomes without this approach, and also because the use of this instrument is not very practical.

The other way to eliminate the vapour is the use of pre-cooled metal surfaces instead of liquid nitrogen for cooling. It can be performed by immersing a metal block into liquid nitrogen [113], or by using a more sophisticated, commercially available version (CMV; Cryologic, Australia).

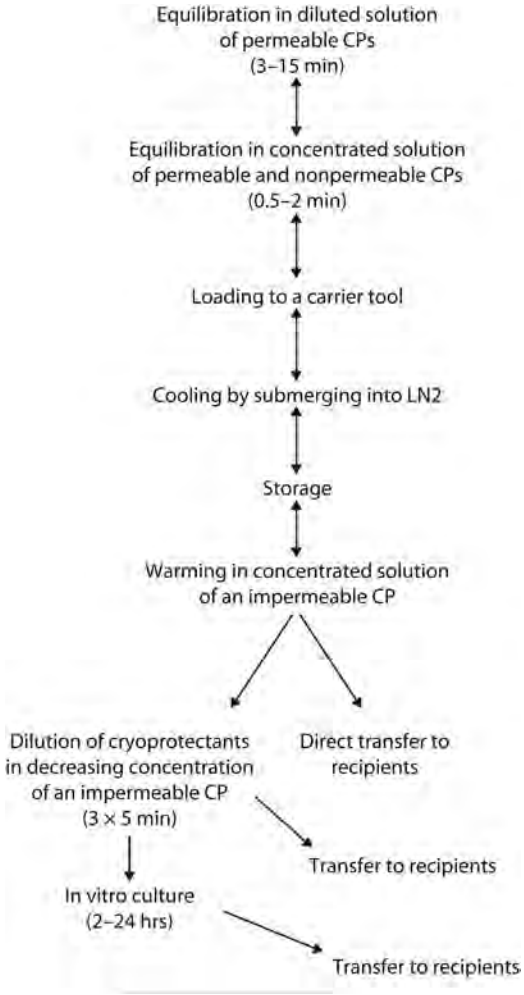

FIGURE 22.3 Flow chart of a typical high-speed vitrification procedure.

The available free comparative data do not provide entirely convincing evidence regarding the superiority of these vapour-minimizing or vapour-free approaches compared to other vitrification procedures.

Transmission of infectious agents

One of the concerns regarding the use of vitrification in human embryology is the potential risk of liquid nitrogen-mediated disease transmission. To understand better, we need to consider the following:

1. Semen and oocyte collection, processing/handling and cryopreservation protocols are not sterile procedures [114]; consequently, the contents of virtually all stored straws and cryovials may be a source of infection.
2. In human embryology, liquid nitrogen may also be contaminated by the surface of straws, cryovials, racks, and other tools that are usually not handled fully aseptically. Accordingly, the presence of infective agents is not strictly related to leaky or open containers.
3. Seemingly sterile containers may not be as safe as supposed. Infection may occur in common straws in slow freezing (through the holes of incomplete sealing, or pores of the plastic walls), and most cryovials do not have secure caps. A possible source of infection may also be the inappropriate decontamination of the outer walls of straws before loading and expelling.
4. Liquid nitrogen in storage tanks likely contains a number of commensal and potentially pathogenic environmental microorganisms [114].
5. Cases of liquid nitrogen-mediated transmission of pathogens [115–117] have been documented but never in relation to cryopreserved oocytes or embryos. Disease transfer has occurred only in one occasion, where two leaky bags containing blood samples were stored in the same dewar [115].
6. According to the experiments of Bielanski [118], cross-contamination may also occur during storage between open embryo storage if one of them is artificially infected. However, the volume of the microbes was artificially extremely high, a concentration that may never happen in a clinical situation.
7. Not a single case of any disease transmission in ART has been found to be related to liquid nitrogen-mediated cross-contamination, in spite of the enormous amount of human sperm samples, embryos, and oocytes stored worldwide, neither related to traditional, supposedly closed (but very often leaky or inappropriately handled) systems, nor with the open vitrification systems, in spite of the enormous focus on the latter. A study published in 2012 provided evidence from a real-case scenario of the lack of risk of cross-contamination among seropositive patients, even using an open device for vitrification [119].

There is no doubt that closed and properly handled systems should always be preferred, provided the outcome is comparable with the open systems. Results achieved by using closed systems for cleavage-stage human embryos and blastocysts are promising [108, 120, 121]. However, the fact that for human oocyte vitrification, open systems are superior than closed ones proves the superiority of the former [69, 122]. A possible solution is to make cooling in sterilized liquid nitrogen [123, 124] and store the samples in pre-cooled, hermetically sealed containers afterwards [83, 125]. Alternatively, open carriers can also be stored in cryo-tanks where instead of liquid, the vapour of nitrogen maintains the low storage temperature [126]. There have been a number of studies demonstrating the efficiency of the vapour storage system for vitrified oocytes/embryos using open carriers both in animal and human systems [126–128]. Concerns may also be raised regarding the applicability of closed systems for other chilling-sensitive objects including cattle oocytes and early-stage embryos, porcine blastocysts, or human oocytes [129], regarding not just *in vitro* survival rates but also *in vitro* development, pregnancies, and birth of healthy offspring.

Warming

Early on, Rall [57] found that high survival of vitrified embryos can be achieved with rather slow warming rates. However, most vitrification methods use rapid warming procedures, and recently it has been demonstrated that warming rates may be even more important than cooling rates [130].

Closed systems are usually immersed into water baths, while open systems can be directly submerged into the medium; this way the warming and the first dilution is performed in a single

step. The seemingly negligible difference may contribute considerably to the inferior results achieved with some closed systems consisting of a simple plastic or glass tube. After warming in the water bath, the surface has to be decontaminated quickly with a non-toxic but perfectly safe disinfectant, then the tube is cut and the sample is expelled into the appropriate medium. It means a significant delay between the warming and dilution; accordingly, the samples in this critical, very fragile phase are exposed for a relatively long period of time (5–10 seconds) to the concentrated cryo-protectants.

Although a slight devitrification (occurrence of ice crystals) may occur, especially when the cryo-protectant level is kept at the minimum level, this transitional change is usually restricted to a part of the embryo-containing medium and most probably does not involve intracellular crystal formation, and consequently does not cause significant harm in the embryos or oocytes [131], especially if the volume of the droplet is minimized (at the time of placing the embryo on the carrier).

In routine warming protocols of vitrified embryos, the dilution is a multistep procedure with decreasing concentration of osmotic buffers (usually sucrose) to counterbalance the swelling caused by the permeable cryo-protectant that leaves the cells relatively slowly. This delicate multistep dilution procedure seems to be indispensable for human embryos or oocytes, although one-step dilution without significant decrease of *in vitro* survival was reported in some animal species including cattle [48, 65, 132] and pigs [133]. Based on this approach, direct transfer methods after ultra-rapid vitrification of embryos resulted in offspring after transfer in cattle [134] and sheep [135]. Interestingly, the commonly used warming protocol (for vitrified samples) may also be used very efficiently for slow-frozen oocytes/embryos—thus providing a simplified and standard warming protocol for all samples, irrespective how they were frozen/vitrified [136].

Factors influencing the outcome

Species, genotype

There are well-demonstrated but poorly understood differences in sensitivity to cryo-injuries between different species in mammals. It appears that transparent oocytes and embryos are usually more resistant, and dense dark ones are more fragile, due to the increased lipid content. Accordingly, cryopreservation of light mouse embryos is a relatively easy task, cryopreservation of darker bovine embryo is a more difficult task, and the cryopreservation of dense pig embryos is truly a challenge in cryobiology. In parallel with the lighter appearance of the cytoplasm, considerably increased survival rates were detected after both slow freezing [137] and vitrification [138–141]. This approach also improves *in vitro* survival of vitrified porcine blastocysts produced by somatic cell nuclear transfer [141, 142].

It should be also noted that apart from the differences between species, in mice, differences between genotypes in the ability to develop after vitrification were also observed [143].

Developmental stage

The change in the size and shape of the cells is unprecedented in the first five to six days of mammalian development. A relatively simple spherical shape protected by an acellular outer layer develops to a complicated multicellular structure without external protection. Predictably, the extreme differences in morphology also correlate considerably with differences in sensitivity to cryo-injuries.

Generally, the earlier the development stage (starting from the germinal vesicle stage), the more sensitive oocytes and embryos are. However, although there is only a minimal difference between the size and shape, the immature oocytes are usually more sensitive to cryopreservation than mature (MII phase) oocytes [25, 129, 144]. Membrane permeability related to the type and expression levels of aquaporin at different stages may also explain differences in cryo-protectant protection efficiency and thus differences in survival [145, 146]. Additionally, a very remarkable difference exists between the chilling sensitivity of unfertilized and fertilized human oocytes. A possible explanation for this phenomenon is the increased chilling sensitivity of membranes: the lipid phase transition at room temperature storage in human germinal vesicle and MII stage oocytes is 10 times higher than that of human pronuclear embryos [129].

In the human, the survival rates after slow freezing are not significantly different between zygotes, cleavage-stage embryos, and blastocysts (between 65% and 85% for each; [147–149]). Similarly, in the human, survival rates after vitrification are not different for zygote-, cleavage-stage or blastocyst-stage embryos (though at each stage, vitrification provides a significantly higher survival rate; [150–152]). The complex structure of blastocysts may give rise to additional problems. In humans, mechanical reduction of the blastocoel by puncturing or repeated pipetting improved survival and pregnancy rates [134, 153–156]. The usual explanation is that the large blastocoel may not be protected appropriately from ice crystal formation [153]. Survival rates of blastocyst-stage embryos using vitrification are extremely high (it is usually above 95%), even without any additional "manipulation" [149]. However, some studies demonstrated that blastocyst survival (and intactness) may be further increased after vitrification, if the blastocoel is punctured, resulting in shrinkage—especially when blastocyst is expanded—in human and in other species as well [156–159].

In vivo- versus *in vitro*-produced embryos

Due to the lack of *in vivo*-derived human embryos, such differences can only be evaluated in domestic and experimental animals [160]. In these species, *in vivo*-produced embryos are more resistant to injuries—including cryo-injuries—than their *in vitro*-fertilized or cloned counterparts. Again, there might be some correlation between the increased lipid content of embryos produced in some *in vitro* systems. In general, the less morphological difference from the *in vivo* counterpart is detectable in the *in vivo*-produced embryos, the smaller the expected difference in survival after cryopreservation [161]. Although total elimination of these differences is still impossible, according to the joint conclusion of many publications, vitrification seems to be especially appropriate to counterbalance this handicap [162].

Outcomes after embryo vitrification

Domestic, experimental, and wild animals

There is an extensive literature of comparative experiments between slow freezing versus vitrification (some examples may include [91, 92, 143, 163–168]). The overwhelming majority of these papers prove the superiority of vitrification for the given purpose. Probably less than 10% of the studies did not find significant differences that were conducted at an early stage; however, the overwhelming majority of more recent studies clearly demonstrated the superiority of vitrification. Moreover, there are situations where vitrification is uniquely or predominantly suitable to achieve the goal: most of these areas are summarized in Table 22.2.

TABLE 22.2 Examples in Mammalian Embryology Where First Success in Cryopreservation Was Achieved by Vitrification.

Species, stage, system	Reference
Bovine immature oocytes for IVF	Vieria et al. [249]
Bovine *in vitro* matured oocytes for IVF	Martino et al. [250]; Vajta et al. [83]
Bovine *in vitro* matured oocytes for somatic cell nuclear transfer	Hou et al. [251]
Bovine cytoplasts for embryonic cell nuclear transfer	Booth et al. [252]
Bovine early-stage IVF embryos	Vajta et al. [84]; *in vitro* study
Bovine zona-included blastocysts generated by somatic cell nuclear transfer	French et al. [253]
Bovine zona-free blastocysts generated by somatic cell nuclear transfer	Tecirlioglu et al. [134]
Bovine transgenic blastocysts generated by somatic cell nuclear transfer	French et al. [254]
Ovine zona included embryos generated by nuclear transfer	Peura et al. [255]
Porcine immature oocytes for ICSI	Fujihira et al. [256]; *in vitro* study
Porcine *in vitro* matured oocytes for ICSI	Fujihira et al. [257]; *in vitro* study
Porcine *in vivo*-derived blastocysts	Kobayashi et al. [258]
Porcine *in vivo*-derived morulae	Berthelot et al. [259]
Porcine *in vitro* produced blastocysts	Men et al. [260]; *in vitro* study
Equine *in vivo*-matured oocytes	Maclellan et al. [261]
European polecat *in vivo*-derived morulae and blastocysts	Piltty et al. [262]
Siberian tiger *in vivo*-derived embryos	Crichton et al. [263]; *in vitro* study
Minke whale immature oocytes for maturation	Iwayama et al. [264]; *in vitro* study

Abbreviation: ICSI = intracytoplasmic sperm injection.

Source: Reprinted from Vajta and Nagy [248] with permission from Reproductive Healthcare Ltd.

Note: Embryos and Oocytes Were Not Treated Mechanically or Chemically to Prepare Them for the Vitrification. Full-Term Developments Were Reported Except Where Otherwise Indicated

Human embryos

In humans, the clinical pregnancy rate from embryo transfer after slow-freezing has been approximately two-thirds that from the fresh transfer of embryos [169], although some techniques have helped to restore (cleavage stage) embryo viability [170, 171]. The theoretical possibility for improvement is supported by the results obtained in cattle, where the difference is no more than 10%–15%.

However, vitrification has a clear superiority for embryo cryopreservation and for this reason, it is the standard in most parts of the world [5, 22, 23, 79, 80, 88, 89, 93, 94, 100, 134, 153, 165, 172–184].

Early on, in 2005, three comparative investigations were published, and all three concluded that vitrification was a more efficient way for cryopreservation of human embryos than slow-rate freezing [69, 185, 186]. More recent comparative studies published in the literature have confirmed that vitrification is clearly more efficient than slow freezing used at different embryonic development stages [187–192]. Accordingly, these representative comparisons have proved that vitrification is more efficient than slow-rate freezing for the cryopreservation of human embryos at all stages [193]. In addition to those comparative studies, other, non-comparative studies on the efficiency of vitrification have been published, applying the technique at different stages of pre-implantation embryo development, including zygote, cleavage, and blastocyst stages [121, 151, 155, 192, 194–198], including also day-7 successful vitrification [199]. Based on these reported improved outcomes, a consensus meeting was organized by Alpha Scientist, to set minimum standards and inspirational outcome parameters (KPIs) following cryopreservation, that today set the standards worldwide [149].

Although several tools (carriers) and kits (vitrification/warming solutions) are currently available for vitrification, two technologies related to the type of carriers have obtained more attention initially: the OPS, predominantly in the animal field, and the Cryotop (and similarly formed other cryo-devices) for human areas. The more delicate Cryotop method may be the preferred choice where extremely high cooling rates are the primary objectives. As written earlier, there are now several new cryotools/carriers available on the market, which have been tested and used more widely for both embryo and oocyte vitrification. Storage time, as expected, had no impact on outcomes of vitrified embryos (or vitrified oocytes), as different studies have established [200, 201], if samples are stored and handled adequately, avoiding accidental warming.

Reproductive Biology Associates (RBA), an IVF Clinic located in Georgia, was one of the very first in the United States (and in the world) to apply embryo (and oocyte) vitrification in routine patient care. Initial data, when employing both techniques (slow freezing and vitrification) on the same time period (2006–2007) demonstrates significantly better outcomes with vitrification compared to slow freezing in comparable patient population (Table 22.3).

Vitrification and ART services

Routine application of vitrification has spread out all over the world in recent years [192, 202, 203], resulting in a paradigm shift in how assisted reproduction treatment is performed. The extreme high efficiency of vitrification applied on oocytes and embryos provides the possibilities for novel patient services. Oocyte and embryo vitrification now can provide the base for fertility preservation for both medical and social reasons [204–208] and for donor egg banking [101, 103], or for various other clinical conditions, such as hyperstimulation, failure to obtain sperm on the day of oocyte collection, or due to moral/ethical reasons for preferring egg preservation instead of embryo preservation [209–212], cryopreserving excess oocytes aspirated from IUI patients with excess follicles [213].

The highly efficient embryo vitrification, has opened up several new possibilities. One of the most important benefits relates to embryo biopsy and PGS/PGD. In the past, survival of embryos after slow-freeze/thaw following embryo biopsy was more than disappointing, strongly limiting the use of biopsy and genetic testing—mainly to be performed on day-3 cleavage stage, or for polar body biopsy [214, 215]. Applying vitrification instead of slow-freezing on biopsied embryos has significantly improved survival rates [185, 216], thus this procedure has become

TABLE 22.3 Outcomes of Embryo Cryopreservation—289 Cycles of Slow Freezing and 108 Cycles of Vitrification (1494 Frozen/Thawed and 418 Vitrified/Warmed Embryos)

Embryo Stage	Outcomes	Vitrification	Slow Freezing	P-Value
2PN	Survival rate	97%	85%	<0.001
	Clinical pregnancy rate	59%	43%	0.1864
	Implantation rate per embryo transferred	28%	23%	0.3921
	Implantation rate per embryo thawed	14%	10%	0.171
Cleavage	Survival rate	90%	75%	0.0044
	Clinical pregnancy rate	50%	43%	0.422
	Implantation rate per embryo transferred	32%	22%	0.0889
	Implantation rate per embryo thawed	26%	12%	0.0021
Blastocyst	Survival rate	93%	76%	<0.001
	Clinical pregnancy rate	65%	55%	0.2802
	Implantation rate per embryo transferred	36%	33%	0.6963
	Implantation rate per embryo thawed	33%	26%	0.1743
All Stages	Survival rate	94%	80%	<0.001
	Clinical pregnancy rate	61%	45%	0.0025
	Implantation rate per embryo transferred	33%	24%	0.0032
	Implantation rate per embryo thawed	23%	12%	<0.001

routine when embryos are to be tested genetically [190, 217]. Additionally, now biopsy timing can be shifted from day 3 (or day 0/day 1) to day 5/day 6, when embryos develop to the blastocyst stage, as there is no more need to use these embryos for fresh transfer, as they will survive cryopreservation much better, usually perfectly. Blastocyst stage embryo biopsy has several advantages compared to earlier stages, specially to day-3 stage biopsy, as embryos are more resistant to the biopsy procedure, more cells can be removed (genetic testing can be more reliable); less likely to encounter mosaicism; and embryos will be transferred in a (possibly) more receptive uterine environment. All these factors in combination result in very high pregnancy rates [217–220]. Pregnancies and live births were reported also when vitrification was repeated on the same embryo at the same (or different) stage or after oocyte vitrification, or even after involving a trophectoderm biopsy, demonstrating the robustness of the technique [104, 220–223]. Because of the extremely high success rates obtained with vitrified embryos after biopsy, it seems a logical extension of the thinking, that other patients with different clinical conditions may also benefit of the "cryopreserve all" embryos and perform transfer in a "cryo-cycle" [224]. Rationally, patients at risk for ovarian hyperstimulation can clearly benefit from vitrifying all embryos [198]. Other studies suggest "cryo-embryos" for women with endometriosis [225], while some may consider to apply this idea for all IVF patients, looking for the benefit of a more receptive endometrium in a cryo-cycle for patients who underwent ovarian stimulation [224, 226]. Additionally, patients with diminished ovarian reserve (irrespective of reproductive age) may also benefit, for the same reasons, from the "cryo-all" strategy. Instead of performing a fresh transfer, all embryos or oocytes (possibly vitrified at an early stage, due to the low oocyte/zygote number) are cryopreserved and then transferred later in a "frozen embryo transfer cycle"/"FER cycle" (either natural or supplemented cycle; [227, 228]). In fact, there are less and less reasons to perform fresh embryo transfer; this is the primary reason that today in most countries the "cryo-all" strategy is applied for most IVF patients—and, consequently, embryo transfers are performed in "FER cycles" [224]. In fact, when comparing live birth rates after fresh and "frozen" embryo transfer, data clearly demonstrates an overall superior outcome after "frozen" embryo transfer. For instance, in the United States, in 2001, the live birth rate following fresh transfer was 33.4%, and after "frozen" embryo transfer it was 23.4% (Figure 22.4). Fifteen years later, in 2016, the live birth rate after a fresh transfer was just very slightly increased to 36.3%; however, the "frozen" embryo transfer rate jumped to 45.9% (Figure 22.4 shows the "power" of vitrification). Moreover, the high efficiency of embryo vitrification also strongly promotes single embryo transfer. For instance, in the United States (where single embryo transfer is optional), the proportion of single embryo transfers increased from 18% (in 2010) to 77% (in 2019), as a direct consequence of having vitrification technology available (Figure 22.5.) In the past, when using slow-freezing, embryo survival was suboptimal, "promoting" higher numbers of embryos to be transferred as fresh, as it was not known how many of the cryopreserved supernumerary embryos would survive upon thawing (the other reason was the suboptimal embryo culture/development). Today, using vitrification, the viability of the embryo post-warming is virtually equivalent to the viability prior to cryopreservation, which in combination with a more "natural" endometrium can benefit patients and offspring [229]. The fact that in a 13-year period the number of "freeze all" IVF cycles increased 60× (from 2000 cycles in 2007 to 121,000 in 2019) is the direct consequence of the impact of vitrification IVF patient care, supported by other changes, such as improved embryo culture and the trend to cryopreserve embryos at the most potent stage, when developed to blastocyst; increasing use of pre-implantation genetic screening to deselect chromosomally abnormal embryos; and the effectiveness of vitrification, as well as taking advantage of a more receptive endometrium.

Safety of vitrification

For a new technique or technology to be fully accepted and applied worldwide, there are two critical points which need to be fulfilled: efficiency and safety. Vitrification of embryos/oocytes has now been clearly demonstrated to provide extremely high

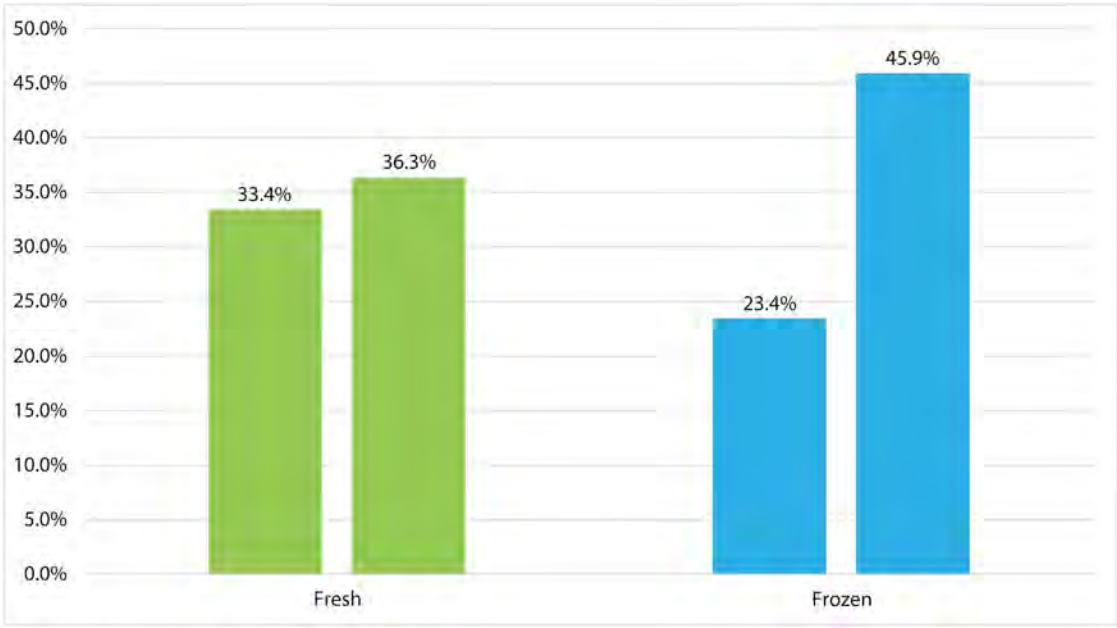

FIGURE 22.4 Live birth rates after fresh and "frozen" embryo transfers in the United States, 2001 and 2016.

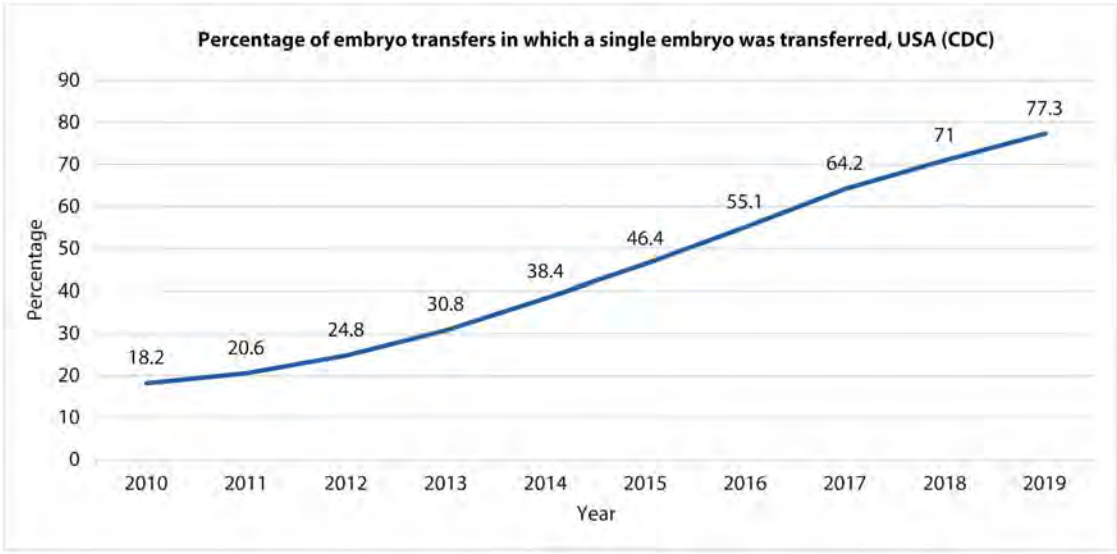

FIGURE 22.5 Proportion of embryo transfers with single embryo transferred in the United States, from 2010 to 2019.

efficiency, demonstrating outcomes similar to those achieved by using fresh oocytes/embryos. However, safety is a point yet to be proven beyond any doubt. Initial studies presenting outcomes on live birth data (mainly gathered after oocyte vitrification) do not indicate any alarming or unexpected results or trends [194, 220, 222, 230–234]. More recent data on babies born after embryo vitrification continue to demonstrate the safety of the technique, not showing increased risks for birth defects or other live birth parameters [235–238]. It would be most prudent for national or international IVF societies to organize the needed data collection through registry(ies). Only a multicentre effort where most IVF clinics participate would be able to provide a sufficient amount of data in a reasonable period of time.

Conclusions

Vitrification as an approach to cryopreserve human embryos or oocytes has achieved remarkable success. Today, vitrification is the "gold standard" in human-assisted reproduction, and most likely all (or virtually all) of IVF clinics are now using vitrification for reproductive tissue cryopreservation. This extraordinary achievement would not have been possible without the constant dedication and hard work of the few early pioneers, mainly coming from the field of veterinary medicine. On safety of vitrification, any currently available data do not indicate a higher incidence of malformation—which is reassuring, but obviously needs to be confirmed on a much larger scale.

The overwhelming majority of the studies/publications support the application of vitrification by emphasizing its advantages: the simple, inexpensive, and rapid procedure leading to higher survival and developmental rates than those achievable with alternative methods. Concerns regarding disease transmission are theoretically justified, but safer methods are now available to mitigate this risk. Outstanding results like the breakthrough in human oocyte vitrification and the excellent (and improved) results on embryo cryopreservation have changed the way how we practice routine IVF, providing more efficient and safer options for the patients.

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Appendix: Embryo/blastocyst vitrification protocol

Vitrification

Materials

Equilibration Solution (ES) is a HEPES buffered medium, 7.5% (v/v) of each DMSO and ethylene glycol and 20% (v/v) serum protein substitute.

Vitrification Solution (VS) is a HEPES buffered medium, 15% (v/v) of each DMSO and ethylene glycol and 20% (v/v) serum protein substitute and 0.5 M sucrose.

Cryolock® *Biodesign*, Columbia

Procedures

1. Bring one vial of each ES and VS to room temperature (20 C –27°C) for at least 30 minutes prior to freezing embryos.
2. Fill the liquid nitrogen reservoir with liquid nitrogen.
3. Determine the number of embryos to be vitrified.
4. Label each Cryolock with necessary information.
5. Prepare four-well dish with 1.0 mL ES and 1.0 mL VS in each well.

6. Transfer the embryos to ES for 15 minutes.
7. Transfer the embryos to VS for 1 minute.
8. Load the embryos onto the Cryolock with a minimal volume.
9. Plunge the Cryolock into liquid nitrogen (cooling at a rate of –12,000°C/minute).
10. Move the plunged Cryolock to the liquid nitrogen freezer for long-term storage.

Warming

Materials

Thawing Solution (TS) is a HEPES buffered medium, 1.0 M sucrose and 20% (v/v) serum protein substitute.

Dilution Solution (DS) is a HEPES buffered medium, 0.5 M sucrose and 20% (v/v) serum protein substitute.

Washing Solution (WS) is a HEPES buffered medium and 20% (v/v) serum protein substitute.

Procedures

1. Bring one vial of each TS, DS, and WS to room temperature (20°C –27°C) for at least 30 minutes prior to thawing embryos.
2. Fill the liquid nitrogen reservoir with liquid nitrogen.
3. Determine the number of embryos to be thawed.
4. Take the Cryolock out of the LN_2 and quickly transfer embryos into TS (3 mL at 37°C), where embryos should stay for 1 minute.
5. Transfer the embryos into 1.0 mL DS for 3 minutes at RT.
6. Transfer the embryos into 1.0 mL WS for 10 minutes at RT.
7. Transfer the embryos into pre-equilibrated culture medium.

23

MANAGING AN OOCYTE BANK

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Introduction

Nowadays, the challenge of the cryopreservation, long-term storage, and successful implantation of the female gamete is feasible thanks to vitrification. There is a large population that is currently benefiting from oocyte banks, such as cancer patients who need an option for fertility preservation before undergoing potentially sterilizing treatment [1] or women who wish to delay their motherhood due to a variety of reasons [2, 3]. Oocyte cryo-storage brings additional advantages to assisted reproduction technology (ART) programs, being helpful in solving different clinical situations such as low-response patients [4], unpredictable availability of semen sample collection from the male partner, risk of suffering from ovarian hyperstimulation syndrome [5], or some other cases in which embryo transfer is not advisable [6]. Undoubtedly, ovum donation programs have also been major beneficiaries of egg banking. Oocyte cryo-storage is very useful for overcoming the most common drawbacks involved in ovum donation as currently applied, such as synchronization between donors and recipients, long waiting lists subject to the availability of a suitable donor, and, most important, the absence of a quarantine period.

In spite of its great value, oocyte cryo-storage has not been a valid option until relatively recently, due to the lack of successful methodologies. The reasons behind the long period of failures in attempts to cryopreserve oocytes are well identified. Among them, the size and shape of the female gamete are two significant reasons. The female gamete is the largest cell of the human body, with a large content of water, leading to a higher probability of ice formation during the cryopreservation process. Chilling injury, defined as irreversible damage to the cytoskeleton [7] and cell membranes [8], following exposure of cells to low temperatures from +15 to −5°C before the nucleation of ice is another major factor responsible for cell death during cryopreservation [9]. Ice crystal formation within the cytoplasm must be avoided at all costs in order to guarantee the survival and integrity of the cells when they are later thawed. Vitrification efficiently avoids chilling injury by direct passage from room temperatures to −196°C and so avoids ice formation [10]. Vitrification employs both high cooling rates and high cryoprotectant concentrations [11]. However, due to the potential toxicity of these compounds, the vitrification protocols have been modified in order to reduce damage. Additionally, efforts have also focused on increasing both the cooling and the warming rates in order to guarantee the viability of the cells [12, 13]. As a result, these days we count on several efficient approaches that are able to provide successful outcomes comparable to those achieved with fresh oocytes, thus making oocyte banking a reliable approach.

In this chapter, we will briefly review the clinical outcomes achieved with the use of vitrified oocytes in ovum donation, but we will primarily focus on the essential issues related to the management of the oocyte bank, including a description of the facilities, the equipment for storage, and liquid nitrogen (LN) supply. We will also evaluate the most relevant clinical aspects involved in the management of the oocyte bank, such as donor selection, preparation of recipients, and the matching process.

Clinical outcome using an oocyte bank for ovum donation

Similar embryo development has been previously shown in embryos that originated from fresh versus vitrified oocytes in a sibling cohort study [14], whereas the clinical validation of using vitrified oocytes for egg donation was later demonstrated in a large randomized controlled clinical trial [15]. Comparable obstetric and perinatal outcomes of the babies conceived using vitrified versus fresh oocytes have been recently demonstrated in a large study involving more than 2000 infants, suggesting the harmlessness of the technology [16].

The use of cryo-stored oocytes in a large ovum donation program has been evaluated recently [17]. The overall survival rate analysed in this large series including over 40,000 vitrified oocytes was 92.6%. The possible effects of storage time on the survival rate and clinical outcome was calculated in different time categories from less than six months until over five years, showing no impact on either survival rate or clinical outcome [17]. We believe that this is very reassuring information since success after long-term storage guarantees the sustainability of the approach. The clinical, ongoing pregnancy and delivery rates were 55.0%, 45.3%, and 37.6%, respectively, thus confirming the consistency of the results as compared to our previous findings [15, 18]. The likelihood of having surplus embryos available for additional cryo-transfers was very high in this series due to the mean number of oocytes donated. The possibility of further cryo-transfers increased cumulative outcomes, and thus maximized the yield of a single-donation cycle, which is precisely what we show herein. The cumulative delivery rate per donation cycle increased to more than 70% after three cryo-transfers and rose to nearly 80% after five cryo-transfers. These results render the donation cycle highly efficient. This finding supports the previous observations we made about the absence of harmful effects of double vitrification (i.e. vitrified embryos developed from vitrified oocytes) [19]. The probability increases exponentially according to the number of oocytes consumed, and the patient can achieve a baby at any number of consumed oocytes with a probability of almost 100% when around three to four donation cycles are completed [17].

To date, we have notification of more than 16,000 babies born (n = 16,739) after above 40,000 ovum donation cycles with vitrified oocytes (41,042), involving nearly 500,000 vitrified oocytes (n = 488,022) in the Instituto Valenciano de Infertilidad (IVI) group (unpublished data), revealing the great scope of this approach. At present, more than 20,000 *in vitro* fertilization (IVF) cycles (n = 20,229) involving the use of own vitrified oocytes (~190,000 oocytes) have been performed at our centres (4384 babies born from whom we have notification), accounting for ~21,000 children born from vitrified oocytes in our group.

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Logistics and technical aspects related to the oocyte bank

Facilities

In accordance with European Directive 2004/23/EC, ART laboratories including centres or clinics, along with banks of gametes, are considered tissue establishments and therefore are under the regulations and standards that were placed to prevent the transmission of infectious diseases of human tissues and cells.

Safety measures need to be implemented not only during procurement, testing, and processing but also during preservation, distribution, use, and, of course, storage. Here, we will describe some of the technical features that an oocyte bank has to meet in order to fulfil the European regulations and so be qualified in the four following aspects: design, installation, operation, and performance.

Regarding the facilities, one of the aspects to be qualified in is related to location, air quality, and construction materials.

Location of the storage room

From the practical point of view, the storage room with the LN tanks should be located close to the IVF laboratory so the cryopreserved oocytes can be easily, rapidly, and successfully transferred to the storage room and into the LN tanks.

Concerning distribution purposes, having your own oocyte bank will be logistically easier for distribution and use. However, oocyte transport is also a feasible and a safe option that will be reviewed in this chapter.

As far as dimensions are concerned, the storage room should be designed to allocate a sufficient number of tanks to the storage of the expected number of samples. Some experts suggest calculating the space based on a linear increment within a 10-year plan basis or to have an off-site storage room in case of urgent need for extra space [20, 21].

Environmental variables

Although storage facilities might not need to strictly follow the same environmental criteria as procurement and processing facilities, it is recommended, at least for oocyte banking in vapour phase and semi-closed systems, to implement preventive measures in order to minimize bacterial and other airborne contaminations during storage. Such preventive measures can be implemented by installing high-efficiency particulate air filters within the air conditioning system to remove small particles (<0.3 mm); positive pressure could also be considered as an option.

Tissue establishments in Europe must achieve grade A-quality environmental air during procurement and processing; however, since fewer critical steps are performed in the storage areas, grade D-quality background air is acceptable.

The effects of volatile organic compounds (VOCs) on cryopreserved human oocytes and embryos have not yet been evaluated; therefore, it is difficult to assess the level of stringency in terms of VOC control in the storage room. Our recommendation would be to control and minimize VOCs by use of fixed or mobile versions of photo-catalytic oxidizing units or similar approaches.

With regards to temperature, even though room temperature (22°C–23°C) should be adequate, setting up the room under a cold temperature might help to minimize the LN evaporation and water condensation that can facilitate microbial growth. Another approach can be undertaken by setting up a humidity controller.

Moreover, low-level oxygen sensors and alarm systems in case of LN leaks have to be put in place for safety reasons. As a part of the clinic's general emergency plan, the storage facility should also have generators or an uninterrupted power supply system in case of loss of electrical power.

Equipment

All our samples are cryopreserved by vitrification. This procedure, as currently performed, is entirely manually operated, making the use of any equipment to carry out the vitrification process itself unnecessary. The ease and efficiency of vitrification have brought about a turning point in the field of cryopreservation, making the whole process take no longer than 20 minutes (vitrification and warming) and involving very simple tools. However, the fact that the samples are vitrified and mostly contained in very low volumes represents a challenge for further handling, storage, and maintenance of the vitrified samples. Here, we describe the material and equipment needed for the proper storage of vitrified oocytes in our oocyte bank facilities.

Storage tank

The storage vessel can be traditional LN tanks or vapour tanks. In our oocyte bank facilities, we use vapour-phase storage tanks (CBS V1500; Custom Biogenic Systems, Bruce Township, MI) which contain an outer jacket with LN (Figure 23.1). This is responsible for cooling the storage area where the oocytes are maintained in a nitrogen gas atmosphere. The cold spreads from the vacuum-insulated jacket by convection and through vents in the storage compartment that expel the nitrogen vapour downwards to the bottom of the freezer, thus creating a flow of extremely cold air through the entire storage area (Figures 23.1 and 23.2). The exceptional uniformity of temperature allows the whole storage tank to be used, achieving temperatures below –180°C at the upper level and –195°C at the bottom. Samples can be manipulated in safe temperature ranges (–180°C) thanks to the working area located on top of the storage area, thus avoiding any risk of accidental warming (Figure 23.2). Figure 23.3 shows the disposition of samples in the storage area. Nearly 11,000 Cryotops can be stored in each tank. An additional advantage of this storage system is that the supply of LN can be programmable, although it also can be performed manually. We have demonstrated the effectiveness of this storage vessel as a strategy for preventing the risk of cross-contamination due to direct contact with the LN, showing comparable results between vapour-stored oocytes versus those stored in conventional LN tanks [22].

For periodical cleaning and due to the more complex and sophisticated nature of these tanks, we recommend regular maintenance, which forces the emptying of the vessels and the temporary location of the samples in a backup tank intended for that purpose. The backup tank must provide the same safe conditions as the storage tank. The emptying for cleaning and maintenance should be scheduled in advance and needs to be performed following strict standard operating protocols.

Construction, nitrogen supply, and gas pipes

The types of construction materials should be similar to those used in procurement and processing facilities, consisting of smooth surfaces and being easy to clean. Perhaps one of the most particular considerations to be undertaken with regard to contraction materials are that the floor should be resistant to large changes in temperature so that it will not easily crack as a result of LN spills.

In our facilities, there are three essential elements for the nitrogen supply to the storage tanks: firstly, a large-scale reservoir of LN (cistern with 2400 L capacity able to supply LN to approximately 10 CBS V1500 vapour tanks) located outside the

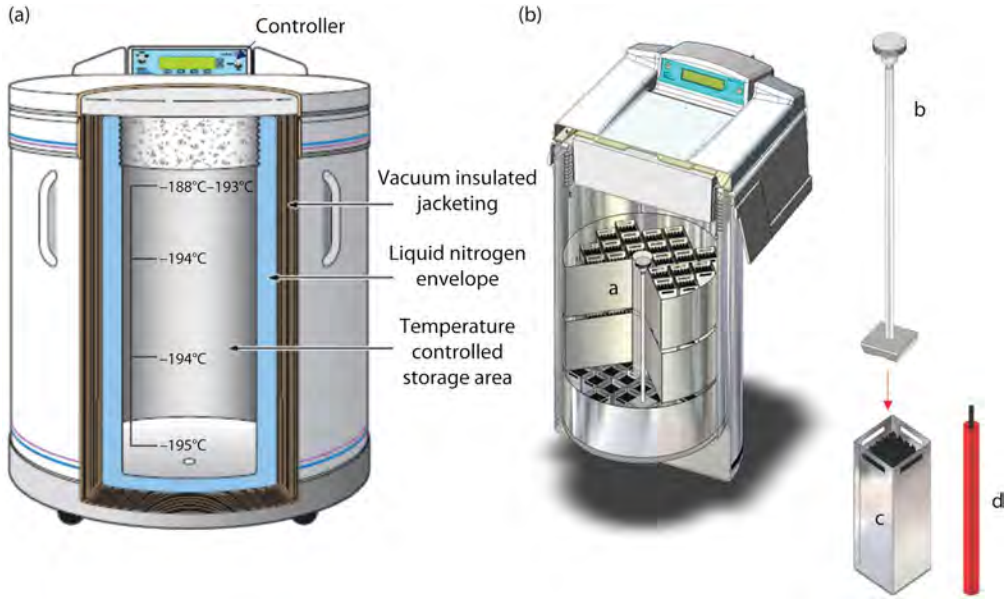

FIGURE 23.1 (A) Diagram to illustrate the inside of the tank, showing the jacket with liquid nitrogen (LN) and the vapour area for storage. (B) (a) Three storage levels assembled on a rotating carousel. (b) Retrieval tool to place and retrieve the canister (c). (d) Goblet containing the samples that are placed into the canisters.

building (Figure 23.4); secondly, a pressurized tank fed by the reservoir; and thirdly, vapour storage tanks that receive supply from the pressurized tank. The circuit is controlled by an automated, programmable system (Simatic Siemens PLC HMI, Nürnberg, Germany). The system is able to control a number of adjustable parameters, such as minimum and maximum permissible levels, pressure of LN filling, and low-level and overfilling alarms.

The conduction system for LN should be completely insulated to avoid loss of temperature and excess condensation and to minimize the evaporation of LN during refilling manoeuvres.

Additionally, individual valves allow the influx of LN into the jackets of the storage tanks. To prevent the impurities that LN may contain entering the storage tank, the use of a pre-filter is strongly recommended, as the presence of "debris" could cause serious problems to the valves of the storage tank (Figure 23.4c).

Nitrogen supply for the vitrification process

With the aim of purifying the LN used during the vitrification process, a specific ceramic filter is coupled to the pressurized tank (Figure 23.5). The Ceralin online filter (Air Liquide

FIGURE 23.2 (a) Working area of the vapour tank showing a storage canister (A arrow) and the vitrification rack (B arrow) at the time of storing oocytes. (b) Display showing the temperature while manipulating the oocytes (–184°C). (c) Storage room.

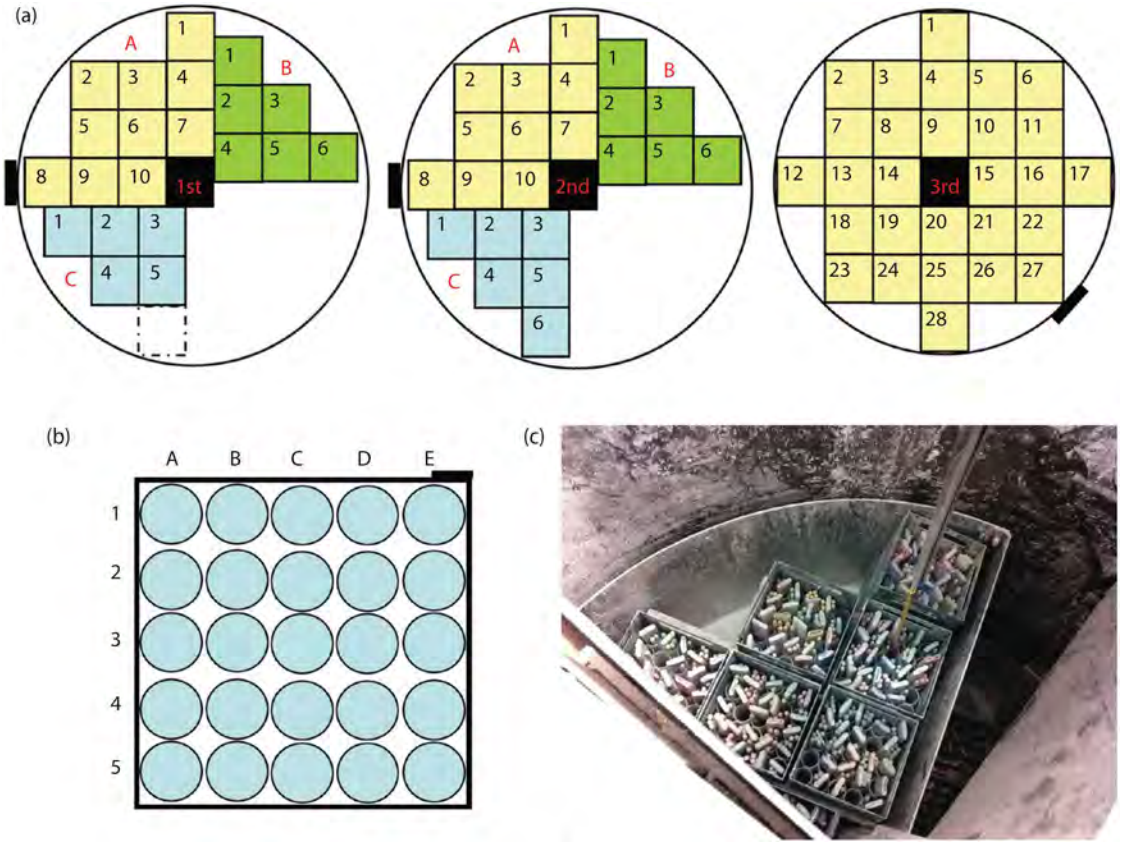

FIGURE 23.3 (a) Schematic drawing showing the arrangement and number of canisters for storage. Each canister is divided into 25 individual alphanumeric positions (b) for a total of 1800 positions in each tank. Each position can hold up to six Cryotops (10,800 Cryotops per bank). (c) Detail of some stored samples showing the canisters containing the goblets and Cryotops. Each goblet contains samples from individual donors. Placing samples from different patients in the same goblet is not allowed.

Medicinal, Paris, France) consists of a 0.1 µm ceramic membrane in accordance with US Food and Drug Administration Guidelines on Aseptic Processing (1987) [23]. The Ceralin online filter consists of two elements of liquid filtration connected in series and inserted into a section of the vacuum transfer line. The ceramic membrane is made from multiple layers formed into a multichannel element. It is housed in a vacuum-insulated pipe, itself installed close to the end-use point. During operation, LN flows through the filter and over the ceramic membrane. The result is high-purity LN with a bacteria count of less than one colony-forming unit (CFU)/L gas. Additionally, the large filtration area of the membrane and low level of contamination of LN means it is likely to be several decades before filter saturation. Periodic sampling for microbial assessment is needed.

Temperature monitoring system during storage

Vitrified samples, especially those loaded in minimum volume in the vitrification device, are extremely sensitive to any change in temperature. For this reason, a temperature monitoring system is strongly advised as a part of the routine quality control (QC) of the cryo-lab. In our facilities, we use a system that allows continuous monitoring of the temperature of every storage tank in our unit (DataCare, ControlTemp, Barcelona, Spain). The system is able to provide numeric and graphic records (Figure 23.6) and display alarms in real time with updates every second. A record of incidents occurring during the alarm can also be easily assessed, differentiating between active alarms or alarms that were active but are no longer in that state. In case of an alarm, the system sends alerts and warning messages to authorized personnel.

Safety during handling of LN

All safety measures for secure handling of LN must be observed. All laboratory personnel, especially embryologists/technicians in charge of the bank, vitrification, and all the related procedures must be aware of the Material Safety Data Sheet for LN and should be informed of the potential hazards of its use. The banking area should be located in a well-ventilated room. The measurement of oxygen levels is highly advisable due to high concentrations of nitrogen potentially reducing the breathable oxygen in the air. Approved personal protective equipment for eyes, cryogenic gloves, lab coats, closed-toe shoes, and long pants are mandatory.

Management of donors and recipients

Egg donor selection

Spanish assisted reproduction law is based on legislation that was passed in November 1988 (Law 35/1988) [24]. Although some countries already had regulations on or recommendations for ART at that time, Spain was the first country to create a specific law to cover this area of medicine. Royal Decree 412/1996 and Ministerial Order of March 25, 1996, established donor requirements, as well as mandatory standard screening procedures, to

FIGURE 23.4 (a) Reservoir tank for liquid nitrogen (LN) storage (Air Liquide, Madrid, Spain). (b) Detail of the touchscreen controller of the system showing the scheme for the filling of the pressurized tank. The filling of the pressurized tank (Apollo 350, Cryotherm, Kirchen (Sieg), Germany) begins at −130°C and is controlled by the system by actuating three solenoids (V1, V2, and V3). All the LN-phase gas coming from the reservoir tank via pipe A is disposed of in order to ensure that the pressurized tank is filled with liquid-phase nitrogen. The LN fills the pressurized tank (nurse tank) when the pre-set temperature is reached. The excess pressure generated during the filling phase is removed via pipe B. The valves automatically close when the filling is completed. LN is supplied to the vapour storage tanks via specific pipe C. In case of failure, the system can be handled manually by the action of the manual solenoids V4, V5, V6, and V7. (c) (1) Pipe with insulating coating for LN; (2) online wire mesh pre-filter; (3) entry valve for each tank. (d) Pressurized (nurse) tank. The arrow shows the ceramic filter Ceralin online.

rule out the transmission of genetic, hereditary, or infectious diseases [25]. In 2006, a new Spanish Law on Assisted Reproduction was approved (Law 14/2006) [26], which determined requirements for gamete and embryo use and regulations on financial compensation. A new Royal Decree 9/2014 established quality and safety standards for the donation, procurement, testing, processing, preservation, storage, and distribution of human tissues and cells.

The following are the most important topics included in Spanish law on egg donation:

- Donation of human gametes is a formal, confidential contract between the donor and the reproductive medicine centre. Identities of donors must remain anonymous.
- The donation cannot be revoked.
- The maximum number of children generated from a single donor's gametes should not exceed six.

To be accepted as egg donors, women must be aged between 18 and 35 years and be healthy. The following steps are necessary to be admitted as an egg donor in our clinics:

- *Medical history*: During the first visit, an interview is conducted to complete the family and personal history.
- *Psychological screening*: Psychological evaluation and counselling by a qualified mental health professional. The donor will be asked to speak with a psychologist to ensure that she fully understands the benefits and risks of egg donation and is properly motivated to become a donor.
- *Gynaecological examination*: Evaluation of the donor's menstrual cycles and vaginal ultrasound is needed to examine ovaries, count antral follicles, and to ensure that there is no pathology in her ovaries. At the same time, body mass index is calculated.
- *Medical screening*: This involves testing for blood type, Rh factor, antibody screening, complete blood cell count, haemostasis, biochemistry, and infectious disease screening, such as HIV, hepatitis C virus (HCV), and syphilis.
- *Genetic screening*: Blood tests for karyotype and carrier screening tests for severe recessive and X-linked childhood diseases based on next-generation sequencing (NGS) are conducted

To begin the egg donation cycle, an oral contraceptive pill is taken for a maximum of 21 days, which starts on days 1 or 2 of the menses of the previous cycle [27]. After a five-day washout period following taking the last pill, donors start their stimulation protocol with 150–225 IU of recombinant follicle-stimulating hormone (FSH), and 225 IU of highly purified human menopausal gonadotropin (HP-hMG), or 150–225 IU of recombinant FSH

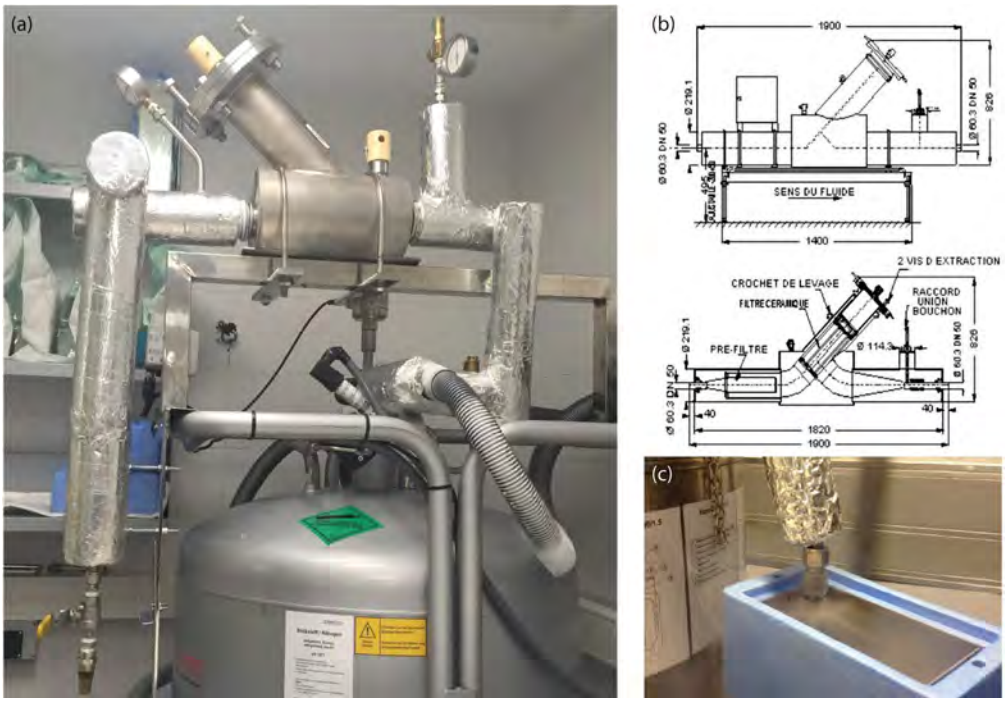

FIGURE 23.5 (a) Ceralin online filter (Air Liquide Medicinal, France). (b) Schematic illustration. (c) Collection of filtered liquid nitrogen (LN) in a sterile container used for vitrification.

plus 75 IU HP-hMG. Medroxyprogesterone acetate is administered orally as a single daily dose throughout stimulation until trigger day for pituitary suppression [28]. Egg donors are monitored regularly during FSH injections to measure follicle growth and to ensure it is within an appropriate range. Vaginal sonograms and blood tests are used to monitor follicle growth. Once follicles have matured enough for retrieval, a single dose of gonadotropin-releasing hormone (GnRH) agonist is administered to trigger final oocyte maturation. Transvaginal oocyte retrieval takes place 36 hours after GnRH agonist administration. Donors receive light intravenous sedation for the egg retrieval procedure to ensure their comfort, and they rest for two hours at the clinic until they are discharged. In some cases, a post-retrieval vaginal scan is scheduled two to three days following egg retrieval [29].

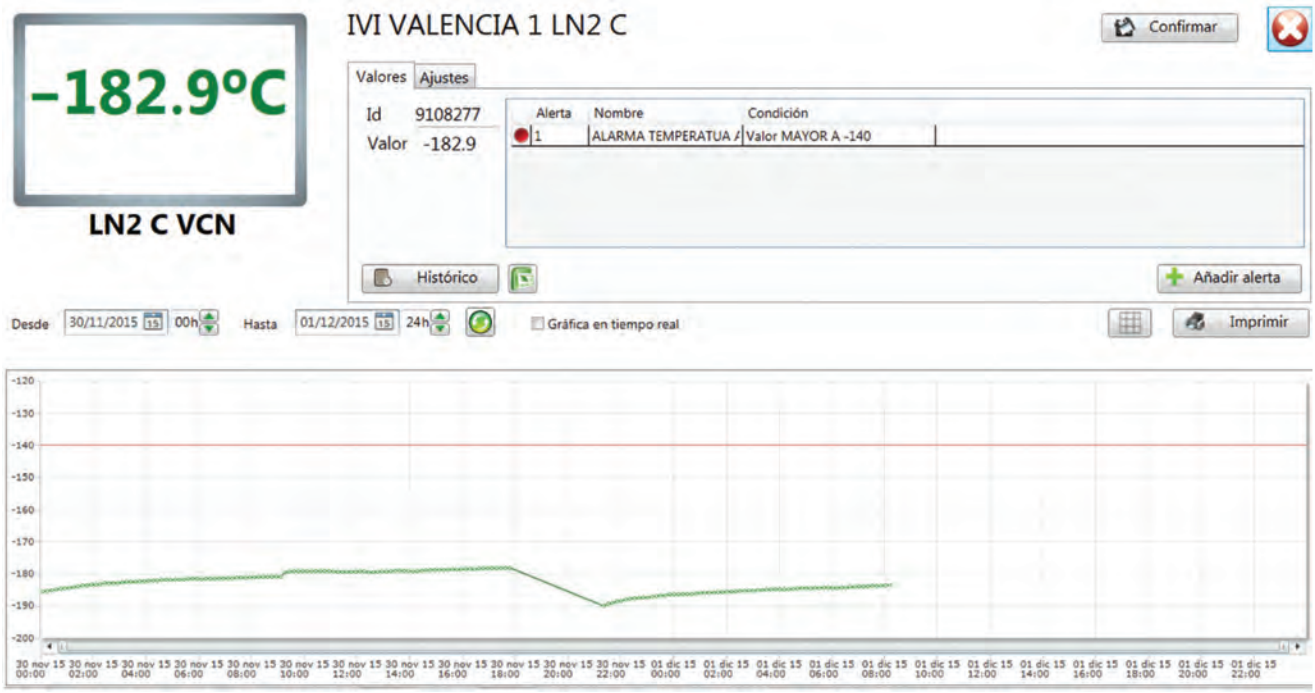

FIGURE 23.6 Datalogger graphic representation of temperature measurement over a time period of one vapour storage tank.

Oocyte recipients

Oocyte recipients enter our egg donation program for one of the following main diagnoses: premature ovarian failure/menopause; failure to achieve pregnancy after at least three cycles of assisted reproduction techniques; genetic or chromosomal disorders; low response to controlled ovarian hyperstimulation; or recurrent miscarriages.

The vast majority of oocyte recipients undergo hormone-replacement therapy (HRT). In patients with ovarian function, depot GnRH agonist is administered in the mid-luteal phase of their cycle, or GnRH antagonist is administered daily with menstruation for five days. HRT is initiated on days 1–3 of the following cycle with oral oestradiol valerate or an oestradiol transdermal patch [30–32]. Recipients without ovarian function are submitted to the same endometrial preparation protocol but are not administered a depot GnRH agonist. On days 15 or 16 of HRT, a transvaginal ultrasound is performed to measure endometrial thickness, and serum E2 and progesterone levels are tested. Most recipients are ready to receive embryos within two to three weeks of starting HRT, although administration of oestradiol valerate can be maintained for a maximum of 50 days until a suitable donation becomes available. Micronized progesterone (800 mg/day vaginally) is initiated on the day of oocyte donation, and embryos are transferred in the blastocyst stage. Progesterone levels are measured on the day of embryo transfer and luteal phase supplementation is adjusted according to progesterone levels [33]. The recipient continues taking oestrogen and progesterone with a positive pregnancy test, and these hormonal supplements are then continued through 12 weeks of pregnancy. Before treatment begins, the recipient undergoes preliminary testing. This assessment phase includes infectious disease screening (e.g. HIV, HCV, and syphilis), blood type, and Rh factor analysis for both parents and TRH. In women older than 45 years, a recent mammogram, full blood cell count, coagulation tests, and blood biochemistry may also be required.

To help the donor team select an egg donor, recipients will be asked to complete a form regarding their physical characteristics, such as hair colour, weight, height, eye colour, and biometrics (facial similarity study), among other traits.

It is advisable to collect a sperm sample if the partner lives far from the clinic.

Ovum donation synchronization

We consider many different factors during donor selection: we take into account race, reproductive history, and the physical characteristics that match those of the female partner, and we match blood type and genetic carrier screening. We call matching the time when we select a donor for a recipient after considering all the aforementioned factors.

The timing for the matching procedure has been improved in the last years thanks to the establishment of egg banking. However, it is important to note that in our current practice, we conduct donations both with fresh and vitrified oocytes, as long as fresh donations are still allowed in our country. Whether to conduct one strategy or another depends on different circumstances related to the availability of oocytes and the needs of the recipient.

Before introducing vitrification into our egg donation program, the numbers of donors and recipients in the clinic are determined at the time of matching: if there are many donors, the matching between donors and recipients is done on the day of the donor's pickup. This means that sometimes recipients are on the waiting list for so many days that they may start bleeding. If, however, the clinic has very few donors needed for special considerations, then donors and recipients are synchronized. This means that recipients and donors start with ovarian stimulation (donors) and HRT (recipients) at the same time. Donor's stimulation may generate certain drawbacks as cycle cancellations due to different causes such as bleeding, etc., or fewer oocytes than expected). Consequently, the date for the donation is only indicative in these cases, therefore cannot be officially scheduled [18]. The likelihood of this happening underlines the importance of having a large egg donor bank with the availability of a large and varied number of stored oocytes that meet different characteristics.

In addition to the previous difficulties, about 65% of our recipients come from foreign countries. As such, compatibility issues, from a medical viewpoint, and the logistics of the process need to be considered.

As our usual medical practice now has an egg bank, the time of matching the donor and the recipient depends on different aspects, such as if recipients need specific characteristics or have requested a specific date for embryo transfer.

- Recipients who need specific characteristics: blood type (O negative, AB negative), specific race, screening for specific genetic diseases, or partners who would like to have another baby with the same donor as they had before:
  - First, we use our donor selection database and select one donor or two with the required characteristics. Sometimes there will be donors under stimulation with the required characteristics, and sometimes we call them to return to our clinic.
  - Second, all the oocytes obtained during pickup are vitrified for the recipient.
  - Finally, the recipient chooses the best time to schedule embryo transfer, and we provide them with instructions to begin HRT depending on embryo transfer.
- Recipients who do not need specific characteristics:
  - Recipients have a date for embryo transfer.
    - First, we make a reservation of oocytes from our egg donor bank.
    - Second, the recipient begins HRT depending on embryo transfer.
    - Finally, we have two options:
      - We use fresh oocytes when we have a donor pickup scheduled on the same date as the donation (with the same characteristics as the partner). The reservation of stored oocytes is cancelled in this case.
      - We use vitrified oocytes.
  - Recipients who do not have a date for embryo transfer:
    - The recipient begins HRT and remains on the waiting list.
    - We use fresh oocytes for the egg donation if we have scheduled a fresh donor pickup.
    - If the recipient stays on the waiting list longer than 20–25 days, we use oocytes from the egg donor bank.

We have created software that allows us to manage the ovum donation program, including all the relevant information about the donors. This application includes donors currently under-stimulation, donors with vitrified oocytes, and vitrified oocytes

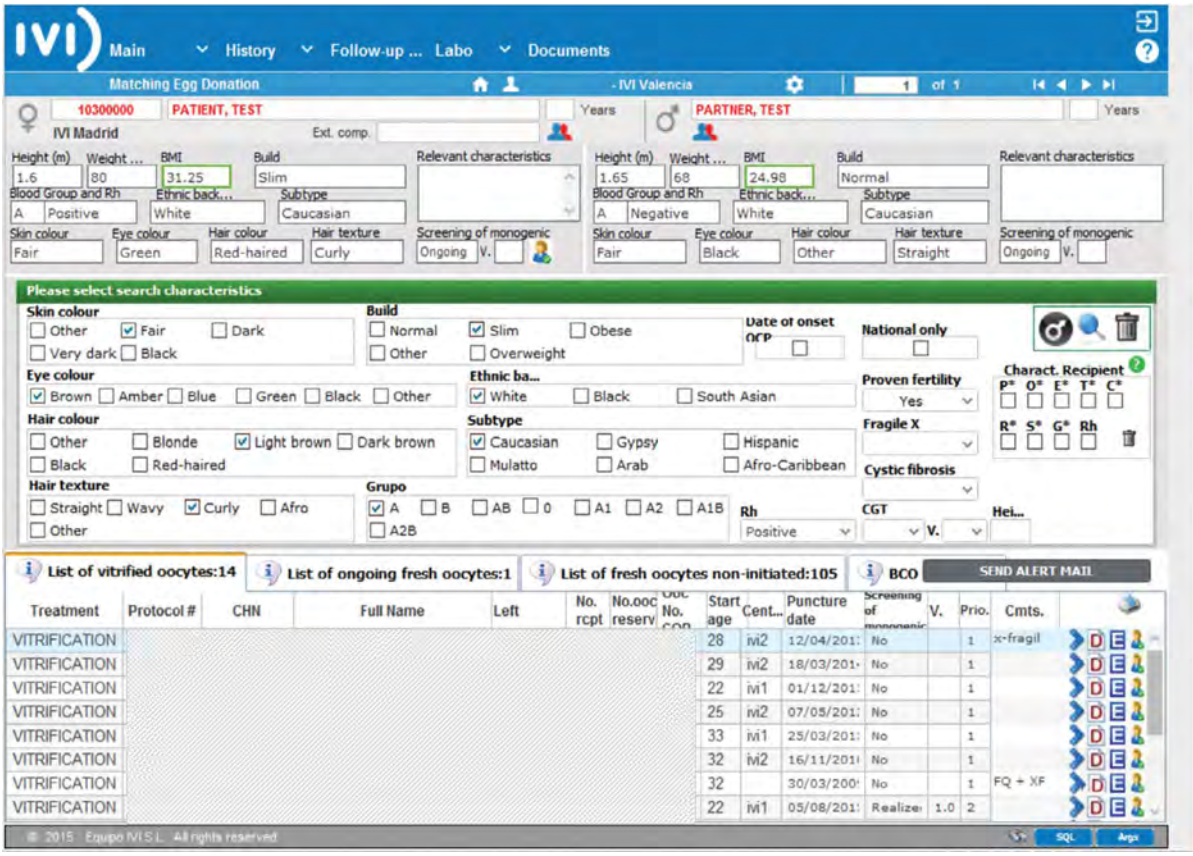

FIGURE 23.7 Matching sheet for donors and recipients. (Equipo IVI S.L.©)

located at different IVI facilities. The software also counts on a matching application that provides a list of the best possible donors after the introduction of recipients' characteristics, including phenotype, blood type, and other special features (Figure 23.7).

QC in the oocyte bank

The cryo-lab, including the bank, is part of the IVF lab, and, as such, it must be subjected to strict QC. In general, the same QC parameters for controlling the IVF lab are useful for the cryo-lab as well [34]. Accordingly, the cryo-lab needs to monitor and document the temperature, pH, osmolarity, and culture media, including vitrification solutions. The temperature of the storage tanks needs to be strictly controlled (Figure 23.6).

On the other hand, unlike other laboratory procedures, vitrification as currently performed is an entirely hand-operated procedure, for which outcomes are usually highly dependent on the embryologist/technician. Thus, in order to ensure efficiency, it should be performed only by highly skilled professionals who have undergone a long learning curve. Therefore, an adequate learning curve is also one of the most important requirements when performing vitrification that requires close attention. Our training program has produced satisfactory results since the introduction of vitrification in our clinical setting. It consists of different phases that gradually increase in difficulty. To pass to the next level, trainees must acquire the necessary skills as well as achieve a pre-set survival rate. Additionally, dynamic database management analysis is routinely performed in order to monitor the maintenance of competence. Periodic analysis of success rates per operator is strongly advised. Figure 23.8 shows survival and clinical pregnancy rates per technician performing the vitrification and warming procedures.

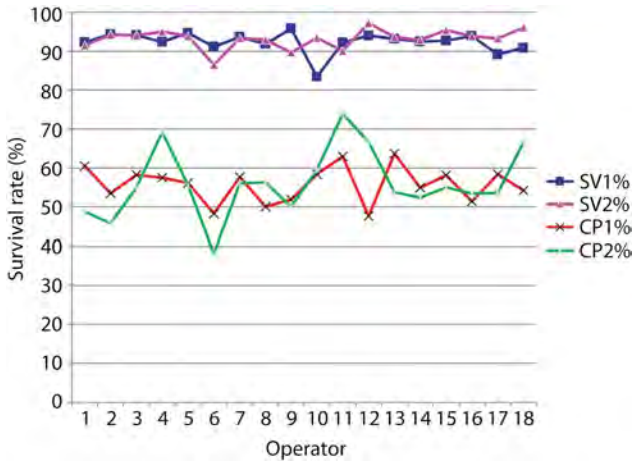

FIGURE 23.8 Survival and clinical outcomes according to the operator. SV1% and CP1%, survival and clinical pregnancy rates for the person doing the vitrification procedure; SV2% and CP2%, survival and clinical pregnancy rates for the person doing the warming procedure.

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24

SEVERE MALE FACTOR INFERTILITY

Genetic Consequences and Recommendations for Genetic Testing

Katrien Stouffs

Overview

Infertility associated with a severe male factor such as oligo-astheno-teratozoospermia (OAT) or azoospermia may be of genetic origin. This means that either the number or the structure of the chromosomes may be aberrant or a gene defect may be present. By knowing the underlying molecular cause of the fertility problems, appropriate genetic counselling can be offered to the patient, his partner, and his family whenever indicated. The role of genetic counselling in case of infertility has increased since the advent of assisted reproduction technology (ART) in general, and certainly since the introduction of intracytoplasmic sperm injection (ICSI), offering the possibility to have children to men with almost no spermatozoa [1–3]. Based on the available data, today a number of genetic tests should also be performed in case of infertility in an otherwise healthy male. For years, the main diagnostic tests have been the analysis of the karyotype in peripheral lymphocytes, a search for the presence of a Yq11 deletion on the long arm of the Y chromosome, and/or an analysis of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene in couples in which the male partner has congenital bilateral absence of the vas deferens (CBAVD). More specific genetic investigations can be done if indicated, especially when the azoospermia or oligozoospermia is part of a more complex disease or syndrome. Although currently not offered on a routine basis for patients with azoospermia or oligozoospermia, next-generation sequencing will be implemented in the near future.

Genetic causes of azoospermia and oligozoospermia

Chromosomal aberrations

It has been known for decennia that the presence of an extra X chromosome in males, resulting in a 47,XXY karyotype, causes Klinefelter syndrome, with testicular atrophy and non-obstructive azoospermia as main features [4, 5]. Since then, many chromosomal studies have been performed in series of infertile males, and the conclusions drawn from these studies are that constitutional chromosomal aberrations increase as sperm counts decrease.

From these studies, it is also clear that the incidence of numerical sex chromosomal aberrations such as 47,XXY and 47,XYY is proportionally higher in males with azoospermia than in males with oligozoospermia, whereas structural chromosomal aberrations of autosomes such as Robertsonian (Figure 24.1a) and reciprocal (Figure 24.1b) translocations are proportionally more frequent in oligozoospermic males (Table 24.1) [6–8].

In azoospermic males, it is also possible to find a 46,XX karyotype. In roughly 90% of these Klinefelter-like males the *SRY* gene, normally located close to the pseudo-autosomal region of the short arm of the Y chromosome, is now, due to a crossing-over event during meiosis, present in that same region on one of the X chromosomes [9–11]. The *SRY* gene, referring to the sex-determining region of the Y chromosome, has to be expressed to induce the sexual development of an embryo towards a male phenotype [12]. In the remaining 10% of XX males, most probably other genes with functions in sexual development are involved. Spermatogenesis seems to be absent in these XX males, whereas in apparently non-mosaic Klinefelter patients sometimes a few spermatozoa can be found in testicular tissue. This can be explained by the absence of the long-arm of the Y chromosome containing the azoospermia factor (AZF) regions in XX males. Spermatozoa obtained from Klinefelter patients have been used in ICSI procedures, and healthy as well as a few 47,XXY children have been born (reviewed in Fullerton et al. [13]).

Microdeletions on the long-arm of the Y chromosome (Yq11)

The first azoospermic male patients in whom a deletion in the q11 region of the long-arm of the Y chromosome (Yq11) was linked to their infertility, were identified through conventional cytogenetic analysis [14]. At that time, the concept of the AZF region, the region lacking factors (genes) necessary for spermatogenesis due to a deletion, was introduced. Since that time, the structure of the Y chromosome, consisting of the gene-containing euchromatic parts (Yp and Yq11) and the polymorphic heterochromatic parts (Yq12), has been studied in detail using more sensitive molecular techniques. These have also helped to define the AZF region better. In fact, the AZF region consists of three subregions: AZFa, AZFb, and AZFc. Deletions in these subregions are most of the time not readily detectable by cytogenetic analysis. The most currently used techniques to reveal detailed information on the presence of deletions in this region are a polymerase chain reaction investigation with in-house developed primer sets or a commercially available kit. In the current guidelines, a two-step approach is recommended. First, the three subregions must be investigated through a multiplex PCR analysis. If a deletion is detected, an extension analysis must be performed to confirm the presence of a deletion and to investigate its size [15]. Also, massive parallel sequencing technologies are currently being applied for the detection of Yq microdeletions. Nevertheless, the investigation of the Y chromosome remains challenging due to the repetitive sequences located on the Y chromosome. In the late 1990s, multiple studies were performed to investigate the frequency of Yq microdeletions. These studies showed a prevalence of around 7.4% of Yq microdeletions, and again the prevalence is higher in azoospermic (9.7%) than in oligozoospermic (6.0%) males [16]. In most patients, the deletions span the AZFb and/or AZFc regions, whereas in a small number the AZFa region is deleted. Most

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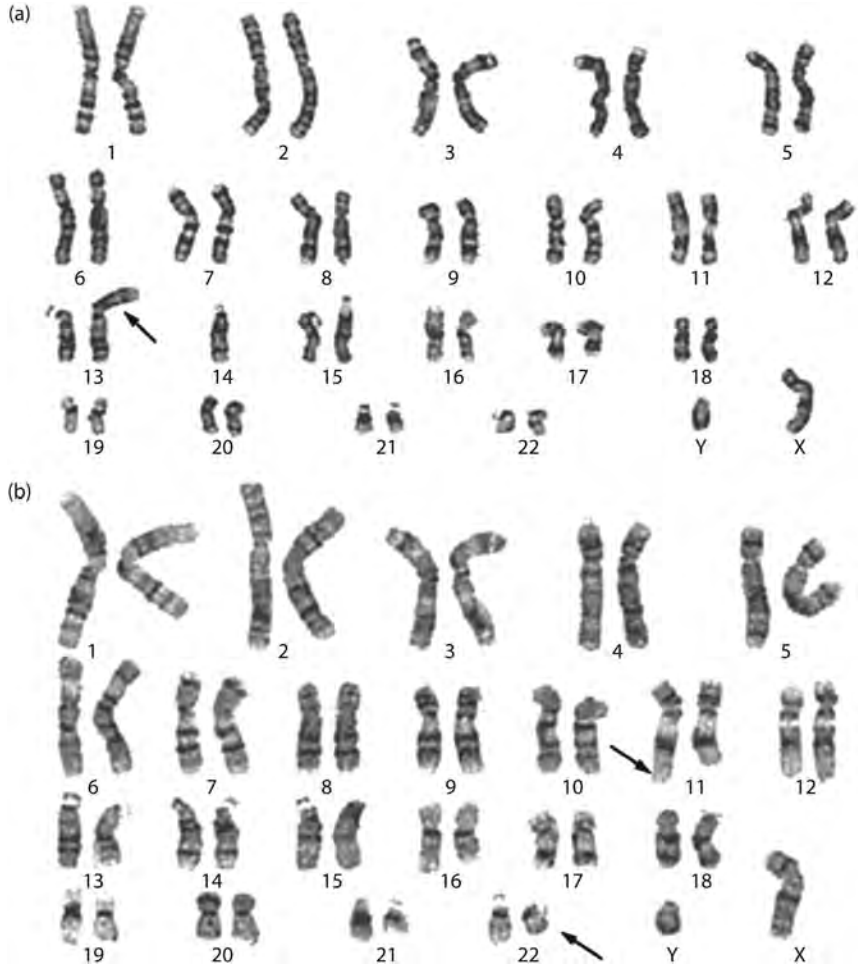

FIGURE 24.1 (a) 45,XY,der(13;14)(q10;q10) karyotype from a phenotypic normal male with a Robertsonian translocation of chromosomes 13 and 14 through centromeric fusion. (b) 46,XY,t(11;22)(q24.3;q12) karyotype from phenotypic normal male with a balanced reciprocal translocation of chromosome 11 and 22 with break points in 11q24.3 (↘) and 22q12 (↖).

deletions occur by intra-chromosomal homologous recombination between repeat sequences spread over the Yq11 region [17–19]. These repeat sequences are either palindromes consisting of inverted repeat arms or intra-chromosomal repetitive sequences. It is evident that if these microdeletions cause a spermatogenic defect leading to a low to very low sperm count present in the ejaculate or a few sperm cells in the testes, these microdeletions will, through the use of ICSI, be transmitted to sons, who most probably will be infertile as well [20]. In a few exceptional cases, fertility has been described in AZFc-deleted fathers who transmitted the deletion to their now-infertile sons [21–23]. Age at investigation may play a role, as observed in one patient with an AZFc deletion being oligozoospermic and later on azoospermic [24].

CBAVD and cystic fibrosis

Men with CBAVD have obstructive azoospermia. Spermatogenesis is usually normal and sperm can be obtained through microsurgical epididymal sperm aspiration, testicular sperm extraction, percutaneous epididymal sperm aspiration, or epididymal or testicular fine-needle aspiration. This sperm can be used to fertilize oocytes *in vitro* through ICSI [2, 25]. CBAVD is known to be present in 97%–99% of male cystic fibrosis (CF) patients. CF is a frequent and by now well-known autosomal

TABLE 24.1 Incidence of Chromosomal Aberrations in Infertile Oligozoospermic and Azoospermic Males Compared with New-borns

Aberrations	Infertile Males (n = 7876)	Oligozoospermia (n = 1701)	Azoospermia (n = 1151)	New-borns (n = 94,465)
Autosomes	1.3%	3.0%	1.1%	0.25%
Sex chromosomes	3.8%	1.6%	12.6%	0.14%
Total	5.1%	4.6%	13.7%	0.39%

Source: Summarized from Van Assche E, Bonduelle M, Tournaye H et al. Cytogenetics of infertile men. *Hum Reprod* 1996; 11: 1–26.

recessive disease in the Caucasian population with an incidence of approximately 1/2500. Many patients, now surviving into their 30s and 40s, suffer from severe lung disease and pancreatic insufficiency. Although they are often too ill to reproduce, improved survival into adulthood generates interest in reproduction [26, 27]. The *CFTR* gene, encoding a protein involved in chloride transport across epithelial membranes, was shown to be responsible for CF due to malfunction of the protein when mutated [28–30].

CBAVD had also been observed in 1%–2% of apparently healthy infertile males, and in 6%–10% of men with obstructive azoospermia [31]. When the *CFTR* gene was studied in these males, pathogenic variants or splice-site variants in intron 8 (comprising the so-called 5T variant and the TG dinucleotide repeat upstream of it) interfering with gene expression were found in 80%–90% of them [32–37]. In the remaining CBAVD patients, no link could be found with aberrant *CFTR* expression. However, in these patients, CBAVD-associated urinary tract/renal malformations were observed [34, 38]. In a small group of patients with CBAVD, pathogenic variants are present in the ADGRG2 gene [39]. When performing ICSI with sperm from CBAVD males carrying *CFTR* mutations, their partners have to be tested as well since the carrier frequency of pathogenic variants in the CFTR gene may be as high as 1/25 in Caucasians. If both partners carry pathogenic *CFTR* variants, the risk of having a child with CF is as high as ¼, or 25%, or even ½, or 50% (Table 24.2). However, since the incidence and the type of *CFTR* mutations vary with ethnic origin as well as with geographical region, counselling and approaches to treatment will have to be adjusted. In high-risk situations, prenatal diagnosis or pre-implantation genetic diagnosis (PGD) is indicated (see later).

Genetic causes of globozoospermia and macrozoospermia

Globozoospermia is a rare (<0.1%) cause of male infertility. A major characteristic of these round-headed spermatozoa is the malformation or absence of the acrosome [40, 41]. The best-studied genes associated with this form of teratozoospermia in humans are *SPATA16*, *PICK1*, and *DPY19L2* [42–44]. In all of these cases, the condition is inherited as an autosomal recessive disease. Variants in the *DPY19L2* gene are the most prevalent and can be detected in 60%–83.3% of patients with (type I) globozoospermia. Around 26.7%–73.3% of these patients are homozygous for a 200 kb deletion of the *DPY19L2* gene [45]. Pathogenic variants in *PICK1* and *SPATA16* have also been observed in patients with globozoospermia, although the prevalence is very low. The number of genes (potentially) involved in globozoospermia is increasing. However, also for these genes, the frequency of pathogenic variants is extremely low.

In another form of morphological abnormal spermatozoa (large-headed, multi-flagellar, polyploid spermatozoa), a condition resulting in male infertility is caused by pathogenic variants in the *AURKC* gene, which is involved in chromosomal segregation and cytokinesis [46]. The first alteration detected in this gene was a deletion of a single base pair (c.144delC). This pathogenic variant has been detected in patients of North African origin. Especially in a Magrebian population, it was estimated that ~1/50 are heterozygous for this variant. A second recurrent pathogenic variant (p.Tyr248*) can be detected in European patients [47].

Male infertility as part of a syndrome

Patients with infertility as part of a syndrome all have a 46,XY normal karyotype. Most of the defects are monogenic and for the majority of the disorders, either the underlying gene defect is known or a chromosomal locus is known or suggested [48]. Nevertheless, part of the male infertility syndromes remains idiopathic at the molecular level, and probably multiple genes are involved.

Myotonic dystrophy is a rather common autosomal dominant syndrome causing muscular dystrophy with an incidence of 1/8000. The presence of an expanded CTG trinucleotide repeat in the *DMPK* gene interferes with its function [49–53]. Symptoms can be very mild and restricted to cataract at an advanced age or, by contrast, very severe, as is the case in the congenital, often lethal form of the disease. Severity is related to the number of CTG repeats [44]. In 60%–80% of male patients, testicular tubular atrophy will develop resulting in OAT. When such spermatozoa are used to fertilize oocytes, the risk of transmitting the disease is ½, or 50%, often in a more severe form due

TABLE 24.2 Risk Calculations for a Child with Cystic Fibrosis (CF) or Congenital Bilateral Absence of the Vas Deferens (CBAVD) in a patient with CBAVD

	Male		Female			Risk
No testing	8/10	×	1/25	×	1/4	=1/125
Testing female						
Carrier	8/10	×	1	×	1/4	=1/5
No carrier	8/10	×	1/150	×	1/4	=1/750
Testing male + female						
Female carrier	CF/CF	×	1	×	1/2	=1/2
Female no carrier	CF/CF	×	1/150	×	1/2	=1/300
Female carrier	CF/5T	×	1	×	1/4	=1/4 (CF) =1/8 (CBAVD)

Note: If the CBAVD patient is not tested for the presence of pathogenic variants in the *CFTR* gene, his risk of having at least one pathogenic CF variant is 8/10; if his partner is not tested and Caucasian, her risk of being a carrier of one pathogenic *CFTR* variant is 1/25. A carrier or heterozygous individual has a risk of 1/2 to transmit the variant. Two carriers have a risk of 1/4 to transmit their mutated gene at the same time. A CBAVD patient with two pathogenic variants will always transmit a mutated gene. Risks for CF can be calculated if none of the partners is tested, if only the female partner is tested, and if both partners are tested. In high-risk situations, pre-conceptional or pre-implantation genetic diagnosis can be offered [91].

to further expansion of the trinucleotide repeat (called anticipation). Prenatal diagnosis or preferential pre-implantation testing should be offered [54, 55].

Hypogonadotropic hypogonadism, characterized by an impaired gonadotropin-releasing hormone secretion, is divided into two major groups based on the underlying pathogenic mechanism. If associated with anosmia, the term Kallmann syndrome is used. X-linked along with autosomal recessive and autosomal dominant inheritance forms exist. The X-linked form of Kallmann syndrome (caused by pathogenic variants in the *KAL1* gene) is the most frequent and the best-known one [56], see Figure 24.2b. An autosomal dominant form of Kallmann syndrome is caused by pathogenic variants in the *FGFR1* gene [57]. A possible interaction between the gene products of the *KAL1* and *FGFR1* genes has been suggested as an explanation for the higher prevalence of Kallmann syndrome in males than in females [58, 59]. However, more than 30 genes involved in hypogonadotropic hypogonadism (including Kallmann syndrome) have been identified and this number is still increasing [60]. Nevertheless, in the majority of patients with a clinical diagnosis of Kallmann syndrome or idiopathic hypogonadotropic hypogonadism, no pathogenic variants can be detected.

Primary ciliary dyskinesia or immotile cilia syndrome is an autosomal recessive disease presenting with chronic respiratory tract disease, rhinitis, and sinusitis due to immotile cilia. Male patients are usually infertile because of asthenozoospermia [61]. If the aforementioned symptoms are associated with situs inversus, the condition is called Kartagener syndrome [62, 63].

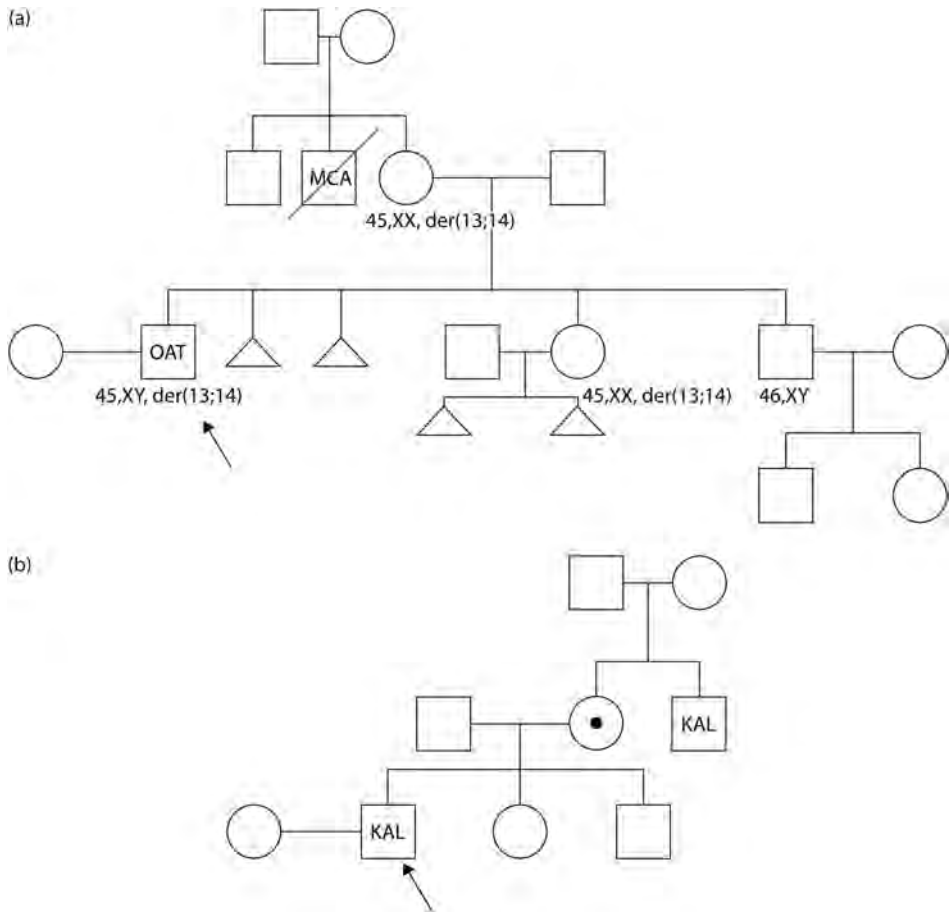

FIGURE 24.2 (a) Segregation of a Robertsonian translocation der(13;14) in a family. "OAT" (our proband ↖) presents with infertility due to oligo-astheno-teratozoospermia. His sister had two miscarriages (Δ); his brother has two healthy children. His mother had two miscarriages (Δ), lost a brother born with multiple congenital anomalies (MCA), and has a healthy brother without children. This story is suggestive of a chromosomal translocation. The karyotype of "OAT" points to a Robertson translocation der(13;14) (Figure 24.1a). His mother and his sister have the same translocation explaining the recurrent miscarriages (Δ). These miscarriages are most probably resulting from a trisomy 14 or monosomy 13 or 14. The brother of "OAT" has a normal karyotype, which is perfectly possible. The MCA brother of the mother died and most probably had trisomy 13. "OAT" should be informed about all these possible risks in case of pregnancy. In case of intracytoplasmic sperm injection, a pre-implantation genetic diagnosis or a prenatal diagnosis should be offered. (b) X-linked Kallmann syndrome in a family: its consequences and recommendations. "KAL" (our proband ↖) has Kallmann syndrome. The family history fits with an X-linked transmission since the brother of the mother of "KAL" has the same disease. This means that the mother of "KAL" must be a carrier ◉. Her daughter, the sister of our proband, therefore has a 50% risk of being a carrier and a 25% risk of having an affected son. Pre-implantation or prenatal diagnosis should be discussed. If the wife of "KAL" becomes pregnant, boys will be healthy and fertile (because they inherit the Y chromosome of their father), while girls will always be carriers.

Men with this condition can reproduce with the help of ICSI. Unfortunately, genetic counselling is hampered because of the lack of knowledge of all genes involved in primary ciliary dyskinesia and Kartagener syndrome [63, 64].

Kennedy's disease, or spinal and bulbar muscular atrophy, is a neuromuscular disease that causes muscular weakness and is associated with testicular atrophy which leads to oligozoospermia or azoospermia. It is an X-linked disease caused by an expanded (CAG) trinucleotide repeat in the transactivation domain of the androgen receptor gene [65, 66]. If treated with ICSI, genetic counselling is again indicated. However, point mutations in the androgen receptor gene might result in androgen insensitivity through impaired binding of dihydrotestosterone to the receptor, which will interfere with sexual development. The resulting syndrome is testicular feminization or androgen insensitivity syndrome, causing a (partial) female phenotype [67, 68]. The presenting problem here will not (only) be male infertility. Similarly, patients with an autosomal recessive 5α-reductase deficiency and therefore unable to synthesize dihydrotestosterone from testosterone may theoretically present at the clinic with azoospermia and pseudo-hermaphroditism [69, 70].

Very rarely, patients with other (mostly) syndrome-associated genetic defects may consult at a male infertility clinic. Up to 80% of patients with Noonan syndrome present with oligozoospermia or azoospermia as a result of cryptorchidism [71]. The diagnosis is often based on other symptoms, including small stature, chest deformity, a rather typical facial dysmorphism, and congenital heart disease. Defects in the *PTPN11* gene are responsible for approximately 40% of patients with Noonan syndrome [72]. Other genes involved in Noonan syndrome have been identified and, altogether, around 60% of cases can be solved. Consequently, more (currently unknown) genes must be involved in Noonan syndrome. The autosomal dominant inheritance pattern asks for genetic counselling. Other possible patients may be affected by Aarskog–Scott syndrome with acrosomal sperm defects [73, 74] or Beckwith–Wiedemann syndrome with cryptorchidism [75]. Syndromes such as Bardet–Biedl syndrome and Prader–Willi syndrome, both presenting with hypogonadism, are associated with other major symptoms, including (severe) mental retardation, which limit procreation [76, 77]. Prader–Willi syndrome is an imprinting syndrome resulting from the absence of expression of the paternal alleles in the 15q11–q13 imprinted region [78–80]. Other causes of male infertility include deficiencies in enzymes involved in the synthesis of testosterone, luteinizing hormone, and luteinizing hormone receptor [81, 82].

Also, defects in energy production by the mitochondria have been implicated in male infertility. Mitochondria are the main sources of energy production for the cells through the process of oxidative phosphorylation. The synthesis of ATP occurs through the action of five enzyme complexes that are encoded by both nuclear genes and the small mitochondrial genome that is exclusively maternally inherited. Mitochondrial diseases usually evolve as multisystem disorders mainly affecting the central nervous system and muscles. In addition, these defects in respiratory function are believed to cause a decline in sperm motility because of the depletion of ATP, which is necessary for the flagellar propulsion of the spermatozoa. Reduced sperm motility and resulting male infertility have been well documented in several patients with mitochondrial encephalopathies caused by mitochondrial tRNA point mutations or (multiple) mtDNA deletions [83].

Genome-wide testing strategies

Since the number of genes known to be causal for male infertility is increasing, there is a great need for genome-wide analyses. For the detection of copy number variations (deletions/duplications), the diagnostic yield and utility of array comparative genomic hybridization has been investigated in multiple studies. Mostly, these results were disappointing. Besides for deletions involving the *MAGEA9* gene, the detected copy number variations were either of unknown clinical significance or not confirmed in subsequent studies [84, 85]. It might, however, be worthwhile to look for copy number variations in regions known to be related to male infertility or containing genes involved in male infertility.

Gene panel analyses have been proven to be successful for conditions/syndromes involving multiple genes, such as hypogonadotropic hypogonadism. Furthermore, gene panel analyses as well as exome sequencing have been applied in view of idiopathic male infertility where azoospermia or severe OAT is the sole symptom [86, 87]. At this moment, however, there is no consensus about the genes to be included in such a gene panel. Again, only a small part of the suggested infertility genes or variants could be confirmed in subsequent studies [88]. When looking at recent literature, it is evident that the number of genes potentially involved in idiopathic male infertility is still increasing, and most likely genes still need to be discovered. In order to avoid updating panels on a regular basis, one might opt to perform exome sequencing. Nevertheless, the more genes that are analysed simultaneously, the more complex the interpretation is.

Consequences and recommendations in the clinic

Genetic evaluation of infertile males before ART use

A personal history from the patient should be taken. In addition, a detailed pedigree should be drawn and completed for miscarriages or children (also deceased) with multiple congenital malformations in first- or second-degree relatives. It is also important to know about the infertility status of siblings or other family members. This information may suggest a possible chromosomal aberration such as a translocation or a monogenic disease like Kallmann syndrome or CF. A thorough inquiry of the proband and his partner may pinpoint other hereditary diseases not necessarily causing infertility but causing morbidity or being lethal to offspring. A complete clinical examination of the proband and his partner is useful for establishing a clinical diagnosis of a disease or a syndrome associated with infertility such as Klinefelter syndrome or CF-linked CBAVD. This examination may also reveal other possible hereditary diseases not identified before. Since the couple is in such a case not aware of a genetic problem, they should be counselled before starting the treatment. Complementary tests—mainly laboratory investigations—will help to confirm a clinical diagnosis. In case of male infertility, the personal history, the clinical examination, a semen analysis, and hormonal tests are sufficient to characterize most of the patients as being:

1. Infertile in association with other physical or mental problems.
2. Infertile but otherwise healthy. These patients can mostly be subdivided into oligozoospermic or eventually OAT males and into males with obstructive or non-obstructive azoospermia. Rarely, patients with teratozoospermia are detected through semen analysis.

Genetic investigations will help to refine the diagnosis and to counsel the patient/couple accordingly. The preceding information will help to select additional tests to be performed. In most cases of male infertility due to severe OAT or non-obstructive azoospermia, a peripheral karyotype should be performed, even if the family history is not suggestive of a chromosomal disorder [6–8]. In the same cohort of patients, microdeletions of the AZF regions on Yq11 should be looked for in DNA from peripheral blood. The possibility of fertility treatment in couples in whom the male has an AZF deletion is strongly dependent on the type of deletion present [89]. Deletions of AZFa or AZFb, or combinations including these regions, have a bad prognosis since no sperm cells will be produced and ICSI will not be possible. In contrast, spermatozoa can be found in about 70% of patients with a complete deletion of the AZFc region [89]. For these patients ICSI will be possible.

In men with non-obstructive azoospermia caused by CBAVD without anomalies of the urogenital tract, pathogenic variants in the *CFTR* gene should be looked for in the patient and, even more importantly, in his partner. At present it is possible to identify 85%–90% of htereozygous individuals ("carriers") in the Caucasian population [90, 91]. Depending on whether *CFTR* mutations have been identified in the male patient and/or his female partner, the risk of conceiving a child with CF can be calculated (Table 24.2). These figures together with the type of alterations may be an indication for prenatal diagnosis or preimplantation genetic testing (PGT)13 [26, 92, 93]. More specific tests should be performed if diseases such as Kennedy disease, Kallmann syndrome, myotonic dystrophy, immotile cilia syndrome, or other syndromes or diseases are suspected. In these cases, it is again important not only to establish a correct diagnosis to treat appropriately, but also to counsel the proband and his family adequately concerning recurrence risks and prenatal diagnosis (PND) or preimplantation genetic testing (PGT).

Genetic testing during ART use for severe male infertility

Genetic tests that can be performed during ART refer to PGT. They involve the genetic testing (PGT) of embryos before implantation [94–99]. The aim is to avoid the birth of a child with a genetic disease. PGT makes conventional prenatal diagnosis, eventually followed by termination of pregnancy, obsolete. PGT is a complex procedure because of the "single-cell" genetic diagnosis. It was developed and first applied in the clinic more than 30 years ago [98]. At first, most of the PGTs performed were for CF, myotonic dystrophy, Huntington's disease, and Duchenne muscular dystrophy, but many others have since been performed for either infertile or fertile couples [96, 97]. For chromosomal aberrations, most PGTs have been done for reciprocal and Robertsonian translocations [94, 99]. An example of a family with a Robertsonian translocation der(13;14) is visualized in Figure 24.2a. In general, the take-home baby rate is of the same order of magnitude of 20%–25% as in ICSI cycles in general [2, 3]. A number of PGTs have been performed for Klinefelter patients in whom spermatozoa found in the testes were used to fertilize oocytes [13].

Genetic evaluation of pregnancies and children conceived through ICSI because of severe male infertility

Follow-up studies of pregnancies established and children born after the use of ICSI have been initiated as soon as this new procedure was applied in the clinic. From these still ongoing studies, it became clear that the number of major malformations was comparable to the number of major malformations in *in vitro* fertilization (IVF) children, and possibly slightly higher than in naturally conceived children. The results of the psychomotor development of these children are also reassuring [100–107]. Current investigations are looking at the reproductive profiles of young adults born after ICSI [108–111].

The *de novo* chromosomal aberrations found at prenatal diagnosis indicate that numerical sex chromosomal anomalies are slightly increased when compared to a large newborn population. The incidence in the newborn after natural conception is 0.2%, but the incidence in ICSI children is 0.8%. This is a fourfold increase, but of course the overall incidence remains low (<1%). Apart from sex chromosome anomalies, *de novo*-balanced translocations have also been observed [105, 107, 112]. These aberrations occurring in children of men with a normal peripheral karyotype could be related to chromosomal anomalies being present in their sperm but not in their lymphocytes [113–116].

Conclusion

In case of severe male infertility, good clinical practice requires genetic evaluation before, during, and after ART in order to properly treat and counsel the proband, the couple, and, eventually, the family. The aim is to inform the patients about possible risks, to improve the success rate of the ART treatment, and to avoid the birth of children affected with a severe genetic disease. Moreover, at present there are still many unknown causes of male infertility. More research in the field of genetics will provide us with a better understanding, along with a better defining of how great the risks are of transmitting infertility or possibly other genetic anomalies to the next generation.

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25

PRE-IMPLANTATION GENETIC TESTING FOR ANEUPLOIDY TO IMPROVE CLINICAL OUTCOMES

Jenna S. Hynes, Eric J. Forman, and Alan H. Handyside

Chromosome aneuploidy is common in human gametes and pre-implantation embryos and is a major cause of pregnancy failure, miscarriage, and still births, with an incidence at birth of <0.3% [1, 2]. Most aneuploidies originate in the oocyte through errors in maternal meiosis and these increase exponentially in women in their late 30s and early 40s [3]. This is associated with a sharp increase in the incidence of miscarriage and a corresponding decline in live birth rates (LBRs) in these women following *in vitro* fertilization (IVF) [4]. In principle, therefore, there is no clinical or ethical justification for the transfer of aneuploid embryos following IVF treatment for infertility, where the aim is to help couples have a healthy baby. The challenge, however, is to identify euploid and aneuploid embryos accurately without compromising their treatment.

Pre-implantation genetic testing for aneuploidy (PGT-A)

Pre-implantation genetic testing for aneuploidy (PGT-A) originally involved polar body or cleavage-stage biopsy and single-cell analysis using multicolour fluorescent in situ hybridization (FISH) with a limited number of chromosome-specific probes, typically 7–9 chromosomes [5, 6] (Table 25.1). However, several randomized controlled trials (RCTs) failed to show a benefit and a large trial in the Netherlands showed a significant reduction in live births [7]. In the 15 years since then, there have been major advances in whole genome amplification and copy number analysis for all 24 chromosomes, which began with microarray comparative genomic hybridization (array CGH), progressed to next-generation sequencing (NGS)-based tests and, more recently, NGS-based targeted sequencing of single-nucleotide polymorphism (SNP) markers to improve accuracy [8, 9]. Over the same period, there have also been significant improvements in culture to the blastocyst stage and cryopreservation by vitrification [10, 11]. Combining the dual selection of blastocyst culture with trophectoderm biopsy and PGT-A, followed by vitrification and transfer of euploid blastocysts in later unstimulated cycles, there have been several reports of exceptionally high implantation rates (IRs) and live births per transfer, demonstrating that this approach is highly effective for embryo selection [12–14]. Furthermore, a recent non-selection study, in which blastocysts were selected based on morphology alone and biopsied before transfer but only tested after clinical outcomes were known, demonstrated this directly [9]. Whereas implantation and ongoing pregnancy/delivery rates per single euploid blastocyst transfers were more than 80% and 64.7%, respectively, only 40% of aneuploid blastocysts were implanted and there were no ongoing pregnancies.

Clinical outcomes following numerous RCTs have been mixed. Five early RCTs demonstrated improved clinical outcomes using different technologies and in various groups of patients with PGT-A [15–19]. Yang et al. examined the clinical pregnancy rates after the transfer of a single blastocyst selected by PGT-A via aCGH plus morphology versus morphology alone in women under the age of 35 years with a tubal factor or male factor infertility undergoing their first cycle of IVF [15]. Among 103 women undergoing frozen embryo transfer (FET), the clinical pregnancy rate (70.9% vs. 45.8%, $p = 0.017$) and ongoing pregnancy rate (OPR) after 20 weeks gestation (69.1% vs. 41.7%, $p = 0.009$) were significantly higher in the PGT-A group.

Scott et al. evaluated the superiority of PGT-A with real-time quantitative polymerase chain reaction (qPCR) for embryo selection vs. traditional morphology [18]. Women in the biopsy group underwent PGT-A followed by fresh transfer of up to two euploid blastocysts vs. transfer of two untested blastocysts. Per embryo transferred, the IR (79.8% vs. 63.2%, $p = 0.002$) and sustained IR (66.4% vs. 47.9%, $p = 0.001$), or those embryos that went on to delivery, were higher in the PGT-A group. Per transfer, the clinical pregnancy (93.1% vs. 80.7%) and LBRs (84.7% vs. 67.5%) were similarly improved in the PGT-A group.

The BEST trial (Blastocyst Euploid Selective Transfer) was designed to compare single euploid transfer with the transfer of two untested embryos. This study enrolled women ≤42 years with at least two good-quality blastocysts [16]. In the PGT-A group, all available blastocysts underwent biopsy with qPCR analysis, and the single best-quality euploid blastocyst was transferred. Patients in the control group underwent transfer of the two best-untested blastocysts based on morphology alone. The OPR, defined as viable pregnancy ≥24 weeks gestation, was similar between groups (60.7% vs. 65.1%), whereas the rate of multiple gestations was significantly higher in the double embryo transfer group (53.4% vs. 0%). Women who underwent single euploid transfer were nearly twice as likely as those undergoing transfer of two untested embryos to have an ongoing singleton pregnancy (60.7% vs. 33.7%). A follow-up analysis of delivery outcomes demonstrated lower rates of preterm delivery (13% vs. 29%), low birth weight (11% vs. 33%), and neonatal intensive care unit stay (11% vs. 26%) in the single euploid transfer group [17]. Since the publication of the BEST trial, transfer of a single euploid embryo has become standard practice and is strongly recommended by the American Society for Reproductive Medicine (ASRM) due to equivalent OPRs with a stark decrease in the risk of multiple gestations and the associated obstetric risks [20].

A meta-analysis published in 2015 combined the results from these original RCTs, confirming increased clinical IRs (1.29, 95% Confidence Interval (CI) 1.15, 1.45) and sustained IRs (1.39, 95% CI 1.21, 1.60) in cycles utilizing PGT-A for identification of euploid embryos for transfer [21]. This same meta-analysis combined the results from eight observational studies, also finding increased clinical IRs (1.78, 95% CI 1.60–1.99) and sustained IRs (1.75, 95% CI 1.48–2.07) in the PGT-A groups. These early RCTs received much criticism for the fact that they included predominantly good prognosis patients with normal ovarian reserve who produced multiple high-quality blastocysts [22]. Consequently,

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TABLE 25.1 Methods for Pre-implantation Genetic Testing for Aneuploidy (PGT-A) with Their Limitations and Additional Capabilities

Method[a]	Chromosome Coverage	Resolution	Whole Chromosome Aneuploidy		Segmental Chromosome Aneuploidy		Abnormal Fertilization	Contamination	Parental Origin	Cost
			Full	Intermediate	Full	Intermediate				
Multicolour fluorescence in situ hybridization (mFISH)	Typically, 13, 16, 18, 21, 22, X, and Y	Probes mostly centromeric	✓		×					Medium
Array comparative genomic hybridization (aCGH)	All 24 chromosomes	Approximately 3000 probes genome-wide	✓	×	✓	×				Medium
Array-based SNP genotyping and karyomapping with parental genotyping	Chromosomes 1–22 and X	1–5 Mb, depending on SNP coverage	✓	×	✓	×	✓	✓	✓	High
NGS-based copy number analysis	All 24 chromosomes	>10 Mb	✓	✓	✓	✓				Medium
Targeted NGS-based copy number and SNP analysis	All 24 chromosomes	Approximately 2500 SNP loci genome-wide	✓	✓	✓	✓	✓	✓	×	Medium
NGS-based copy number and SNP analysis with parental genotyping	All 24 chromosomes	>10 Mb	✓	✓	✓	✓	✓	✓	✓	High

Note:

[a] General guide only to the methodologies and not intended to be an accurate representation of specific commercially available tests.

patients who did not produce blastocysts were not randomized. Additionally, the aCGH and qPCR methods did not report on putative embryo mosaicism.

Rubio et al. sought to evaluate the effect of PGT-A in an older population (38–41 years old) with higher baseline rates of aneuploidy [19]. Women in the PGT-A group underwent cleavage-stage blastomere biopsy with aCGH analysis and subsequent fresh transfer of euploid blastocyst(s). The delivery rate per transfer was increased in the PGT-A group (52.9% vs. 24.2%, $p = 0.0002$) with a shorter time to pregnancy (7.7 vs. 14.9 weeks) and number of necessary transfers (1.8 vs. 3.7). Although this trial included cleavage-stage transfer, after the publication of a trial by Scott et al. that demonstrated a potential detrimental effect of blastomere biopsy, the field of PGT-A has shifted to almost exclusive use of trophectoderm biopsy at the expanded blastocyst stage [23].

In contrast to the Rubio trial, the STAR trial (Single-Embryo Transfer of Euploid Embryo), designed to compare outcomes when selecting embryos for transfer using PGT-A via NGS, found no significant improvement in clinical pregnancy or miscarriage rates [24]. This large RCT included women 25–40 years old, recruited from 34 clinics in the United States, the United Kingdom, and Australia, all with at least two blastocysts available for biopsy or transfer. The overall OPR per transfer (at ≥20 weeks' gestation) was similar between groups with no improvement in the PGT-A group. However, the OPR in the older subset of the cohort (35–40 years old) was significantly higher in the PGT-A group (50.8% vs. 37.2%, $p = 0.035$). Embryos with an intermediate copy number call, also called "mosaic" embryos, were deemed aneuploid and not eligible for transfer. Subsequent retrospective and non-selection studies have demonstrated that embryos with a mosaic report can perform similar to euploid, non-mosaic embryos and result in healthy live births [25, 26]. The inability to transfer mosaic embryos may have lowered the OPR in the group randomized to PGT-A.

To date, the largest RCT evaluating the impact of PGT-A via NGS technology on ART outcomes was published in 2021 [27]. This ambitious multicentre RCT randomized 1212 women aged 20 to 37 years who were undergoing their first IVF cycle and had three or more high-quality blastocysts. Women in the PGT-A group had their top three blastocysts biopsied. The primary outcome was cumulative LBR following sequential FETs until either live birth or all euploid (PGT-A group) or three best-untested embryos (control group) had been transferred. The cumulative LBR following a maximum of three transfers was similar between groups (PGT-A: 77.2% vs. control: 81.8%). However, more women in the control group required a second (192 vs. 119) or third (49 vs. 5) transfer. This study design likely underestimates the true cycle potential, as less than half of the available blastocysts were biopsied.

Given these mixed results, the use of PGT-A, therefore, remains controversial [28, 29]. One reason is that with highly effective vitrification protocols becoming increasingly routine, the emphasis on measuring IVF success rates has shifted to cumulative pregnancy and LBRs per cycle started or intention to treat. Clearly, any form of embryo selection in this context, including PGT-A, cannot change cumulative outcomes and embryo biopsy could potentially reduce them.

Also, the possibility that viable embryos could be discarded because of false positive results is a concern, particularly in poor prognosis patients [30]. To this end, non-selection studies, in which PGT-A tested embryos are transferred without prior knowledge of the genetic test results, allow us to retrospectively analyse the outcomes of aneuploid transfers. The first such study was performed in 2012, examining both cleavage-stage and blastocyst biopsies analysed using SNP array technology [31]. A total of 255 morphologically selected embryos were chosen and biopsied shortly before transfer. Of 232 embryos with interpretable results, 99 were deemed aneuploid with four of these leading to live births, for a negative predictive value of 96%.

Going forward, a similar non-selection trial was performed to validate NGS technology [9]. The authors evaluated the transfer outcomes of 484 blastocysts in women aged 18–44 years. Biopsy results were analysed using NGS after knowledge of the clinical outcome was available, and were reported as euploid, whole chromosome aneuploid, whole chromosome mosaic, and segmental. Aneuploidy was detected in 102 embryos, leading to 41 (40.2%) positive pregnancy tests, 24 (23.5%) clinical pregnancies, and no sustained implantations beyond 13 weeks' gestation, for a 100% negative predictive value of aneuploid embryos.

An additional non-selection study from China divided PGT-A results first into euploid vs. aneuploid, and then into subcategories (euploid, euploid mosaic, euploid segmental, aneuploid, and aneuploid segmental) [32]. The LBR was 49.6% (67/135) in the euploid group vs. 7.5% (4/53) in the aneuploid group. Of the four live births in the aneuploid group, two were from embryos deemed to be uniformly aneuploid and two were thought to be segmental aneuploid. There were no differences in the LBRs between the uniformly euploid embryos vs. any other group except the uniformly aneuploid ($p < 0.0001$), although the segmental aneuploid group did trend towards worse outcomes.

Chromosome mosaicism

Unlike earlier methods used for PGT-A, including, for example, aCGH, low read depth, NGS-based copy number profiling has a linear relationship with chromosome copy number in the DNA amplified from the sample and increased resolution [33]. With multiple trophectoderm cell samples (typically 3–10 cells) biopsied at the blastocyst stage, this has enabled the identification of both whole and segmental chromosome aneuploidies with copy numbers ranging from those expected for trisomies or monosomies (full changes) to low or intermediate copy number changes (Figure 25.1). Intermediate copy number changes are generally interpreted as resulting from chromosome mosaicism between the cells of the biopsied trophectoderm cells, which can arise through non-disjunction and other mechanisms, including spindle abnormalities [34]. Whereas low copy number changes may be technical artefacts related to the amplification and NGS protocols used.

The clinical significance of intermediate or mosaic, whole or segmental copy number changes is not fully understood, since the PGT-A results are based on only a small sample of cells from the embryo and may not be fully representative, particularly of the inner cell mass lineage from which the fetus is formed. Indeed, transfer of mosaic embryos, following appropriate genetic counselling, has resulted in healthy live births [35–38]. Analysis of clinical outcomes following the transfer of more than 1000 mosaic blastocysts has confirmed that high-level mosaics (between 50% and 80%) have significantly lower ongoing pregnancy and LBRs compared with uniformly euploid blastocysts, whereas the outcomes with only low-level mosaicism (20%–50%) are similar [26]. Also, the number and type of aneuploidies present also affected the outcomes.

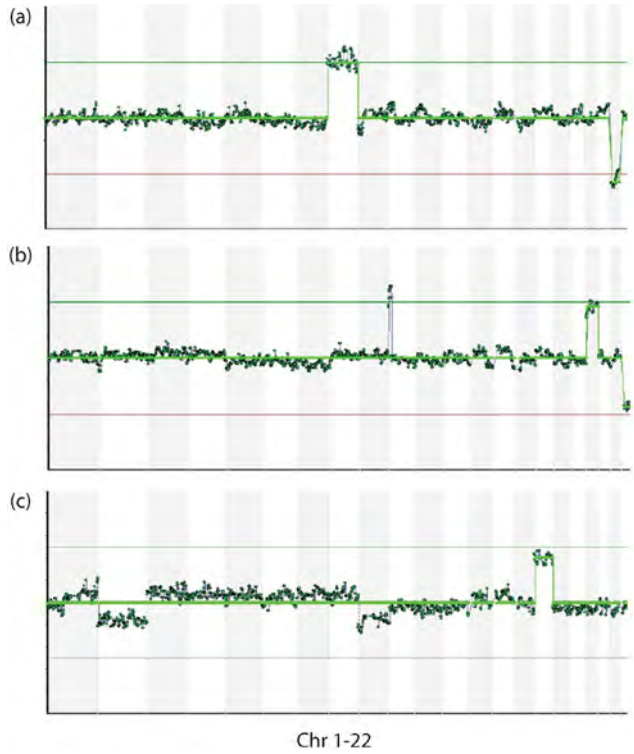

FIGURE 25.1 NGS-based chromosome copy number profiles for three trophectoderm biopsies following whole genome amplification, in a cycle from a patient aged 43 years, demonstrating the range of copy number changes which can be identified: (a) trisomy 8 and monosomy 22, (b) duplication of the terminal region of the short arm of chromosome 10 (p10dup), (c) mosaic loss of chromosomes 2 and 9 and trisomy 16. The dots represent the normalized distribution of fragments mapped to successive bins across each chromosome, and the bright green line indicates the best fit for two copies. The upper and lower green and red lines represent the theoretical displacement for three copies (trisomy) and one copy (monosomy), respectively.

Furthermore, a prospective non-selection trial confirmed that blastocysts with only low or moderate levels of mosaicism in the trophectoderm have equivalent developmental potential as those which are fully euploid [25]. Embryos with 20%–30% and 30%–50% aneuploid cells were labelled "low grade" and "medium grade" mosaic, respectively. A total of 897 embryos were available for transfer: 484 uniformly euploid, 282 low mosaic, and 131 moderate mosaic. The pregnancy, live birth, and miscarriage rates were similar across groups, demonstrating equivalent reproductive potential between euploid and low- to moderate-level mosaic embryos. The authors further performed karyotype analysis on 27 neonates born after mosaic embryo transfer and found that they all had normal karyotypes without persistence of the original mosaic findings.

The upper and lower thresholds for defining intermediate, mosaic changes from full aneuploidies will depend on the protocol used. By identifying meiotic errors in polar bodies and trophectoderm biopsies using SNP genotyping and karyomapping [39] in parallel with NGS-based PGT-A, it has recently been demonstrated that, with one exception, all female meiotic aneuploidies resulted in copy number changes exceeding 70% of full changes in the corresponding trophectoderm biopsies or whole arrested embryos [40]. In contrast, most non-meiotic (presumed mitotic origin) aneuploidies had copy number changes ranging from 30% to 70%, although a minority exceeded the 70% threshold and may have resulted from chromosome mal-segregation in the first mitotic cleavage divisions. Furthermore, some samples originally reported as having mosaic, whole, or segmental chromosome copy number changes only were identified as having meiotic aneuploidies or, conversely, were euploid when reanalysed by karyomapping. Thus, one explanation for the differences in clinical outcomes between high- and low-level mosaic embryos may be the incidence of meiotic versus localized mitotic aneuploidies affecting the whole or only part of the embryo, respectively. Only a minority of otherwise euploid blastocysts have mosaic copy number changes, but the uncertain clinical significance of these abnormalities makes it good practice to require that patients have genetic counselling before considering these embryos for transfer [41].

Non-invasive PGT-A

The need to biopsy embryos for PGT-A increases the resources required and the cost for patients. Delaying biopsy to the blastocyst stage on days 5 to 7 post insemination, and limiting biopsy to good quality, clinical grade blastocysts, which can be considered for transfer, minimizes this. Biopsy could also potentially damage the embryos, though recent evidence demonstrates that limiting the number of trophectoderm cells removed (typically 3–10 cells) does not reduce either implantation or OPRs following transfer of euploid blastocysts (9). Nevertheless, non-invasive PGT-A (niPGT-A) would eliminate the possibility of damage, broaden access, and lower costs. Reports that cell-free DNA is present in blastocoel fluid and spent culture medium at the blastocyst stage [42, 43] therefore have led to numerous reports of niPGT-A protocols which aim to amplify and test this cell-free DNA [44–46].

Ideally, niPGT-A should be minimally invasive, accurate, and efficient [47]. Blastocentesis, in which blastocoel fluid is aspirated by penetrating the trophectoderm layer with a sharp pipette to sample cell-free DNA, presumably from dead cells in the blastocoel cavity, is clearly less invasive than biopsy of multiple trophectoderm cells, and is already used in some laboratories to collapse the blastocoel cavity to improve survival after vitrification [42, 48]. In contrast, sampling of spent culture medium is only "invasive" to the extent that embryos need to be cultured individually and, to avoid maternal contamination, carefully denuded of cumulus cells, washed extensively, the media changed before sampling, and culture extended to at least day 6 post insemination [45].

For niPGT-A, various protocols have been used to optimize whole genome amplification from fragmented cell-free DNA, and use the amplified DNA for NGS-based copy number analysis. However, copy number analysis alone does not allow the origin of the cell-free DNA to be tested, and a recent study, using whole genome methylation sequencing, demonstrated that differentially methylated regions characteristic of blastocyst, cumulus cell, and oocyte/polar body DNA could be detected [49]. This confirms a previous report that DNA from the second polar body can persist to the blastocyst stage and could potentially give false results at a chromosomal and single gene level [50]. Protocols that include analysis of genome-wide SNP markers, for example, may therefore be necessary to avoid false negative results caused by maternal contamination [9].

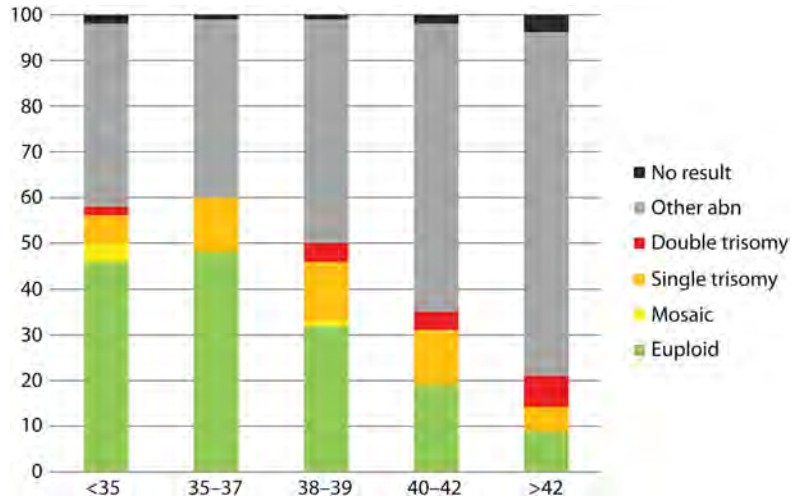

FIGURE 25.2 Distribution of euploid, full copy single trisomies, double trisomies, mosaic, and other complex aneuploidies based on the results of single trophectoderm biopsy of all biopsied blastocysts [14]. The groupings are based on the chromosome complement observed in an analysis of retained products of conception following single blastocyst transfer [53].

The accuracy of niPGT-A tested in concordance with corresponding trophectoderm biopsies, whole blastocysts and, in some cases, the isolated inner cell mass has varied widely [51]. However, a recent multicentre prospective study of concordance between cell-free DNA and trophectoderm biopsies from 1301 human blastocysts in which the medium was changed on day 4 and harvested ≥40 hours later demonstrated a concordance of 78% with a 12% and 8% false positive and false negative rate, respectively, 12.5% maternal contamination and failed in about 12% of samples [45]. Thus, for clinics willing to change their embryology protocols, niPGT-A may be useful for prioritizing blastocysts for embryo transfer.

Prospects for integration into routine clinical practice

The methods used for PGT-A continue to evolve, and several now include SNP markers to improve accuracy and extend diagnostic capabilities, allowing the detection of, for example, abnormally fertilized embryos and contamination. Alternatively, niPGT-A offers the possibility of avoiding embryo biopsy and can be used for prioritizing embryos for transfer but with a lower accuracy. PGT-A remains challenging, with uncertainties around intermediate chromosome copy number changes and the requirement for genetic counselling [52], and costs are still high. Nevertheless, PGT-A is now in widespread use and has a range of clinical and laboratory benefits (Table 25.2). Most importantly, these include optimizing pregnancy rates following single embryo transfer to avoid the complications of multiple pregnancies, reducing the risk of miscarriage and abnormal pregnancy, and improved clinical and laboratory management. The use of PGT-A has confirmed the high incidence not only of single aneuploidies but also multiple aneuploidies and other abnormalities known to result in pregnancy loss and which increase exponentially with maternal age [14, 53] (Figure 25.2). Accurate and cost-effective methods of PGT-A are therefore essential to improve clinical outcomes and increase our understanding of the causes of infertility and IVF failure beyond chromosome aneuploidy.

TABLE 25.2 Clinical and Laboratory Benefits of Combined Time-Lapse Analysis, Vitrification, and PGT-A at the Blastocyst Stage

- High implantation and clinical pregnancy rates and low miscarriage rates with single euploid, vitrified, warmed blastocyst transfers
- Cryo-storage of euploid blastocysts only
- Reduces the risk of chromosomally abnormal pregnancy or live birth
- Improves clinical management and embryology standards
- Enables patients to make informed decisions

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26

DIAGNOSIS OF ENDOMETRIAL RECEPTIVITY, EMBRYO–ENDOMETRIAL DIALOGUE, AND ENDOMETRIAL MICROBIOME

Maria Ruiz-Alonso, Inmaculada Moreno, Feilpe Vilella, and Carlos Simon

Introduction

Implantation is the first major physical embryo–maternal interaction. Successful implantation requires synchronized and bidirectional communication followed by embryo adhesion and invasion of the decidualized endometrium [1]. The human endometrium is a complex tissue composed of predominantly epithelial and stromal cells, which are cyclically regulated by steroid hormones (oestrogens and progesterone [P]). Endometrial receptivity is a self-limited period when the endometrium is transiently receptive to implantation of a blastocyst, a process that is mediated by immune cells, cytokines, growth factors, chemokines, adhesion molecules, and several other molecules through different pathways (for review, see [2–4]). A receptive endometrium, a viable euploid blastocyst, and an exquisitely coordinated cross-communication between them are required for successful implantation and invasion of the underlying maternal endometrium.

In this chapter, we discuss the molecular characterization of endometrial receptivity through transcriptomics with special attention to its clinical translation. We also examine embryo–endometrial dialogue that plays an important role in transmitting information from mother to embryo during peri-implantation. We close by discussing the endometrial microbiota and its impact on reproduction. Together, we provide a complete view of the diverse events that take place in the pre-conceptional space and how they may impact embryo implantation.

Molecular diagnosis of endometrial receptivity

Advances in endometrial biology fall closely on the heels of the genomics revolution. Early endometrial transcriptomic profiling studies identified the window of implantation (WOI) during natural cycles and yielded four simultaneous reports on the human secretory endometrium transcriptome (for review, see [5]). Later efforts characterized transcriptomic profiles across the menstrual cycle, under controlled ovarian stimulation (COS), and in patients with refractory cycles (for review, see [5]). Studies on the endometrial transcriptome have since proliferated (for review, see [6]), and additional work has identified transcriptomic differences relating to endometrial pathologies and factors that may affect fertility [7–13]. These findings catalysed the acceptance of the importance of molecular endometrial factors in fertility and health in addition to anatomical factors.

Our group first defined human endometrial receptivity in terms of its molecular (transcriptomic) signature. The signature comprised 238 differentially expressed genes, which, when combined with a computational algorithm, provided a predictor to classify endometrial samples into proliferative (PRO), pre-receptive (PRE), receptive (R), or post-receptive (POST) phases [14]. This work prompted a new approach to assessing endometrial receptivity beyond classical histological dating. Further, our 2020 study confirmed the transcriptomic signature across endometrial phases at a single-cell resolution [15]. By analysing more than 70,000 individual cells falling into six distinct endometrial cell types, we identified that transcriptomic activation in the epithelial cells opens the WOI [15].

This molecular evidence base provided the impetus for applying endometrial receptivity analysis (ERA) to diagnose and treat recurrent implantation failure (RIF) of endometrial origin [16]. The goal of this approach is to time embryo transfer while the endometrium is receptive (i.e. personalized embryo transfer [pET]). ERA revealed critical information about the WOI. The WOI lasts only 30–36 hours, and, depending on the patient, it can occur at different moments during the cycle [usually between five and ten days after luteinizing hormone (LH) surge in natural cycles (LH+6 to LH+9) or between three and eight days of P exposure in hormonal replacement therapy cycles (P+4 to P+7)] [17]. Because the WOI is not the same for all women, results of the ERA after five full days of P exposure (P+5; the standard WOI) reveal how the embryo transfer should be personalized for each case (Figure 26.1).

We tested whether ERA could produce accurate and reproducible results across the menstrual cycle by using the histological gold standard. To achieve this, data for 49 endometrial biopsies were assessed using the quadratic weighted Kappa index [18]. We also determined whether ERA results were reproducible across cycles in the same individual by analysing biopsy pairs collected 29–40 months apart under the same conditions. No inter-cycle variation was detected [18]; however, specific variables could affect the endometrium, increasing the probability of a displaced WOI. One factor is body mass index (BMI). In a prospective cohort study stratifying patients by BMI, WOI displacement was more common among those with a BMI of >30 kg/m^2 than in those with a BMI of <30 kg/m^2 [19].

The final proof of concept to demonstrate the utility of the endometrial receptivity testing is how personalizing the embryo transfer impacts in the clinical outcome (for review, see [20]). To date, 27 publications describe clinical outcomes for ERA-guided pET; these studies occurred at different fertility centres, encompass specific clinical indications, and report different outcomes (Table 26.1). These studies show how synchronizing the transfer of a viable blastocyst with a receptive endometrium, can increase the success rate in patients with previous implantation failures.

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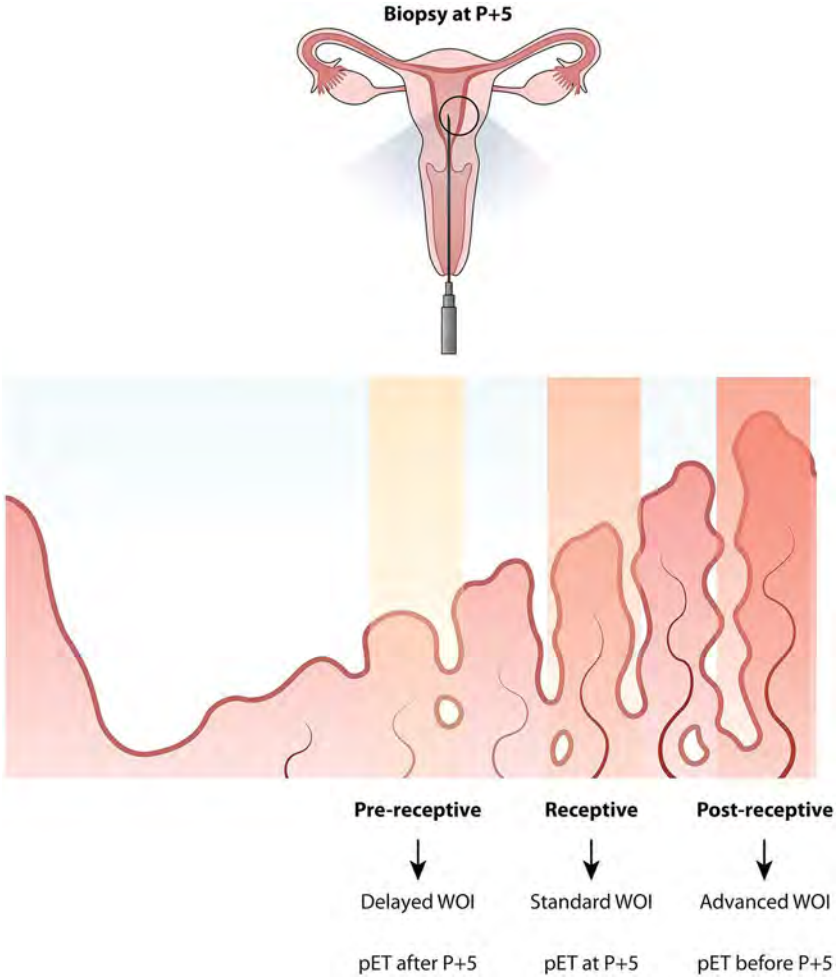

FIGURE 26.1 Representation of different displacements of the WOI. ERA identifies endometrial status from an endometrial biopsy collected during the standard WOI (at P+5). If there is no displacement, the ERA result will be receptive. If a pre-receptive result is obtained, it means that the WOI is delayed, so pET should be performed after P+5. However, if a post-receptive result is obtained, the WOI has already passed, and pET should be performed before P+5.

Embryo–endometrial dialogue

Intercellular communication is established during cell-to-cell contact that permits the release and uptake of different chemicals, hormones, or growth factors [21]. This process is essential for multicellular organisms and for relationships between unicellular organisms and the environment or their hosts [22]. Signalling during implantation represents the first major physical embryo–maternal interaction and requires synchronized, bidirectional communication [1, 23–25]. Human pre-implantation embryos produce soluble ligands and receive signals through their receptors in an autocrine/paracrine fashion, including soluble factors of maternal origin [26]. Numerous ligands (mainly cytokines and growth factors) are produced by the human endometrium during the receptive phase [27]. An embryo–maternal "cross-talk" or molecular dialogue is therefore postulated to exist during the peri-implantation period. These signalling pathways are highly complex and are often described as regulatory "circuits" [28]. Recently, evidence has emerged to support the existence of cross-talk between the mother and the embryo.

Mother-to-embryo communication

Extracellular vesicles (EVs) are critical factors in intercellular communication [29, 30]. EVs function by direct interaction with cell surface receptors or by transmission of their contents via endocytosis, phagocytosis, or fusion with target cell membranes. Recipient cell specificity seems to be driven by receptors present in cells and EVs [30, 31]. Communication between the mother and embryo appears to be mediated through EVs that are present in the endometrial fluid [32–35] and released by endometrial epithelial cells [32, 33, 36, 37].

Our laboratory demonstrated effective communication between the mother and the pre-implantation embryo through EVs. EVs are present in human endometrial fluid [33], and exosomes containing microRNA (miRNA) has-miR-30d are actively transferred from endometrial epithelial cells to embryo trophoblast cells, where the miRNA is internalized. Murine embryos treated with a synthetic analogue of human miR-30d show altered gene expression, with higher expression of genes encoding molecules involved in embryo adhesion, such as *Itgb3*, *Itga7*,

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA

Study type	Year	Title	Authors	Journal	Sample size	Main findings
RCT	2020	A five-year multicentre randomized controlled trial comparing personalized, frozen and fresh blastocyst transfer in IVF	Simon C. et al.	Reproductive BioMedicine Online, 2020; 41(3):402–415	458 patients with blastocyst transfer at first appointment were randomized to pET guided by ERA, FET, or fresh ET	Per ITT: No differences except cumulative PR that was significantly higher in the pET (93.6%) vs. FET (79.7%) (p = 0.0005) and fresh ET (80.7%) (p = 0.0013). Per protocol: LBR at first embryo transfer were 56.2% in PET versus 42.4% in FET (P = 0.09), and 45.7% in fresh embryo transfer groups (P = 0.17). Cumulative LBR were 71.2% in PET versus 55.4% in FET (P = 0.04), and 48.9% in fresh ET (P = 0.003). PR in PET, FET and fresh embryo transfer arms were 72.5% versus 54.3% (P = 0.01) and 58.5% (P = 0.05), respectively. IR were 57.3% versus 43.2% (P = 0.03), and 38.6% (P = 0.004), respectively. Obstetrical outcomes, type of delivery, and neonatal outcomes were similar in all groups.
Prospective	2013	The endometrial receptivity array for diagnosis and personalized embryo transfer (pET) as a treatment for patients with repeated implantation failure	Ruiz-Alonso M. et al.	Fertil Steril. 2013; 100(3):818–24	RIF group: n =8 5 Control group: n = 25	WOI was displaced in 25.9% of patients in the RIF group vs. 12% in the control group. RIF patients after pET reached PR 51.7% and IR 38.5% that is similar to good prognosis patients
	2021	Role of endometrial receptivity array for implantation failure in in-vitro fertilization & intracytoplasmic sperm injection	Nafees et al.	Biomedica. 2021; 37(4):220–226.	Patients with ≥1 IF (n = 16)	Displaced WOI in 25% of patients. PR of 75%.
	2021	Routine endometrial receptivity array in first embryo transfer cycles does not improve live birth rate	Riestenberg C. et al.	Fertil Steril. 2021; 115(4):1001–1006.	ERA group n = 147 Standard ET group n = 81	Displaced WOI in 59.2% of ERA group patients. LBR was not different between pET and standard ET group (56.6% vs. 56.5%).
Retrospective	2014	What a difference two days make: "personalized" embryo transfer (pET) paradigm: a case report and pilot study	Ruiz-Alonso M. et al.	Hum Reprod. 2014; 29(6):1244–7.	Case report and Series with 1–6 failed transfers (n = 17)	Case report: clinical case of successful pET after seven previous failed IVF attempts. Case series: After pET these patients reached 60% PR vs 19% PR after ET in a non-receptive endometrium diagnosed by ERA.
	2015	Endometrial receptivity array: Clinical application	Mahajan N.	J Hum Reprod Sci. 2015; 8(3):121–9.	RIF group (n = 80) Control group (n = 93)	In RIF Indian population, WOI displacement was 27.5% vs. 15% in control non-RIF group (P = 0.04). Both groups have pET guided by ERA, reaching similar outcomes: RIF: OPR 42.4% and IR 33% vs. Non-RIF: OPR 56% and IR 39% (p > 0.1).
	2017	Efficacy of the endometrial receptivity array for repeated implantation failure in Japan: A retrospective, two-centers study	Hashimoto T. et al.	Reprod Med Biol. 2017; 16(3):290–296.	RIF group (n = 50)	In RIF Japanese population, WOI displacement was found in 24% of patients. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET: PR 35.3% in receptive (R) patients vs. 50% in non-receptive (NR) patients (p = 0.9).

(Continued)

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA *(Continued)*

Study type	Year	Title	Authors	Journal	Sample size	Main findings
Retrospective	2017	Window of implantation (WOI) transcriptomic stratification reveals different endometrial subsignatures associated with live birth and biochemical pregnancy	Diaz-Gimeno P. et al.	Fertil Steril. 2017; 108(4):703–710.e3.	Fertile donors (n = 79) ERA patients (n = 771)	Ongoing PR ranged from 76.9% and 80% in the late pre-receptive and receptive (R), respectively, versus 33.3% when ET is performed in late-receptive. The biochemical pregnancy was 7.7% and 6.6% in late pre-receptive and R, respectively, but 50% when ET was performed in late-receptive.
	2018	The role of the endometrial receptivity array (ERA) in patients who have failed euploid embryo transfers	Tan J. et al.	J Assist Reprod Genet. 2018; 35(4): 683–92.	ERA group with ≥1 previously failed euploid transfer (n = 88)	WOI displacement in 22.5% of patients. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET. In the group of RIF patients with euploid transfer, IR and OPR were apparently higher after correcting the WOI in NR vs. R patients (76.5 vs. 53.8% and 64.7 vs. 42.3%, respectively) although not statistically significant.
	2018	Does the endometrial receptivity array really provide pET?	Bassil R. et al.	J Assist Reprod Genet. 2018; 35(7):1301–1305.	ERA group with 0–2 failed transfer (n = 53) Standard ET group with 0–2 failed transfer (n = 503)	WOI displacement in 64.15% of ERA groups patients. No statistically significant differences in PR between pET and ET group (39% vs. 35.2%)
	2018	WOI is significantly displaced in patients with adenomyosis with previous implantation failure as determined by endometrial receptivity assay	Mahajan N. et al.	Journal of human reproductive sciences. 2018; 11(4):353.	Adenomyosis group (n=36) Control group (n = 338)	In adenomyosis, WOI was significantly displaced (47.2%) vs. controls (21.6%) ($p < 0.001$) The incidence of RIF in adenomyosis was 66.6% versus 34.9% in controls ($P < 0.001$). PR after pET in adenomyosis group was 62.5%.
	2019	pET Helps in Improving IVF/ICSI Outcomes in Patients with Recurrent Implantation Failure (RIF)	Patel JA. et al.	J Hum Reprod Sci. 2019; 12(1):59–66.	RIF group (n = 248)	WOI displacement in 17.7% of RIF patients. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET Ongoing PR 41.7% vs. 42.9% ($p = 0.93$).
	2019	Endometrial Receptivity Analysis – a tool to increase an implantation rate in assisted reproduction.	Hromadova L. et al.	Ceska Gynekol. 2019; 84(3):177–183.	ERA group (n = 85)	WOI displacement in 36.5% of patients. PR after pET in NR patients was 69.2%.
	2019	What is the clinical impact of the endometrial receptivity array in PGT-A and oocyte donation cycles?	Neves AR. et al.	J Assist Reprod Genet. 2019; 36:1901	pET group with ≥1 previously failed euploid transfer (n = 24) or with ≥2 previously failed donor transfer (n = 32) Standard ET group with ≥1 previously failed euploid transfer (n = 119) or ≥2 previously failed donor transfer (n = 158)	After euploid embryo transfer no differences in pET vs ET groups were found (IR 55.6% vs. 65% and PR 58.3% vs. 70.6%). Significant lower PR (34.4% vs. 65.2%; $p = 0.001$) in donor pET group vs. donor standard ET group. RIF patients with displaced or non-displaced WOI reached similar outcomes after pET in both euploid and donor arms.

(Continued)

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA ***(Continued)***

Study type	Year	Title	Authors	Journal	Sample size	Main findings
Retrospective	2020	Evaluation of the endometrial receptivity assay and the pre-implantation genetic test for aneuploidy in overcoming RIF	Cozzolino M. et al.	J Assist Reprod Genet. 2020; 37(12):2989–2997.	Moderate RIF group: (n = 2110) Severe RIF group (n = 488)	Patients with euploid embryo transferred in the moderate RIF group, had higher IR and ongoing PR than those without PGT-A. The use of the ERA test did not appear to significantly improve clinical outcomes in either group.
	2020	Comparing endometrial receptivity array to histologic dating of the endometrium in women with a history of implantation failure	Cohen AM. et al.	Syst Biol Reprod Med. 2020; 66(6):347–354.	RIF group (n = 97)	WOI was displaced in 47.4% of patients. The concordance between ERA and histological dating was 40.0%. RIF patients with displaced (22.5%) or non-displaced WOI reached similar clinical PR after pET (26.7% vs. 22.5%) (p = 0.66).
	2020	Does pET based on ERA improve the outcomes in patients with thin endometrium and RIF in self versus donor programme?	Selvaraj P. et al	Gynecological Research and Obstetrics 6.3 (2020): 076–080.	RIF self oocyte ERA (n = 179) RIF self non-ERA (n = 180) RIF donation ERA (n = 181) RIF donation non-ERA (n = 182)	Displaced WOI in 35–39% of patients. Clinical outcome not statistically different between patients with self-oocytes with and without ERA (due to embryo factor) but significant higher in ovum donation patients with ERA (CPR 59.4%) than without ERA (CPR 43.4%)
	2021	Clinical utility of the endometrial receptivity analysis in women with prior failed transfers	Eisman LE. et al.	J Assist Reprod Genet. 2021; 38(3):645–650.	ERA group with ≥1 previously failed transfer (n = 131) Control group (n=91)	WOI was displaced in 45% of patients with ≥1 failed transfer, 40% of patients with ≥3 previously failed transfer and 52% of control patients. The pregnancy outcomes did not differ between women with ≥1 prior failed ET and controls. In women with ≥3 prior failed ETs, there was a lower ongoing pregnancy/LBR (28% vs. 54%, p = 0.046).
	2021	Evaluation of Pregnancy Outcomes of Vitrified-Warmed Blastocyst Transfer before and after Endometrial Receptivity Analysis in Identical Patients with RIF	Kasahara Y. et al.	Fertility & Reproduction. 2020; 3(2):35–41	RIF group (n = 95)	In RIF patients, comparison of previous ET and pET demonstrate a significant increase in PR for pET per patient and cycle (5.3% vs. 62.8%, 4.4% vs. 47.9%, respectively). PR, IR at the first pET were significantly higher in patients with displaced WOI vs. non-displaced.
	2021	The use of propensity score matching to assess the benefits of the endometrial receptivity analysis in frozen embryo transfers	Bergin K. et al.	Fertil Steril. 2021; 116(2):396–403.	ERA group (n = 133) Non-ERA group (n = 353)	No statistically significant differences were found between ERA and non-ERA group (LBR 49.62% vs. 54.96%)
	2021	Do clinical outcomes differ for day-5 versus day-6 single embryo transfers controlled for endometrial factor?	Stankewicz T. et al.	Reprod Biomed Online. 2021 Nov 18:S1472-6483 (21)00581-2.	Day 5 blastoc: 183 Day 6 blastoc: 77	Clinical outcomes were similar when transferring day-5 blastocysts versus day-6 blastocysts: PR 75.4% and 70.1% (P = 0.465); IR 67.8% and 63.6% (P = 0.476); and OPR 57.9% and 58.4% (P = 0.728).

(Continued)

TABLE 26.1 ERA Clinical Publications. Scientific Publications Showing the Clinical Outcome Obtained after Performing pET Guided by ERA ***(Continued)***

Study type	Year	Title	Authors	Journal	Sample size	Main findings
Retrospective	2022	Role of endometrial receptivity array in RIF	Samadhiya R. et al.	Fertility Science and Research 8.2 (2021): 180.	RIF patients (n = 34)	WOI displacement in 38.2% of patients. PR and IR after pET in the NR group, achieved 50% and 45.5%, similar to the 55.4% obtained in general patients (non RIF).
	2022	Comparison of the Effectiveness of Endometrial Receptivity Analysis (ERA) to Guide pET with Conventional Frozen Embryo Transfer in 281 Chinese Women with RIF	Jia Y. et al.	Medical Science Monitor: International Medical Journal of Experimental and Clinical Research 28 (2022): e935634–e935634.	RIF ERA group (n = 140) RIF Non-ERA group (n = 141)	The ERA test identified 35% of samples as R and 65% as NR in the ERA group. Higher Clinical PR and IR were found in the ERA group than in the non-ERA group (Clinical PR with ERA 50% vs 24.8% without ERA and IR with ERA 41.7% vs 18.8% without ERA; $P < 0.01$), while no significant differences were detected between the two groups in terms of miscarriage rates ($P > 0.05$).
Case report	2014	Live birth after embryo transfer in an unresponsive thin endometrium	Cruz F. & Bellver J.	Gynecol Endocrinol. 2014; 30(7):481–4.	Case report	WOI found in an endometrium with 3.5mm with subsequent live birth achieved after pET.
	2018	Different Endometrial Receptivity in Each Hemiuterus of a Woman With Uterus Didelphys and Previous Failed Embryo Transfers	Carranza F. et al	J Hum Reprod Sci. 2018; 11(3):297–299.	Case report	ERA showed Receptivity in the right-sided hemiuterus while the left-sided hemiuterus was NR. Live birth achieved after pET in the right-sided hemiuterus.
	2019	Why results of endometrial receptivity assay testing should not be discounted in RIF?	Simrandeep K. et al.	The Onco Fertility Journal. 2019; 2(1): 46–49.	Cases report (n = 3)	Three severe cases of RIF patients; two of them had a previous ERA performed at a different centre, but pET not followed, resulting in failure. Once pET was implemented, successful clinical pregnancy was achieved in both patients.
	2019	The Reproductive Outcomes for the Infertile Patients with RIFs May Be Improved by Endometrial Receptivity Array Test	Ota T. et al.	Journal of Medical Cases. 2019; 10(5), 138–140.	Case report	Patient who achieved pregnancy with pET guided by ERA after 11 previous failed attempts.

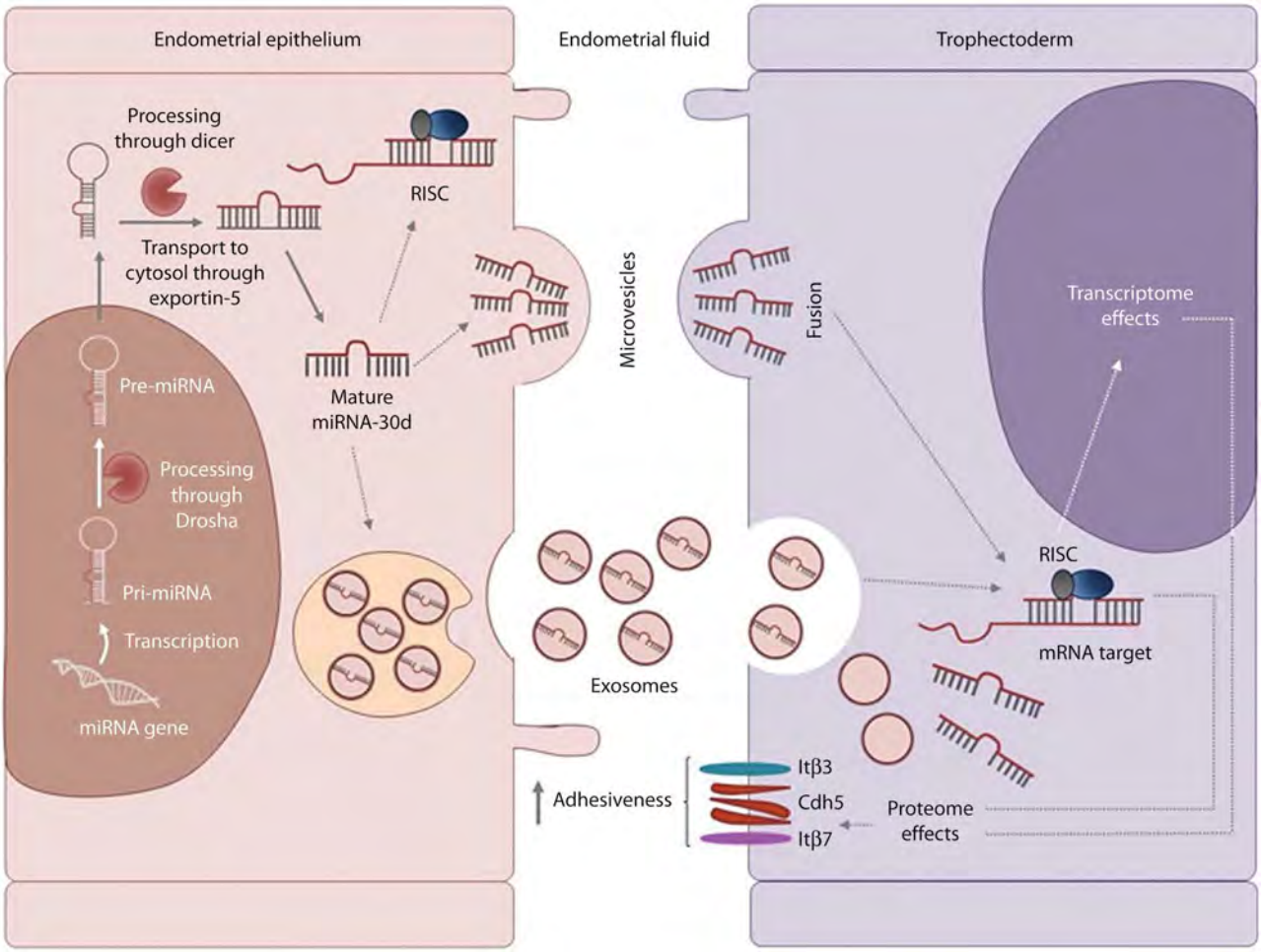

FIGURE 26.2 Schematic representation of cross-talk between the mother and embryo. Endometrial epithelial cells encapsulate miRNAs in EVs. Specifically, miRNA-30d secreted by epithelial cells are encapsulated in EVs and internalized in trophectodermal cells that produce effects to increase the adhesiveness of the embryo (from [32], reprinted with permission).

and *Cdh5*, highlighting the importance of miR-30d transference via exosomes [32] (Figure 26.2). EV proteomes are regulated by steroid hormones, and exosomes produced during the receptive phase may potentially impact embryo implantation. For example, exosomes are internalized by human trophoblastic cells, enhancing their adhesive capacity partially through the focal adhesion kinase cascade [37].

DNA transmission between cells may also occur via EVs. Single-stranded DNA and double-stranded DNA are present in different types of vesicles [38], and horizontal transfer of EV DNA is a possible new mechanism for the transfer of genetic material across species [39]. Exosomes and micro-vesicles are also transporters of mitochondrial DNA (mtDNA) and may therefore transmit altered mtDNA [40]. Vertical transmission of maternal mtDNA cargo to the embryo may be a mechanism to modulate embryo bioenergetics during the peri-conceptional period [41].

Embryo-to-mother communication

The pre-implantation embryo releases specific molecular effectors to the extracellular milieu, which influence endometrial cell gene expression (e.g. trypsin released by embryos before implantation raises calcium signalling in endometrial epithelial cells) [42–44]. Decidualized stromal cells recognize incompetent embryos and act as biosensors of embryo quality by assessing these embryo-derived soluble factors that are involved in implantation. Decidualized stromal cells of patients with recurrent miscarriages cannot discriminate between low- and high-quality human embryos [42, 45]. Therefore, disruption of this biosensing process can result in recurrent miscarriage, and natural killer cells play an important role as modulators of these biosensors [46].

To analyse molecules released by embryos, laboratories can use culture medium from *in vitro* fertilization (IVF) cycles, allowing for the characterization of paracrine/autocrine processes. Secretion of miRNAs during early embryo developmental stages may mediate such dialogue between an embryo and the mother. miRNAs are detected at a much higher scale at the blastocyst stage, indicating that this type of signalling begins soon after blastulation in a period concomitant with endometrial invasion [47].

Embryos also produce EVs that participate in cross-talk with the endometrium [35, 48] and in autocrine regulation [49]. The presence of specific embryonic exosomes significantly increases

from the cleavage stage to the blastocyst stage [50]. Endometrial cells may take up different types of vesicles that contain miRNAs and other molecules [27, 32]. In *in vitro* models, EVs isolated from a trophectoderm cell line stimulate the proliferation of endothelial cells, showing that EVs can act as potential regulators of endometrial angiogenesis [36]. Additional investigations of this potential communication between embryos and implantation sites are needed to better understand the processes underlying implantation.

The endometrial microbiome

The existence of an endometrial microbiome was demonstrated recently through the use of culture-independent techniques (fingerprinting, targeted amplification, DNA microarrays, metagenomic sequencing, etc.) [51]. The most common method for bacterial profiling is 16S rRNA gene sequencing. This gene is conserved in every bacterium and contains numerous species-specific hypervariable regions that can be used like "fingerprints" to identify bacterial taxa based on reference sequences [52, 53]. This technique can detect minute amounts of microbial DNA in the environmental or in biological specimens and has helped reveal the indigenous microbiota of numerous body sites [54], including those classically considered sterile, such as the uterine cavity.

The female reproductive tract possesses a microbiota continuum that increases in diversity and decreases in abundance from the outer to the inner organs [55]. Thus, the endometrial microbiota, like others in the upper reproductive tract, has a low biomass, which is estimated to be 100–10,000 times lower than the bacterial load in the vagina [55]. The uterine cavity may be colonized via several mechanisms, including sexual activity, gynaecological procedures, hematogenous spread of oral or respiratory bacteria, and translocation of gut or urinary microbiota to the reproductive tract [56, 57]. The most plausible route of endometrial colonization is the ascension of bacteria from the vagina due to its close proximity and direct communication with the uterine cavity. This hypothesis is supported by the similarity between bacterial profiles found in the endometrium and vagina of women at different ages, the presence of polymicrobial biofilms adhered to the uterine lining of women with vaginal infections, the results of animal studies of bacterial translocation, and the increasing convergence of endometrial and vaginal microbiota across the lifespan [58–61].

Increasing evidence shows that *Lactobacillus* is the most common genus in the endometrium of reproductive-aged women. Consistent with the vagina, the dominance of *Lactobacillus* spp. is considered the reproductive tract physiological flora [58, 62–65] (Figure 26.3). Other bacterial genera, such as *Gardnerella*, *Prevotella*, *Streptococcus*, *Clostridium*, *Bacteroides*, *Atopobium*, etc., have also been detected in the endometrium [58, 61, 65–67]. However, despite global similarities between the bacterial composition of endometrial and vaginal microbiota, some studies analysing paired endometrial and vaginal samples collected from reproductive-aged women showed that the microbiota of these two body sites are not identical in every woman. This finding shows that potential pathogenic bacteria may reside in the endometrium and are absent in the vagina and vice versa [58, 62].

Deviations from a *Lactobacillus*-dominated endometrial microbiota are associated with gynaecological conditions, including endometrial polyps [68], endometrial cancer [69], endometriosis [55, 70], menorrhagia and dysmenorrhea [71], and infertility [64, 72]. Specifically, chronic endometritis (CE; persistent inflammation of the endometrium caused by a subclinical bacterial infection with common pathogens) is significantly associated with RIF and recurrent pregnancy loss (RPL) [73–75]. Comparative studies showed worse reproductive outcomes in RIF [implantation rate (IR): 15% CE versus 46% no CE] and RPL patients [live birth (LB) rate: 7% before treatment versus 56% after treatment] with concomitant CE than in patients without CE [76, 77]. Importantly, antibiotic treatment in RIF patients improves IR, clinical pregnancy rate (CPR), and LB rate after eliminating CE, and clinical results in patients with cured CE were comparable with those in patients without CE [78].

The reproductive tract microbiota may fluctuate in response to endogenous and exogenous factors, including hormones [57, 79]. A study conducted in 392 RIF patients revealed that 44.9% had a non-*Lactobacillus*-dominated endometrial microbiota with high abundance of *Gardnerella*, *Atopobium*, *Streptococcus*, and *Prevotella* [72]. The content of *Lactobacillus* in endometrial fluid increases with follicular development, starting with <50% of *Lactobacillus* after menstruation and gradually increasing to an average of >70% in the luteal phase [72]. In the context of IVF, the endometrial microbiota may change following COS cycles and P supplementation [67, 71], with decreased *Lactobacillus* content and increased abundance of reproductive tract pathogens, such as *Atopobium*, *Escherichia*, and *Prevotella* [67]. Because of this, when managing patients with infertility undergoing assisted reproductive technology (ART) treatment, the endometrial microbiota should be assessed during the WOI in a mock cycle before embryo transfer to accurately assess the microbial environment that the embryo may encounter during implantation. Failure to assess the microbiome under these conditions may result in misdiagnosis of the endometrial microbiota in these patients.

Several studies analysed the association between composition of the endometrial microbiota and clinical results in ART patients, with variable results [80]. Patients with positive culture for pathogens such as anaerobic bacteria, Enterobacteriaceae, *Enterococcus* spp., *Escherichia coli*, *Haemophilus* spp., *Klebsiella pneumoniae*, *Staphylococcus* spp., and *Streptococcus* spp. in the uterine cavity at the time of embryo transfer had lower IR and CPR and higher clinical miscarriage rates than patients with negative cultures [81–86]. Results from molecular studies using 16S rRNA sequencing that analysed the whole endometrial microbiota have led to consideration of the endometrial microbiota from an ecological point of view. The impact of the endometrial microbiota on IVF outcomes was first demonstrated in a cohort of 35 RIF patients in which a *Lactobacillus*-dominated microbiota (>90% *Lactobacillus*) was associated with increased LB rate, while the presence of pathogenic bacteria, to the detriment of *Lactobacillus*, was significantly associated with reproductive failure [58]. These results were corroborated in a recent international observational prospective study conducted in 342 patients that confirmed the significant association between composition of the endometrial microbiota and reproductive outcomes after receiving pET at the time of the maximum endometrial receptivity, as determined by ERA [65]. Women with a higher abundance of lactobacilli are more likely to achieve a live birth, while the pathogenic profile associated with reproductive failure (no pregnancy, biochemical pregnancy, or clinical miscarriage) consisted of *Atopobium*, *Bifidobacterium*, *Chryseobacterium*, *Gardnerella*, *Haemophilus*, *Klebsiella*, *Neisseria*, *Staphylococcus*, and *Streptococcus* [65] (Figure 26.3).

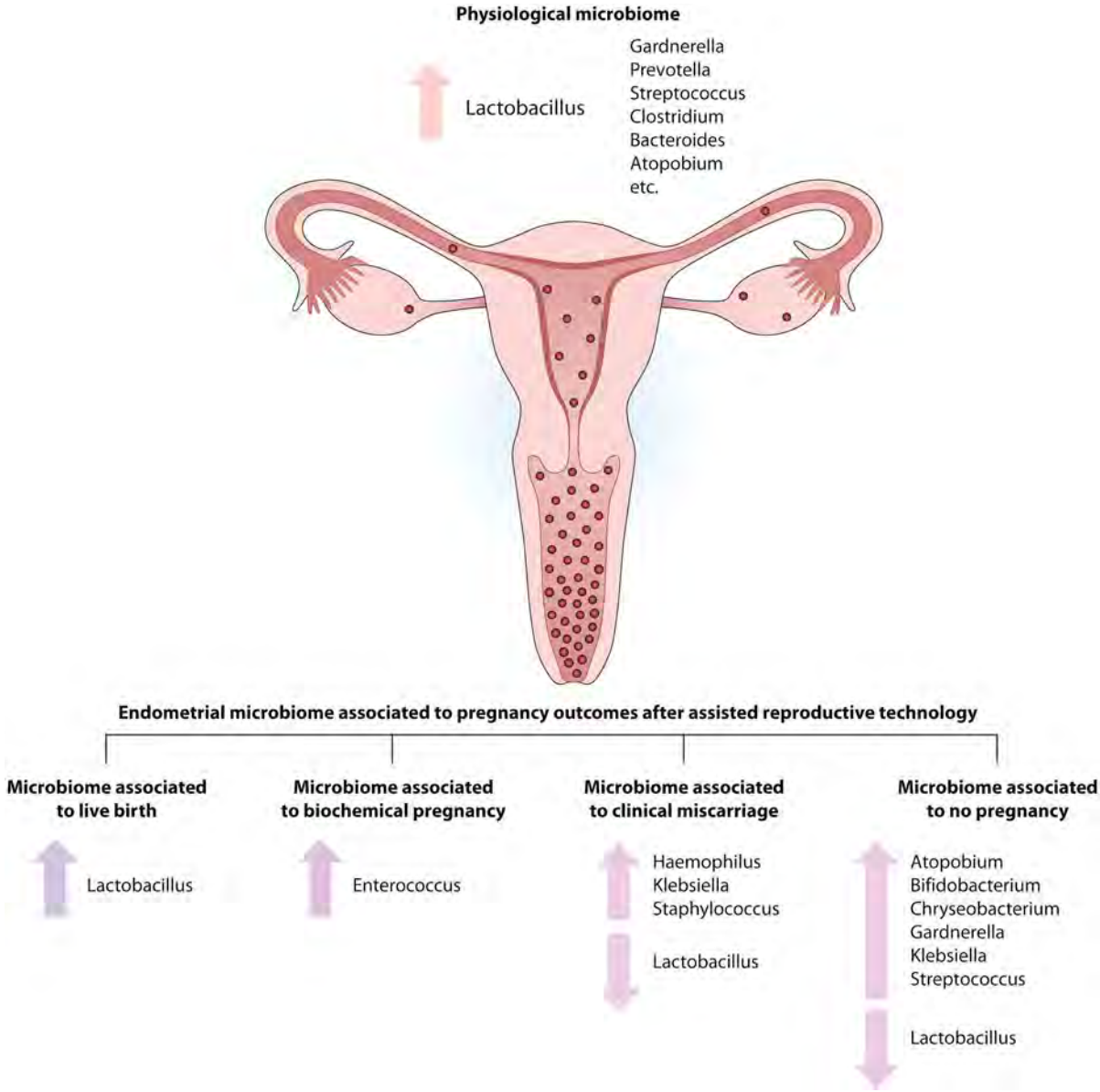

FIGURE 26.3 Endometrial microbiome composition in physiological and pathological conditions. The endometrial microbiota in reproductive-aged women is mainly composed of *Lactobacillus* species under physiological conditions, although other bacterial taxa are identified at lower abundances. Pathological conditions, such as infertility, are characterized by a shift in the endometrial microbiota towards increased abundance of bacterial pathogens. In the context of IVF, dominance of *Lactobacillus* is associated with successful ART, while implantation failure or pregnancy loss is associated with a concomitant decrease in *Lactobacillus* and increase in bacterial pathogens.

The mechanisms behind the detrimental effects of dysbiotic microbiota in embryo implantation are still unknown, but the secretion of bacterial metabolites may disrupt the endometrial epithelial barrier, causing exacerbated immune and inflammatory responses, deregulation of metalloproteinases and other structural proteins relevant for embryo implantation, and secretion of pathogenic molecules that may produce toxicity in the ready-to-implant uterus. However, while these hypotheses warrant further testing, endometrial microbiota composition can be considered as a complement to ERA in cases of infertility of endometrial origin.

Conclusion

New transcriptomics-based molecular methods to assess endometrial receptivity have been introduced into the clinic in recent years, providing us with molecular tools to time the WOI and improve IVF success. Several other "omics" approaches are now being applied to unravel the complex process of endometrial receptivity. Although some technical limitations still exist, we believe that integrative sciences are the future of diagnosing the correct timing for embryo implantation. Furthermore, the application of these new technologies should be used to improve

our knowledge of endometrial receptivity, providing additional knowledge on critical dialogue between the embryo and endometrium and the endometrial microbiota composition. This knowledge will enable the discovery of the main causes of implantation failure and open new avenues of investigation into interceptive molecules to aid in the diagnosis and treatment strategies to improve embryo implantation.

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27

ARTIFICIAL GAMETES

Oocytes

Evelyn E. Telfer and Yvonne L. Odey

Introduction

Improvements in human-assisted reproductive techniques (ART) have been hindered by our lack of knowledge of the mechanisms regulating human oocyte development from formation through to maturation. Whilst there is an increasing understanding of these processes in rodent models, there is a need for tractable human model systems. The capacity to follow human oocyte development entirely *in vitro* would provide insights into the basic science of oogenesis, folliculogenesis, and meiosis and would lead to the development and improvement of ART. The production of *in vitro*-derived gametes, whether from immature gametes (primordial follicles) or from stem cells, has been described as "artificial gametes." Apart from providing important models for research, if they are shown to be safe, they would reduce the need for donor eggs and sperm as well as provide more fertility preservation options for a wider group of people. This chapter will cover the progress in producing so-called "artificial oocytes" from a range of cell types and consider the technology of growing oocytes *in vitro* from the most immature stages to maturity.

Source of artificial oocytes

Formation of oocytes *in vivo* occurs during fetal life in the human ovary. Primordial germ cells (PGCs) migrate to the presumptive ovary where they become oogonia and upon entering meiosis become oocytes that are enclosed within somatic cells (granulosa cells) to form primordial follicles. Primordial follicles consist of an oocyte arrested at the dictyate stage of prophase 1 of meiosis enclosed within flattened somatic (granulosa) cells and form the non-proliferating pool of germ cells from which recruitment for growth will take place throughout a woman's reproductive life [1]. The number of follicles formed, the rate at which they are utilized, and exposure to gonadotoxic substances are all factors that determine female fertility. For women who have a high-risk of premature ovarian insufficiency (POI), fertility preservation options such as cryopreservation of ovarian tissue for subsequent re-implantation have been developed [2, 3]. This technique has resulted in the birth of more than 130 babies [4], but reimplantation is not suitable for all patient groups. For women who have few oocytes remaining, alternative strategies such as making new oocytes from stem cells and developing them *in vitro* are being considered as future therapies.

The *in vitro* differentiation (IVD) of oocytes from stem cells has clear application for fertility preservation, and there has been rapid progress in this field particularly using mouse models. Stem cells have the potential to provide a source of new oocytes that could be utilized to achieve fertility in women who are infertile or have an exhausted ovarian reserve. Research has focused on obtaining artificial oocytes from pluripotent cells, either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Another potential source is from unipotent oogonial stem cells or germline stem cells [5, 6].

Stem cells are undifferentiated or differentiation-limited, self-renewing cells within a distinct niche. Pluripotent stem cells have the ability to differentiate into all the cells of a mammalian embryo and therefore have the potential to generate germ cells. Mammalian oogenesis *in vivo* is a tightly coordinated process which requires the transient switching on and off of regulatory genes and molecular processes, of which we still have limited knowledge (Figure 27.1).

Oocytes from pluripotent stem cells (ESCs and iPSCs)

The inner cell mass of the developing blastocyst forms the pluripotent epiblast cells which give rise to somatic tissues and germ cells. Derivation of two types of stem cell lines from the mouse epiblast has been achieved: (i) embryonic stem cells (ESCs) [7] and (ii) epiblast stem cells (EpiSCs) [8]. These cell lines can differentiate into somatic and germline lineages [8]. Embryonic stem cells from human blastocysts (hESCs) have been derived [9] and have been considered candidate progenitor cells for *in vitro* oogenesis [10].

Differentiating germ cells have been derived from mouse embryonic stem cells (mESCs) [11]. Isolation of these cells based on Oct4 and cKit expression showed a range of germ cell developmental stages (migratory primordial germ cells and post-migratory germ cells) (Figure 27.1). Oocyte-like cells (OLCs) were identified from cultured mESCS but these were not functional oocytes as they did not undergo meiosis [11]. These experiments highlighted that the formation of OLCs occurs independently of the process of meiosis as confirmed by experiments in mice on Stra8-deficient ovarian germ cells where oocytes are formed, but meiosis does not take place [12].

Identifying early germ cells within culture shows that the earliest stages of the complex pathway for germ cell development can be recapitulated *in vitro*. Before germ cell migration, germ cell fate is induced in the epiblast cells in mice via bone morphogenetic protein 4 (BMP4) signalling from the surrounding soma [13]. Epiblast-like cells (EpiLCs) have been induced from mESCs with a gene expression profile consistent with pre-gastrulating epiblasts. BMP4 induced expression of Blimp1 in EpiLCs and led to upregulation of Nanos3, Dppa3, and Prdm14 associated with primordial germ cell specification and downregulation of somatic markers Hoxa1, Hoxb1, and Snai1. These changes occurred alongside epigenetic changes replicating *in vivo* differentiation of epiblast cells into primordial germ cells [14]. These results demonstrate successful differentiation of EpiLCs to primordial germ cell-like cells (PGCLCs) *in vitro* comparable to that occurring *in vivo*.

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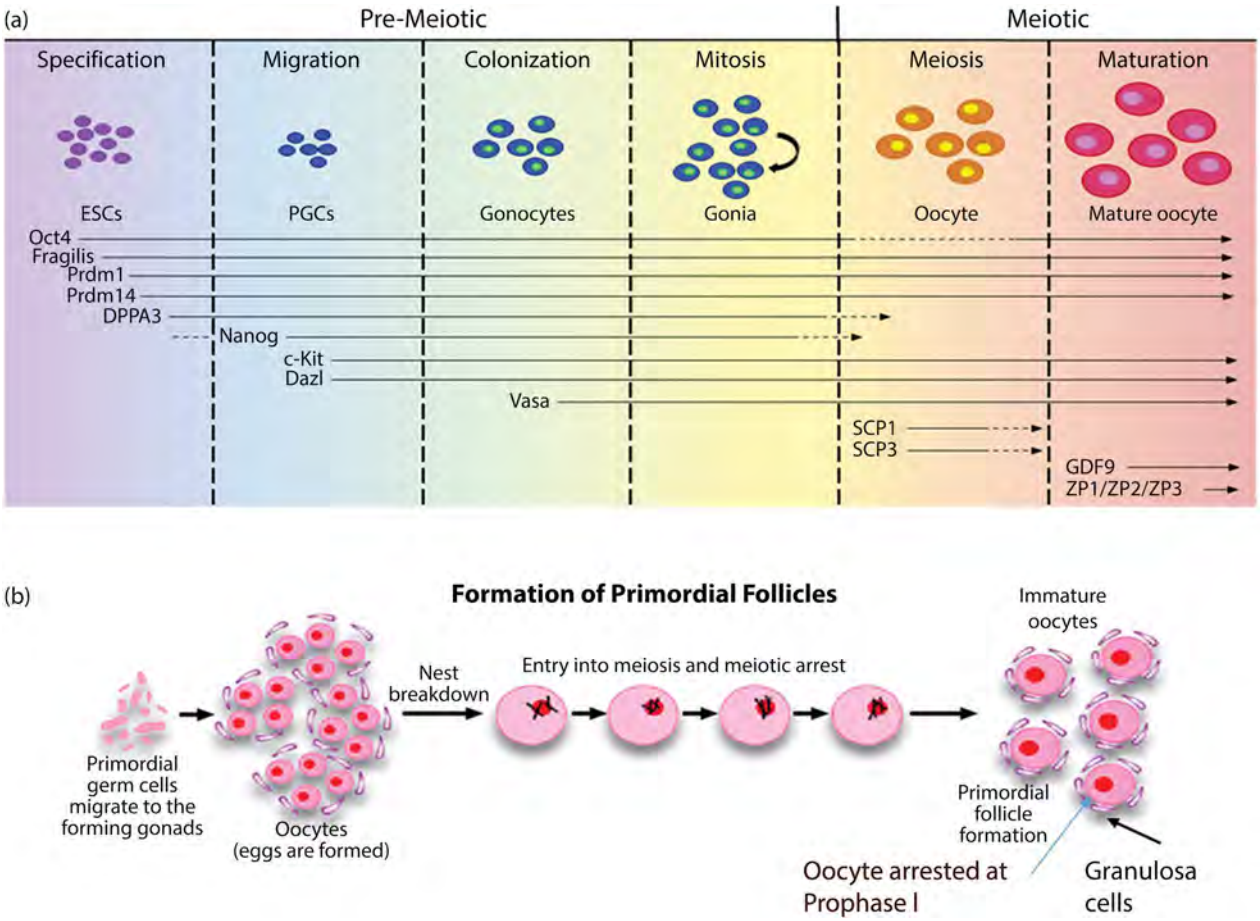

FIGURE 27.1 (a) Stages of germ cell formation/development and expression levels of germ cell markers. (b) The process of follicle formation from PGCs to oogonia entering meiosis to form oocytes that are arrested at prophase I of meiosis, surrounded by somatic cells to form primordial follicles. *Abbreviations*: ESC, embryonic stem cell; PGC, primordial germ cell.

BMP4-dependent differentiation of PGCLCs could be inhibited by Noggin (BMP4 antagonist), whereas another mesoderm promoting factor (Wnt3a) also induced PGCLCs in culture [15], illustrating the importance of somatic factors in *in vitro* differentiation of germ cells. PGCLCs derived from embryoid bodies (EB) differentiated into oocyte-like cells with expression of oocyte-specific genes (Figα, GDF9, ZP1, ZP2, and ZP3) and early meiotic marker (SCP3) when co-cultured with granulosa cells [16], similar results were observed when PGCLCs were co-cultured with Chinese hamster ovary cells [17]. However these results could not be replicated with granulosa cell conditioned medium [16], confirming the importance of cell–cell interactions with ovarian somatic cells (Figure 27.2).

When PGCLCs were combined with embryonic ovarian somatic cells and xenotransplanted to the ovarian bursa of immune-deficient recipient mice, oocyte-like cells enclosed within follicles were formed. The oocyte-like cells were capable of being matured and fertilized *in vitro* and embryos were produced. The resultant offspring were healthy, fertile, and showed normal imprinting patterns [18]. These studies demonstrated the potential of mESCs to undergo differentiation to all stages of oogenesis and subsequent embryonic development, and, as with *in vivo* oogenesis, interactions with surrounding somatic cells are essential for successful *in vitro* oogenesis (Figure 27.2).

Germ cell differentiation of human ESCs has been investigated and PGCLCs have been derived from hESCs with gene expression patterns similar to PGCs [19]. The differentiation of hESCs to germ cell precursors occurs spontaneously but it has been shown that the addition of BMP4 increases the rate of differentiation [20]. Several growth factors and feeder layers have been utilized to improve the differentiation rate cells with germline and meiotic markers have been obtained (reviewed by [21]). Follicle-like structures that express oocyte-specific markers (ZP1 and GDF-9) were formed in EBs derived from hESCs; however, a zona pellucida could not be detected in the presumptive oocyte [22]. More recently, hESCs have developed into oocyte-like structures [23], but meiosis has not been observed.

Whilst research using ESCs gives us insight into cell lineage development, the use of human ESCs clinically is fraught with practical difficulties and ethical concerns. A major concern is that derivation of oocytes from human ESCs for clinical application would be dependent on somatic cell nuclear transfer as the cells would not be biologically related to the recipient [24] and many ethical concerns surround this. Therefore, it is unlikely that derivation of gametes by this route would be applied clinically and a more likely route would be to utilize induced pluripotent stem cells (iPSCs) derived from adult cells, which overcomes the difficulties associated with hESCs [10].

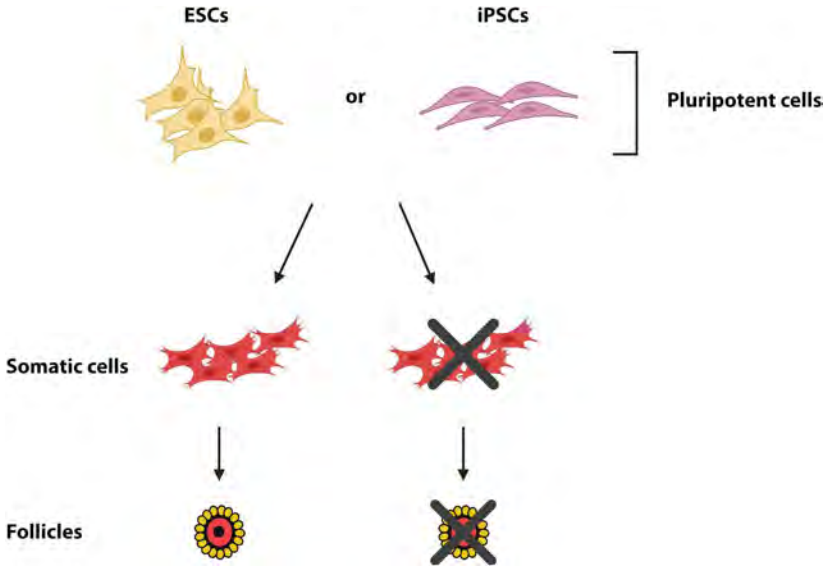

FIGURE 27.2 Illustrating the essential step of combining somatic cells with primordial germ cell-like cells to form follicles *in vitro*. If somatic cells are absent, no follicles will be formed. *Abbreviations*: ESCs, embryonic stem cells; iPSCs, induced pluripotent stem cells.

Oocytes from iPSCs

Methodology to dedifferentiate and induce pluripotency in adult cells was developed by Takahashi and Yamanaka and led to the generation of the first iPSC lines [25, 26]. Mouse fibroblasts under the expression of four crucial pluripotency genes—Oct3/4, Klf4, Sox2, and c-Myc—were induced to a pluripotent state and termed induced pluripotent cells (iPSCs) [26], and iPSCs have now been derived from several species including humans [25]. These cells have been used to regenerate several tissue types and are becoming clinically viable [27]. Given that iPSCs do not raise the same ethical concerns as ESCs they are more likely to provide a clinical option for artificial gametogenesis.

Using a tetraploid complementation assay, mouse iPSCs have demonstrated their ability to generate all cell types including germ cells [28, 29], similar to ESCs. Mouse iPSCs can be derived *in vitro* to EpiLCs and PGCLCs and have been combined with somatic cells to form a reconstituted ovary, after xenotransplantation oocytes have been generated (reviewed by [30]). Some of these oocytes are meiotically and developmentally competent, and offspring have been produced [18]. Following on from this, it has been demonstrated that competent oocytes can be derived from stem cells entirely *in vitro*, avoiding the need for a transplantation stage [31] (Figure 27.3). Hikabe et al. developed a multistep system that supports *in vitro* differentiation, *in vitro* growth, and *in vitro* maturation to produce developmentally competent oocytes entirely *in vitro*.

Each stage of development is supported using medium containing a combination of factors specific for each stage [31]. Stage one supports the generation of PGCLCs from iPSCs or mESCs using media with a mixture of growth factors then combining the PGCLCs with embryonic ovarian cells to make an ovarian organoid which supports the formation of oocytes/follicles within 21 days of reaggregation [31]. Stage two supports follicle/oocyte growth *in vitro* in media containing BMP15, GDF-9, and FSH to produce fully grown oocyte–cumulus complexes that can be matured in stage three. Stage three utilizes standard IVM protocols to mature these *in vitro*-derived oocytes. Some of the oocytes reached Metaphase II, were fertilized, and offspring were produced [31]. Whilst the offspring produced were healthy and epigenetically normal, the success rate was low, and many unhealthy oocytes were produced with less than 4% resulting in the formation of embryos (31). A greater understanding of the factors regulating oocyte formation/early development is required, and recently a group of transcription factors that can produce oocyte-like cells *in vitro* from embryonic stem cells has been identified [32]. These findings represent major progress in defining the mechanisms required to produce good oocytes [33].

Clearly, the ability to derive oocytes from iPSCs entirely *in vitro* is a huge step, but this protocol [31] relied on using embryonic tissue as a source of somatic cells to support germ cell development. The use of embryonic/fetal tissue is not a viable option if these protocols are ever to be applied to humans and utilized clinically. Another major advance in this field has shown the development of ovarian somatic cell support from PSCs [34]. Under defined culture conditions, mESCs can be differentiated into fetal ovarian somatic cell-like cells (FOSLCs) [34], and these can be combined with PGCLCs derived from mESCs to form aggregates that support the formation of follicles with functional oocytes capable of being fertilized, developing embryos leading to healthy offspring [34] (Figure 27.3). The ability to form functional oocytes/follicles without the need to utilize embryonic somatic cells is a major advance and sets the scene for developing support cells from iPSCs derived from adult cells thus improving techniques for human and other species [30].

Induced pluripotent stem cells (iPSCs) from human cells (hiPSCs) have been developed in to germ-like cells with post-meiotic cells being induced [35]. PGCLCs have been induced from human iPSCs [36, 37], and more recently PGCLCs and oogonia have been derived from hiPSCs and combined with human fetal-derived somatic cells to form follicle-like structures [38]. These developments bring us closer to human oocytes being derived entirely *in vitro* from hiPSCs.

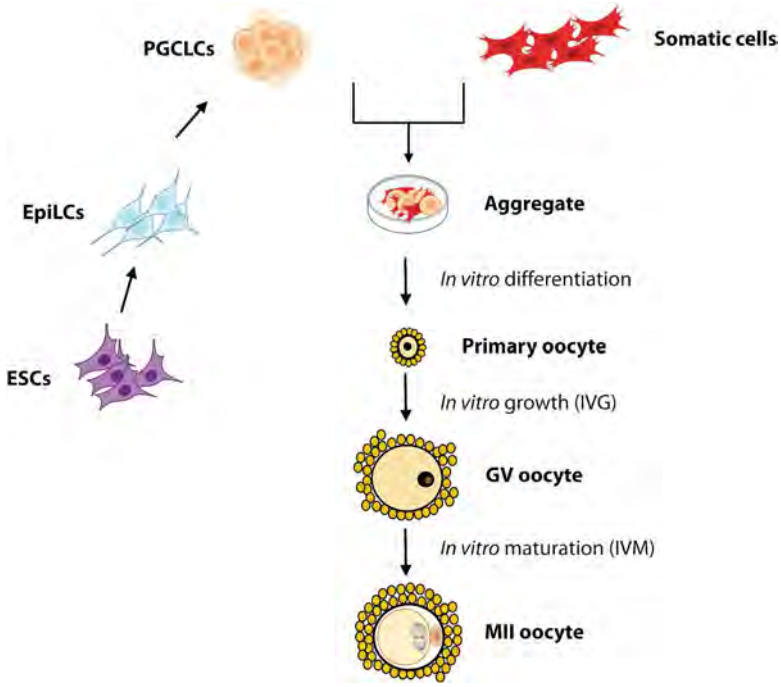

FIGURE 27.3 Steps to achieve complete *in vitro* formation of ovarian follicles from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). [31] used embryonic tissue to obtain somatic cells to support germ cell development, whereas [34] derived somatic support cells from pluripotent stem cells (PSCs) demonstrating the complete recapitulation of germ cell development *in vitro* forming competent oocytes capable of being fertilized and forming embryos. *Abbreviations:* ESCs, embryonic stem cells; EpiLCs, epiblast-like cells; PGCLCs, primordial germ cell-like cells; GV, germinal vesicle; MII, metaphase II.

There has been tremendous progress in the development of oocytes from mESCs and iPSCs in mice. With the birth of apparently healthy offspring, there is now proof of principle that artificial oocytes can be produced from mESCs and iPSCs. Human studies are making good progress but there is still much work required to determine the long-term safety of these methods and to translate it to human oocyte development.

Oocytes from somatic cell transformation

The hierarchical stem cell differentiation model has recently been challenged by the cell plasticity model, which describes that cells possess the ability to cross traditional lineage barriers [39]. The ability to manipulate a somatic cell to transdifferentiate (conversion of a differentiated cell of one lineage to a differentiated cell of another lineage without reinstating pluripotency) by direct reprogramming could also allow the *in vitro* generation of new oocytes. Skin-derived stem cells (SDSCs) isolated from neonatal mice have been shown to differentiate into PGCLCs *in vitro* [40, 41]. SDSC-derived PGCLCs underwent epigenetic changes similar to PGCs *in vivo* and activin A promoted PGCLC differentiation [41] similar to human ESCs. SDSCs also generated aggregates, morphologically similar to follicles, containing large cells with expression of oocyte-specific markers, when cultured alone and with ovarian cells derived from neonatal mice. The large oocyte-like cells expressed meiotic markers, but SCP3 showed discontinuous staining patterns, consistent with the cells' inability to progress through meiosis. SDSCs aggregated with neonatal ovarian somatic cells and transplanted to the kidney capsule of recipient mice generated oocyte-like cells in developing follicles through to antral stages of development [40], suggesting SDSCs can differentiate and generate cells that have morphological similarities to oocytes, again confirming the separation of formation of oocyte structures from meiotic capacity [12].

Fetal porcine SDSCs have also demonstrated a potential for germ cell differentiation *in vitro*. PGCLCs derived from fetal porcine SDSCs express germ cell markers (Dppa3, Dazl, Vasa, and cKit) and also show imprint erasure [42]. Further development of these PGCLCs to OLCs in aggregates demonstrates the presence of a zona pellucida and expression of oocyte and meiotic markers. Rat pancreatic stem cells have also generated OLCs in culture with a structure similar to a zona pellucida, in aggregation with smaller cells, and share gene expression patterns with oocytes, with the presence of oocyte and meiotic markers expressed [43]. Human amniotic fluid stem cells have also been able to recapitulate this differentiation pathway *in vitro*, generating aggregates with large central cells (OLCs) surrounded by a zona pellucida structure and smaller cells which produced oestrogen during culture. Analysis of the OLCs showed the expression of oocyte-specific and meiotic markers and underwent parthenogenetic activation during prolonged culture [44, 45], consistent with previous reports from other cell types and species.

A recent study has shown that functional oocytes with genomic stability can be generated from adult mouse ovarian somatic granulosa cells [46]. Using a chemical reprogramming approach granulosa cells could be induced to form PSCs through reprogramming using crotonic acid. These gPSCs (granulosa pluripotent stem cells) acquired germline potential and could form PGCLCs which produced functional oocytes that could be fertilized and produced fertile offspring [46].

It is clear that some cells can be reprogrammed under certain conditions and can form morphological oocytes, but in most cases these do not enter meiosis. As emphasised earlier, the process of oocyte differentiation can be dissociated from meiosis [12], so if functional artificial oocytes are to be obtained then it will be essential to understand the connection between oocyte differentiation and entry into meiosis.

Oocytes from oogonial stem cells (OSCs)

In recent years there have been some exciting and controversial developments in female germ cell biology relating to an increasing body of evidence that shows oocytes may be formed by a rare population of putative germline stem cells that can be isolated from the adult ovary [5, 47–49]. These cells are proposed to be germ lineage-specific rather than being pluripotent cells but their contribution to the pool of oocytes is still unclear. This chapter will not deal with their potential physiological role and will only consider their potential utility *ex vivo*.

The isolation and identification of oocyte-producing germline stem cells, also referred to as oogonial stem cells (OSCs) from adult mammalian ovaries was reported in 2009 when putative germline stem cells were isolated from adult mouse ovaries [50]. Isolation of similar cells from adult human ovaries followed [51]. These cells have now been isolated from the ovaries of adult mice [50, 51], rats [52], and humans [51, 53–55].

The isolation of these cells has been based on magnetic or fluorescent (FACS) cell sorting, utilizing an antibody to a germ cell marker DEAD box polypeptide 4 (DDX4) [50, 51, 53]. The isolation process has led to controversy, as DDX4 is assumed to be localized internally rather than being expressed on the surface, although there is evidence that DDX4 can be expressed on the cell surface [56]. Some groups have failed to isolate the cells using similar methodologies [57, 58], whereas others have isolated a population of cells which have a molecular signature which includes germ and stem cell markers [50, 51] in rats [52] and humans [51, 53–55]. In human ovarian tissue, this is a rare cell population, comprising 0.014% of the total cell population which can stably proliferate *in vitro* for months and spontaneously generate oocyte-like structures, as determined by morphology and gene expression [51], and will also form follicle-like structures *in vitro* when combined with fetal somatic cells [53].

Injection of fluorescently labelled mouse OSCs into recipient fertile and infertile mouse ovaries has generated GFP-positive oocytes within host somatic cells and these have been capable of ovulation, fertilization, and embryonic development [50, 51], and in some cases live young have been produced [50, 52]. Human OSCs (hOSCs) have also generated OLCs enclosed in host somatic cells, as assessed by morphology and expression of oocyte-specific markers after injection into adult human ovarian cortical tissue and xenotransplantation into an immune-deficient mouse for seven days [51, 59]. Putative OSCs isolated from adult human ovaries combined with human fetal ovarian-derived somatic cells (FODSCs) *in vitro*, form oocyte/follicle-like structures in 57% of the aggregates [53]. Figure 27.4 summarizes results to identify the oogenic potential of OSCs from human ovaries.

In addition to putative OSCs, another population of stem cells have been isolated from the adult human ovary that have characteristics of very small embryonic-like stem cells (VSELs) [60, 61]. These cells have been isolated and comprise a population of small cells of less than 5 microns that express germline and stem cell markers and appear to have oogenic potential [62]. It is thought

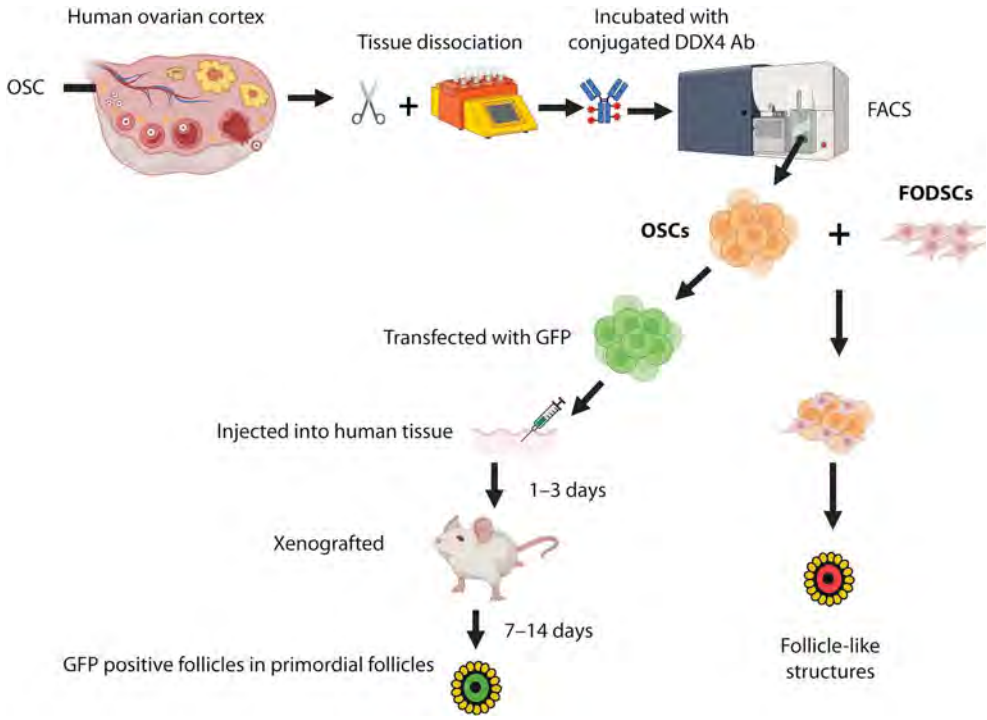

FIGURE 27.4 Oogonial stem cells (OSCs) isolated from adult human ovaries form oocyte structures within follicles if injected into human ovarian cortical tissue and xeno-transplanted to immune-deficient mice [51]. Oocyte/follicle structures can also be formed *in vitro* if OSCs are combined with human fetal ovarian-derived somatic cells (FODSCs) [53].

that these VSELs are precursors to OSCs [63], but there remains a great deal of work to be conducted to determine cell lineages in the adult human ovary.

Whilst identifying cells with apparent germline potential in the human ovary represents a major development, it has also led to a great deal of controversy. The methods used to isolate these cells still need to be clarified, but given the evidence from several groups they should be investigated further. These cells present an opportunity to learn more about germ cell development and the processes involved. The "oocyte-like" cells derived from each of the cell types discussed require somatic cell support of paracrine and junctional communication to form follicles and to support development into functional oocytes. Combining these "oocyte-like" cells with ovarian culture models may facilitate follicle formation and growth [64].

Supporting oocyte formation and growth *in vitro*

Obtaining viable oocytes by growing immature oocytes *in vitro* has been the subject of a great deal of research for more than 30 years. Whilst this is not the production of "artificial gametes," the techniques to support *in vitro* oocyte growth are essential to support the development of a gamete being developed from any source. Complete growth *in vitro* from the most immature oocytes (primordial stages) with subsequent *in vitro* fertilization (IVF) and production of live young has only been achieved in mice [65, 66]. Early work on this two-step culture system resulted in only one live offspring being obtained, and this mouse had many abnormalities as an adult [65]. Following improvements in the technique and after alterations in the culture medium several mouse embryos and offspring have been obtained using oocytes that have been *in vitro* grown (IVG) combined with *in vitro* maturation (IVM) and IVF [66]. This work has provided proof of concept that complete oocyte development can be achieved *in vitro* and has facilitated the work on mouse ESCs and iPSCs described earlier. This has led to the development of culture systems that could be applied to other species, particularly human. Advances in culturing follicles from humans, non-human primates, and domestic species had been made; bringing the prospect of achieving an *in vitro* system that supports complete human oocyte development closer [64, 67].

In vitro growth systems

A defining feature of primordial follicles formed *in vivo* is that they enter a resting phase and must be activated to initiate growth (Figure 27.5). Activation and growth of primordial follicles is marked by the transformation of the flattened epithelial cells surrounding the oocyte into cuboidal cells which proliferate, forming a multilaminar structure in which the germ cell will develop. Normal follicle/oocyte development is critically dependent upon intercellular communication between the growing oocyte and the developing granulosa cells, therefore support and maintenance of these connections are essential [68]. During follicle development, the oocyte is held in meiotic arrest, but as it grows it must acquire the ability to resume meiosis (meiotic competence) and the ability to support fertilization and embryonic development (developmental competence). The development of culture conditions to support germ cell development is an enormous challenge, and an understanding of the physiological requirements of each component of the developing follicle is needed.

Initiation of primordial follicle growth

Primordial follicles represent the dormant store of follicles, and their activation is regulated via complex interactions of paracrine factors mediated by oocyte–somatic cell interactions, all of which are influenced by biomechanical forces [69]. Supporting this complex multi-layered process *in vitro* is technically challenging, but complete human oocyte development from primordial/unilaminar follicles to meiotic maturation has been achieved [70, 71]. All systems being developed to support human oocyte development start with either ovarian cortex or whole ovaries that have been removed for fertility preservation [64]. If ovarian cortex is being used then this tissue will contain mainly primordial follicles, whilst growing follicles can be isolated from whole ovaries.

Primordial follicles isolated from human ovarian tissue are not activated to grow *in vitro* [72], but the activation of human primordial follicles occurs spontaneously within ovarian cortex [73–76] and occurs in higher numbers over a shorter timeframe if the tissue has been mechanically loosened [70, 77]. Activated follicles can develop to multilaminar (secondary) stages within six days [77]. The density of stromal cells and tissue architecture are emerging as critical factors contributing to the regulation of activation of growth *in vitro*. The fragmentation of ovarian tissue that

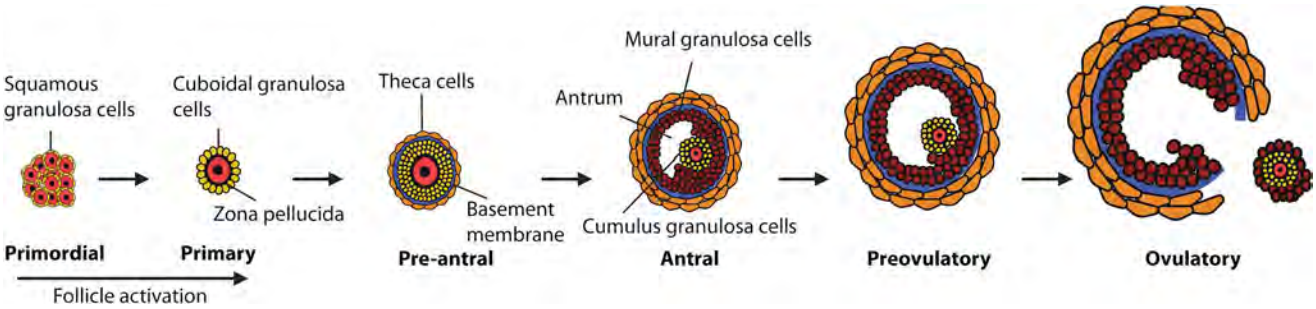

FIGURE 27.5 Stages of ovarian follicle development. Primordial follicles represent a pool of non-growing follicles which are continually initiated to grow throughout reproductive life. Once follicles are activated to grow (primary stage) granulosa cells proliferate to form multi-laminar structures (pre-antral) and then form a fluid-filled cavity (antral) which undergoes expansion to reach preovulatory stages and the oocyte–cumulus complex being released at ovulation in response to luteinising hormone (LH) signalling.

occurs during the preparation of tissue into fragments of micro-cortex affects the Hippo signalling pathway which controls organ size through regulating cell proliferation and cell death [78]. In addition to the Hippo signalling pathway, a key cell signalling pathway involved in regulating primordial follicle activation is the phosphatidylinositol-3'-kinase (PI3K-AKT) pathway [79, 80].

IVG of preantral follicles

Once follicles have been initiated to grow within ovarian micro-cortex (Figure 27.6), they develop to the preantral/secondary stage but when they reach this stage the cortical environment that they are embedded within becomes inhibitory to further growth, resulting in a loss of follicle integrity and oocyte survival [77]. This inhibitory effect can be overcome by removing growing follicles from the micro-cortex and placing them in individual culture wells to limit the effect of follicle interactions [77].

Culture systems have been developed to support the growth of multilaminar (preantral) follicles that have been isolated as growing follicles from human ovarian cortex [81–85] or developed *in vitro* from primordial stages [70, 71, 77, 86]. Maintaining the structure of isolated human ovarian follicles is challenging as they can grow up to several millimetres. Tissue engineering principles have been applied to tackle this problem and several groups have encapsulated human preantral follicles within biomatrices such as alginate to support their structure and promote their growth *in vitro* [82, 84, 85]. In addition to alginate there has been development of a range of scaffolds to support human follicle growth *in vitro*. These include de-cellularized ovarian tissue [83, 87] and three-dimensional micro-porous scaffolds [88, 89].

A supporting matrix is not required to promote the development of isolated follicles and the multi-step culture system that has been developed for human follicles does not use them (Figure 27.6) [70]. Individual multilaminar follicles are placed within v-shaped micro-well plates with serum-free medium containing a low dose of FSH, Activin-A, and ascorbic acid [70] (Figure 27.6). Growth and differentiation of preantral follicles takes place within this system and three-dimensional architecture is maintained *in vitro* in human [77, 86, 90] and cow follicles [91], with antral formation occurring within 10 days (Figure 27.6).

Once antral cavities have formed, oocyte–granulosa cell complexes can be retrieved by applying gentle pressure to the follicle [70]. Complexes with complete cumulus and adherent mural granulosa cells are then selected for further growth on membranes in step three of the multi-step system (Figure 27.6).

The aim of this stage is to promote oocyte growth given that oocyte size is an indicator of meiotic potential, and following this step oocytes of at least 100 microns can be obtained and selected for further maturation. Some IVG oocytes derived from the multi-step culture system undergo meiotic maturation following an IVM protocol with approximately 30% of oocytes that survive the entire culture period forming Metaphase II spindles [70]. Polar bodies formed by the IVGM oocytes are significantly larger than normal [70], but it is not known if this impacts developmental potential.

An important application of IVG would be to prepubertal girls who have few options for fertility preservation [92], but significant differences exist in the follicle population with age and stage of pubertal maturation [86]. Follicles derived from prepubertal girls grow at a different rate *in vitro* compared to those derived from adults, therefore culture systems developed for adult tissue may not be suitable for prepubertal girls. Adaptations and refinement for specific age groups and origin of oocytes will be required.

Final stages of growth and maturation

The end point of an *in vitro* system is to produce oocytes that can be fertilized and produce developmentally normal embryos. In order to achieve this, *in vitro*-grown human oocytes need to be matured *in vitro* to resume meiosis and reach Metaphase II (MII). The production of meiotically competent MII oocytes from human IVG follicles has been achieved [70, 93]. Whilst both systems supported oocyte growth to a diameter of >100 µm which could be selected for IVM, and oocytes reached Metaphase II, the polar bodies in the oocytes grown from the primordial stage were larger than expected [70]. More recent work utilizing a multi-step culture system over a prolonged period (nine weeks) has resulted in successful maturation of IVG oocytes to the MII stage following IVM, and all with normal-sized polar bodies [71]. Whether these IVG mature oocytes are developmentally competent remains to be assessed. Additionally, studies comparing IVG mature oocytes derived from fast or protracted culture systems are urgently needed to determine which one will provide the most adequate support for oocyte function, chromosome arrangement, epigenetic imprinting, and health.

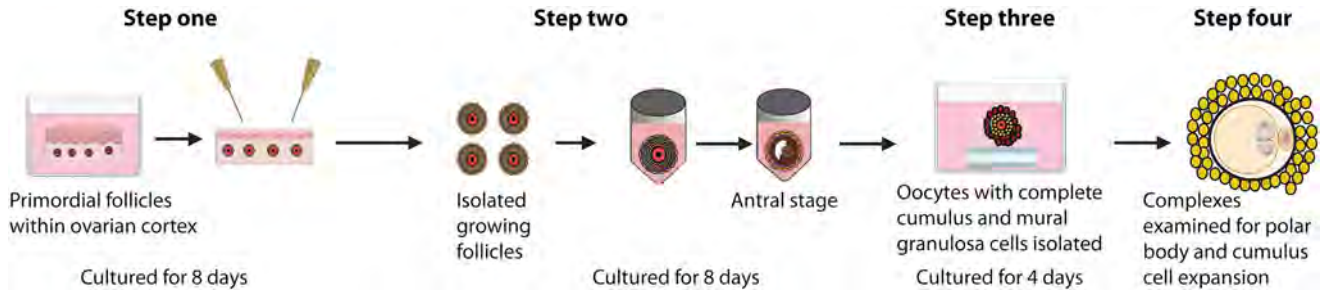

FIGURE 27.6 A multi-step culture system for human follicle/oocyte development [70]. Step one: Pieces of ovarian tissue containing primordial/unilaminar follicles are prepared for culture. Once follicles have reached multi-laminar stages, they can be mechanically isolated using needles. Step two: Isolated follicles are cultured individually from preantral to antral stages. Step three: Cumulus–oocyte complexes (COCs) are retrieved from the antral follicles and further cultured until oocyte diameter is >100 µm. Step four: COCs are placed within medium for *in vitro* maturation (IVM) and then examined for cumulus cell expansion (yellow), metaphase II spindle formation, and the presence of a polar body.

Whilst these studies provide proof of concept that complete IVG of human oocytes is possible, the clinical use of IVG culture systems is still limited by low MII rates, ambiguous fertilization capacities, and unknown epigenetic safety. As well as improvements in IVG systems there is also a need to utilize more precise IVM methods. Advances in IVM techniques that involve a pre-maturation phase have been made [94, 95]. This technique helps to prevent spontaneous meiotic maturation that occurs *in vitro* whilst maintaining synchronization of oocyte nuclear and cytoplasmic maturation. This pre-maturation is carried out in the presence of C-type natriuretic peptide ("capacitation" step – CAPA), followed by conventional IVM (CAPA-IVM). There is now accumulating evidence that CAPA-IVM leads to increased oocyte maturation rates, enhanced embryo quality, and higher pregnancy rates [96–98]. Nevertheless, further refinement and optimization of IVM protocols are still required to (i) develop and validate a standardized, efficient and safe IVM system and (ii) improve the maturation rate and developmental potential of IVG-derived oocytes.

Conclusion

Improvements in ART require a greater understanding of the mechanisms regulating human oocyte development from formation through to maturation. The capacity to follow human oocyte development entirely *in vitro* would provide insights into the basic science of oogenesis, folliculogenesis, and meiosis, potentially leading to the development and improvement of ART. The generation of healthy progeny from stem cells in mice has brought new hope for restoring female fertility. Nevertheless, advances in the development of the human germline *in vitro* have been modest compared to mouse, and the genetic stability and functionality of the cells is still uncertain. Developing these techniques into mature, safe, and replicable processes following International Society for Stem Cell Research guidelines [99] is ongoing, but there is still a long way to go before artificial oocytes could be used clinically.

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28

HOW MICROFLUIDICS AND MICROFABRICATION WILL IMPROVE DIAGNOSIS AND TREATMENT IN HUMAN ART

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Introduction

Microfluidics is the science of manipulating small volumes of fluid typically in the range of microlitres to picolitres [1, 2]. Microfluidic components and platforms are being applied to many biomedical applications, by leveraging the precise levels of environmental control, cellular manipulation, and automation that these systems provide [3–6]. The term "lab-on-a-chip" is often applied to microfluidic platforms, which is due to the truly multidisciplinary nature of microfluidics, requiring equal contributions from fields such as biology, chemistry, physics, and computer software development, which are made possible thanks to the appropriation of traditional skills in microfabrication and the advent of new microfabrication methods from modern manufacturing [7–10]. Microfluidics arose initially with the development of inkjet printers in 1951, which harnessed the Hagen–Poiseuille equation, 1840, which was used to describe the characteristics of laminar flow of fluid through a tube [11]. Some decades later, in 1979, the merging of microfabrication with analytical methods resulted in the creation of the field of microfluidics [12]. Fabrication of devices using soft lithography for malleable and lightweight structures was adopted shortly thereafter in the early 1990s. Since then, microfluidic platforms have been used in numerous research and industrial applications within biotechnology, such as for point-of-care diagnostics (lateral flow immunoassays), tissue engineering and modelling (organ-on-a-chip), nanoparticle synthesis, drug discovery, and on-chip cellular analysis [13–16]. Compared to conventional approaches, these microfluidic platforms enable a higher quantity and quality of data to be gathered at a speed and price that is unmatched. Microfluidic devices also afford researchers excellent visualization of live cellular and physical reactions which are particularly valuable for the study of cells.

The modern assisted reproduction technology (ART) clinic consists of a myriad of highly regulated micro-environments with optimized layers of quality control and assurance to minimize external and internal stressors imposed on gametes and embryos throughout fertilization, embryo culture, and cryopreservation. These processes are all subject to the skill of the embryologist and successful operation of equipment. Microfluidics and microfabrication, in recent years, have attracted considerable attention for the many applications in which microlitre or nanolitre volumes of fluid could apply to the modern IVF laboratory or clinic. In the context of fertility and assisted reproduction, microfluidics has made several contributions, most notably in the areas of infertility diagnostics, gamete processing and analysis, embryo culture, on-chip fertilization, cryopreservation, and reproductive organ modelling (Figure 28.1) [1, 17, 18]. Microfluidics allows researchers to selectively mimic the geometry and environmental conditions present within reproductive systems, presenting opportunities for biomimetic emulations of *in vivo* processes such as temperature, chemical, and physical environment. This has proven extremely useful for applications such as the assessment of oocyte and sperm quality and dynamics [17, 19, 20], and sperm selection for IVF, from both neat and frozen semen as well as testicular tissue from surgical sperm retrieval operations [17, 21, 22]. These platforms may employ "active" or "passive" approaches which apply either external forces (such as heat, current, or flow) or physical phenomena derived from a controlled geometry at the microscale. Microfluidics also offer precise control over heat transfer, which is an essential requirement for cryopreservation technologies [23] and has proven useful in sperm selection approaches [24]. Furthermore, paper-based microfluidic technologies offering simple, low-cost, and rapid diagnostic platforms are already available as pregnancy tests and are commonly employed outside ART [25].

More recently, microfluidics has also been applied in the modelling of complex tissue and organ micro-environments for reproductive science. These models can recapitulate the organ environments and endocrine signalling present naturally and may be able to provide reliable models for studying reproductive and whole-body health, *in vitro* drug screening, toxicity testing, and tissue transplantation [26–28]. Where 2D models lack the physiological relevance and three-dimensional (3D) architecture within tissue-tissue and multi-organ interactions microfabrication and microfluidics have been extensively utilized to develop better cell culture platforms than the existing conventional *in vitro* models [29]. However, despite impressive advances in the application of microfluidics for ART, translation of these technologies into clinical practice has been limited. Since the landmark first-generation work in lab-on-a-chip systems by Terry et al. in 1979 [12] and later by Manz et al. in 1990 [30], most systems are not yet realized as commercial products for research-grade instrumentation outside of specialist laboratories [31]. There are, however, notable exceptions, which this chapter will cover, illustrating a potential paradigm shift in laboratory processes and research for ART.

Here we consider how microfluidics, and more recently microfabrication, can be used for the analysis and diagnosis of sperm, and to enhance ART procedures including ICSI, *in vitro* maturation, embryo culture, and cryopreservation.

Microfluidics for semen analysis and diagnostics

The future of personalized infertility treatments will be bolstered by reliable and accurate diagnostics prior to clinical ART. In male infertility, semen analysis is the cornerstone of clinical diagnostics due to its relative simplicity and non-invasive nature; however, men often avoid voluntary proactive clinical assessment due to social stigma, leaving women to bear the burden of infertility in the initial stages [32]. Furthermore, notable biological variation

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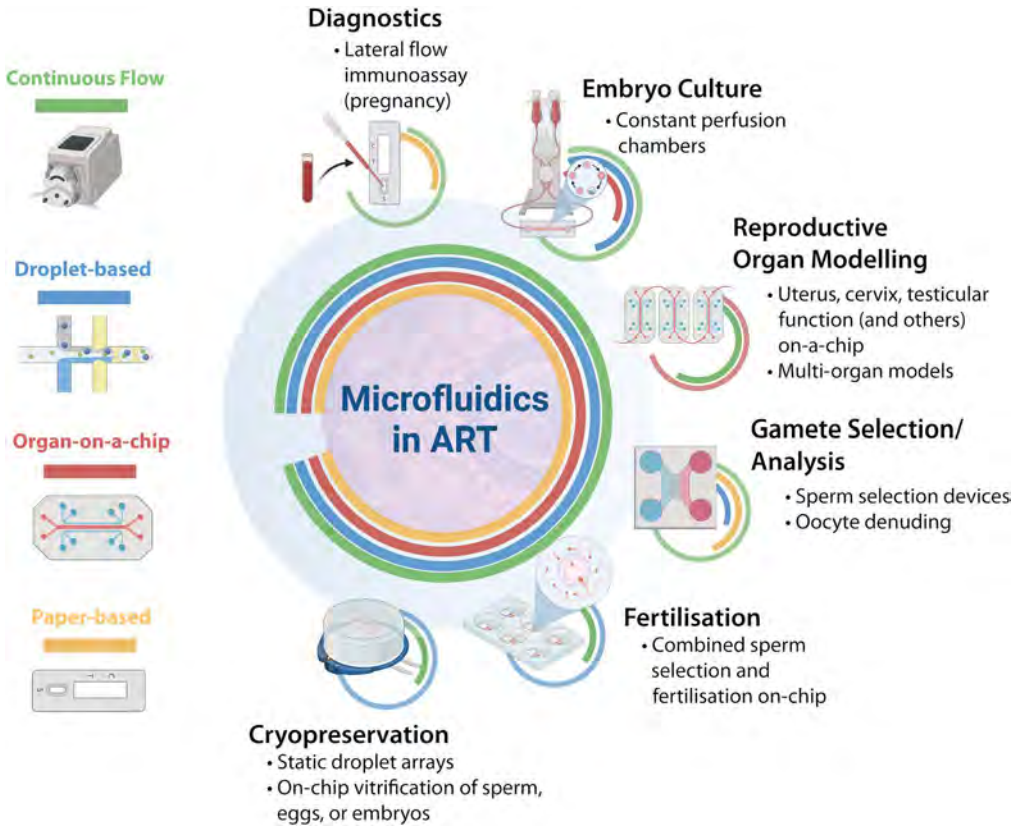

FIGURE 28.1 Summary of the applications to date of microfluidics in assisted reproductive technologies.

occurs between semen samples regarding basic semen parameters, thus semen analysis requires a robust, repeatable approach to standardize analysis by minimizing operator variability, while providing precise and accessible metrics indicative of patient fertility status [33]. Conventional clinical approaches to semen analysis have marginally improved with advancing technology, utilizing microfabricated counting chambers [34]; computer-aided sperm analysis (CASA) [35]; and assays for sperm viability [36], morphology, and DNA integrity [37]. These methods are nevertheless limited by factors such as poor standardization, high complexity, high cost, user variability, and extended processing times.

Concerted efforts to provide accessibility and de-stigmatization of male infertility diagnostics has seen several commercially viable systems developed for semen analysis in recent years. Traditional microfluidic devices, with miniaturized geometry and physics, consist of fabricated microchannels using materials such as polydimethylsiloxane (PDMS) and glass, enabling modular systems comprised of a series of chambers and valves, capable of incorporating multiple basic semen analysis techniques into a single device [38]. This can facilitate these devices to provide standardized metrics attempted by many computer-aided semen analysis CASA platforms. These proposed devices have the potential to reduce hands-on time and operator variability. Current CASA systems can assess sperm concentration, motility, kinematics, morphology, and vitality, which could theoretically be integrated with pH, viscosity, DNA fragmentation, and biomarker analysis using microfluidics. Alternatively, paper-based microfluidic approaches, which function by passively wicking fluids via capillary action through paper that has selectively patterned hydrophobic boundaries [18], are specifically accessible and affordable formats for performing diagnostics.

Basic semen analysis

There have been several recent microfluidic methods of assessing sperm concentration, motility, and vitality which vary in both practicality and user-friendliness. Traditional microfluidic platforms based on sperm migration have shown promise for raw semen motility and concentration analysis (Figure 28.2a) [39] using parallel microchannels to separate motile sperm from immotile sperm and debris, and by measuring pellet size from each channel, concentration and motility is comparable to counting chambers. Fluorescently labelled sperm in microchannels have also been used with comparable results to CASA for total and progressive motility assessment, indicating sub-fertility based on WHO parameters [36, 40].

A commercial, at-home, paper-based rapid test such as the SpermCheck® Fertility device (SpermCheck, Fairfield, OH, US) provides a basic result indicating whether sperm concentration is normal, low, or very low using immunodiagnostic colorimetric signals (Figure 28.2b) [41]. Fertell (Genosis Ltd, Boston, US), another immunodiagnostic test used a swim-up chamber connected to a nitrocellulose strip trapping sperm labelled with anti-CD59 colloidal gold conjugate progressively motile sperm, indicating if progressive motility is over 10 million sperm per mL (Figure 28.2c) [42]. The Men's Rapid Fertility Test (LabCorp OnDemand, Burlington, VT, US) is another at-home test kit, using a compact, low-speed centrifuge and disposable microfluidic

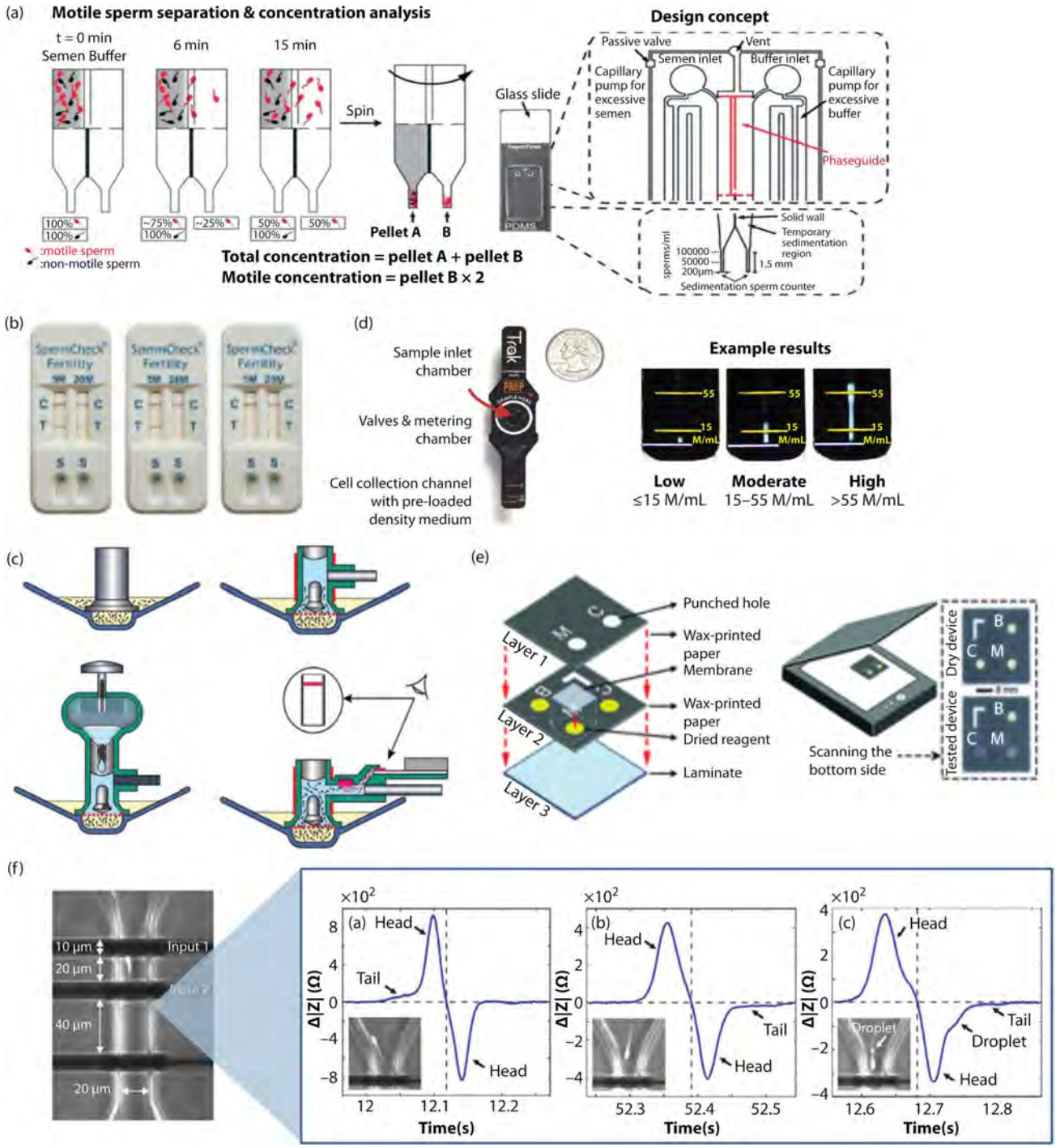

FIGURE 28.2 Microfluidic semen analysis examples showing a device (a) using parallel microchannels for motile sperm separation and assessing concentration based on pellet size; (b) the SpermCheck® Fertility device for sperm concentration assessment; (c) the Fertell immunodiagnostic test for sperm motility assessment; (d) the Men's Rapid Fertility Test for sperm concentration assessment; (e) an experimental paper-microfluidic device for sperm vitality assessment; (f) sperm morphology assessment using impedance. ([a] From [39] with permission; [b] from [41] with permission; [c] from [42] with permission; [d] from [43] with permission; [e] from [13] with permission; [f] from [45] with permission.)

chips, which funnel sperm into a microchannel column whereby column height indicates sperm concentration categorized into three broad ranges (Figure 28.2d) [43].

At-home tests for sperm motility, integrated with cloud-based analyses and mobile health strategies provide the ability for users to take control of their treatment and receive medical advice without stepping foot into a clinic. CASA-based, at-home systems using microchamber slides such as the Yo Male Fertility Sperm Test (Mira, Pleasanton, CA, US) and the smartphone-based Sperm Test Kit (ExSeed Health, London, UK) aim to provide users with

digestible and simplified information by comparing sperm motility and concentration to reference values, and providing personalized advice from a cloud-based platform. CASA-based systems also have great potential for automating and standardizing basic semen analysis in the future, yet global adoption is currently hampered by cost, complexity, and standardization issues with calibration as a key factor which needs to be addressed.

Experimental microfluidic platforms for the analysis of sperm motility and concentration have been proposed using an easy-to-use paper-based approach to quantifying live and motile concentrations of sperm as well as sperm motility using colorimetric reaction of tetrazolium to purple formazan by an enzyme found in metabolically active sperm called diaphorase flavoprotein enzyme (Figure 28.2e) [13]. Paper-based microfluidics have also been applied to analysing DNA integrity of sperm using ion concentration polymerization (ICP) effects on nanoporous membranes on paper with a strong correlation to clinically performed flow-cytometry-based sperm chromatin structure assay (SCSA) [44]. Electrically integrated microfluidic chips have been developed to determine sperm concentration and morphologically normal sperm using sperm impedance cytometry and separating sperm from collateral cells and other sperm with large cytoplasmic droplets using dielectrophoretic sorting and counting (Figure 28.2f) [45]. This concept was explored using a planar platinum electrode pair between a microchannel with flowing sperm, which identified and counted sperm in a heterogenous sample using electrical impedance via a homemade impedance analyser [46]. Furthermore, another group showed an accurate differentiation of sperm with normal morphology versus those with cytoplasmic droplets using this technology [45].

Liquid biopsies

Microfluidics has also garnered attention recently in the field of liquid biopsies, providing non-invasive testing of disease states with high accuracy and actionable results. This complete control over fluid volumes and flow rates allows for high-resolution cell and particle separation within these platforms as well as enabling other parameters such as temperature, geometry, and mechanical stimuli [38]. Paper microfluidics, for example, has become widely used in point-of-care diagnostics as lateral flow immunoassays to rapidly test for Covid-19. The most common targets of this research and technology have been circulating tumour DNA (ctDNA), circulating tumour cells (CTCs), and small extracellular vesicles (S-EVs or exosomes) [47, 48]. In the context of infertility, S-EVs have been proposed as novel targets for diagnostics and point-of-need application, with extensive biomarker candidates within seminal plasma S-EVs being identified and proposed [49–53]. These observations have been complemented by an ever-growing body of evidence correlating S-EVs with many important physiological functions, pathophysiological states, and ubiquitous intercellular communication owing to their valuable cargo of proteins, lipids, metabolites, functional messenger RNA (mRNA) and microRNA (miRNA), and double-stranded DNA (dsDNA) [48, 54–58]. Seminal plasma is composed of secretions from the testes, epididymis, seminal vesicles, prostate, and bulbourethral glands, which provides a highly diverse cohort of S-EVs, free-floating proteins, nucleic acids, minerals, and other vesicles [59]. Isolating S-EVs however, due to their size, presents unique challenges and requires complex and laborious methods to isolate in pure populations.

The current gold standard for isolating S-EVs is differential ultracentrifugation, which requires high starting volumes (millilitres), large complex equipment, and dedicated operators, and leads to significant loss of S-EVs during processing [48, 60]. Microfluidics, however, has been shown to effectively isolate S-EVs from microlitre starting volumes with high specificity and purity. Microfluidic approaches appropriate conventional methods such as immunoaffinity capture (using antibodies conjugated on nanobeads or surfaces to bind specifically to target exosomes) and filtration, but also employ methods unique to microfluidic geometry and physics such as acoustic isolation (using acoustic field radiation waves to focus and isolate particles with different size, density and compressibility) [61, 62] and nanowire trapping (trapping of S-EVs on surface-bound nanowires acting as a filter for S-EVs while allowing larger particles to pass through) [63]. Microfluidic devices can also incorporate multiple methods of S-EV isolation to improve both capture or detection efficiency as well as purity of target S-EVs, increasing the accuracy of measurements and conclusions made thereafter. Most microfluidic S-EV isolation platforms are purpose-built for cancer diagnostics with a blood-based or urine-based liquid biopsy approach and are designed for point-of-care applications. Applying microfluidic S-EV isolation and detection to infertility diagnostics and liquid biopsies would be better served using a point-of-need approach to direct and personalize treatments in ART, such as what type of insemination (IVF or ICSI) would lead to the greatest of successful live births based on the expression of biomarkers detected [53].

Microfluidic technology can enable new methods of analysing sperm quality as well as simplifying this process to provide accessible options for patients and clinicians. The unique advantage microfluidics offers is the ability to integrate multiple procedural steps within a single automated platform, highly relevant to semen analysis and liquid biopsies of an easily obtained fluid such as seminal plasma. Furthermore, with innovative development and validation, this technology can enable portability and cost-effectiveness in diagnostics and remove the need for large and expensive technology. This technology has applications in other biofluids obtained during ART such as ovarian follicular fluid and blood, and could open a new avenue of actionable diagnostics prior to, during, and after clinical infertility treatment.

Microfluidics for sperm selection

Sperm selection forms a core facet of ART that can considerably influence embryo development, miscarriage rates, and live birth rates [64–67]. In particular, the use of sperm with DNA fragmentation in IUI, IVF, or ICSI can negatively impact ART outcomes. However, the most commonly employed method of sperm selection, density gradient centrifugation (DGC) and swim-up (SU), remain largely unchanged and concerns around their safety and efficacy for ART have been reported with increasing frequency [68, 69]. Both DGC and SU have the potential to induce sperm DNA fragmentation through the production of reactive oxygen species (ROS) and iatrogenic damage as a result of centrifugation [70, 71]. In the female reproductive tract, a stringent series of selection mechanisms will filter all but a minute percentage of the starting population of ejaculated sperm [72, 73]. These natural mechanisms that have evolved in nature are able to discern the quality of sperm, yet are still poorly understood. The presumption that sperm are able to traverse the female reproductive tract to the oviduct are fecund is plausible but not certain. However, understanding the mechanisms that select sperm *in vivo* will clarify the properties of these sperm and inform the next generation

of sperm selection technologies, and may improve treatment outcomes when compared to the conventional methods widely used today [73, 74]. Additionally, the requirements for sperm selection can vary depending on the ART being performed, and this should be considered during the development of alternative sperm selection technologies.

Microfluidic sperm selection technology has progressed since the first peer-reviewed publication in 2003 [18, 72], although very few attempts have resulted in commercial products. Attempts at microfluidic sperm selection can be categorized into active and passive methods. Passive microfluidic devices rely upon their inherent geometry to manipulate fluids and the cells and compounds they may contain. Passive microfluidic devices typically select sperm by leveraging their motility and behaviour in confined microchannel environments [17, 21, 75–78]. These systems often make use of hydrostatic pressure (gravitational pressure from a column of fluid) and capillary forces (fluid movement in narrow micro or nanochannels) to forego the use of pumping systems to push fluids. This simplifies devices and makes them more accessible to researchers unfamiliar with microfluidics. Cho and Schuster [76] used such a system to select motile sperm capable of crossing laminar flow streams into fresh media, leaving behind dead and non-motile cells [76, 79] (Figure 28.3a). This device was reportedly able to select sperm with 97% motility and improved morphology (9.5 ± 1.1% normal forms prior to sorting to 22.4 ± 3.3% normal forms after sorting). Later the same group went on to test clinically infertile samples and the device's ability to select sperm with improved DNA integrity, achieving a DNA fragmentation index (DFI) of less than 1% [80]. The treatable population for this device (like many existing ARTs) is limited to those with motile sperm cells and may not be applicable to those with low thresholds of motility. Asghar, Velasco [75] proposed a microfluidic chip consisting of a microchannel ending in a polycarbonate membrane (8 μm) (Figure 28.3b). This device processes neat semen samples, and achieved a separation of 85% and normal morphology averaging 30% [75]. While this device did display lower ROS than conventional SU methods, the use of a polycarbonate membrane may pose issues in clinical applications considering the potential for cell aggregates and tissue debris causing membrane blockage. The devices exhibited saturation at 30 minutes of operation, about half the time of a conventional DGC, but was limited in its throughput considering the use of a single channel. A similar design is shown in Figure 28.3c [81], which used a space-constrained model for sperm racing. Interestingly, the testing of both mouse and human sperm cells revealed a motility exhaustion of 30 and 60 minutes, respectively. Again, this study was benchmarked against SU methods but not DGC. However, over 30 minutes of sorting showed a 1.9-fold and 1.3-fold increase in velocity and motility, respectively, from raw samples [78]. Both devices have been commercialized, with the device by Asghar et al. receiving greater success due to its simplicity and robustness, and leading to the development of the Zymōt Fertility sperm selection device (Zymōt Fertility, Gaithersburg, MD, US) (Figure 28.3d).

While many microfluidic attempts at sperm selection suffer from low throughput, Nosrati and colleagues [21] developed a system of more than 500 radial microfluidic channels to process a large number of cells quickly. This device performed a one-step sperm purification and selection by processing 1 mL of raw semen, guiding sperm through boundary-following behaviour into a viscous media reservoir in 20 minutes (Figure 28.3e). This device also used a viscoelastic media, similar to the viscosity of the mucous secretions of the oviducts [82]. This process achieved an 89% increase in sperm vitality and 80% improvement in DFI in clinically infertile samples. The group then went a step further and discovered that sperm able to follow the boundary of varying degrees of corners (without losing the wall) presented higher DNA integrity than normal straight-swimming cells (17). Although the exact biological mechanism for this behaviour is not understood, turning sperm exhibited more than 50% better DNA integrity than straight-swimming sperm. To go further still, Yazadan Parast and colleagues appropriated the same concept to provide a 3D network of more than 560 micro-channels within a familiar syringe format [83]. This "sperm syringe" was able to recover 41% of sperm from diluted semen samples in under 15 minutes, and provided a considerable improvement in DNA integrity and morphology (Figure 28.3f). These studies demonstrate how controlled microfluidic geometries coupled with a detailed understanding of sperm kinematic behaviour may benefit ART. There is potential for this process to be improved using computer vision and improved media such as with the inclusion of AI or the use of molecular makers for sperm function [80, 83].

A wider goal of microfluidic devices is to parallelize or integrate several ART functions into one platform, thus conducting sperm selection, oocyte trapping, fertilization, and embryo culture on a single device. One example of this approach is a microfluidic device utilizing chemotaxis to guide mouse sperm towards oocytes through four perpendicular channels [84]. Sperm motility increased from 60% to 96% (at 15 minutes) when first measured at the inlet then near to the central wall, although motility did decline over time. However, the use of one interconnected fluid network with no fluid boundaries meant that several media exchange steps were necessary. This limits the clinical viability of this chip as it does little to alleviate the manual handling of media exchange, although it does remove the need for centrifugation to isolate sperm prior to use in insemination. Several other studies have developed microfluidic technologies for the investigation of chemotaxis and sperm but without the necessary ports for sperm selection, and are therefore purely investigative tools [24, 85].

Active microfluidics involves the use of external forces such as thermal, acoustic, or electromagnetic forces to influence fluids and reactions. Active microfluidic devices for ART have typically employed electrophoresis, thermotaxis, or light-induced dielectrophoresis [45, 86–88]. Thermotaxis is an established method of long-range sperm guidance [89]. The fallopian tube itself exhibits a thermal gradient of approximately 1.4°C, warming as the sperm travels in the direction of the oocyte. Several attempts have been made to introduce temperature as a means of sperm guidance; one such attempt by Li et al. investigated the thermotactic responses of motile sperm diverting into reservoirs adjacent to a primary channel after a constriction of the channel, mimicking the uterotubal junction (where *in vivo* thermotaxis begins) [87]. The channel shown in Figure 28.3g, uses an interfacial valve-closing mechanism to trap sperm once they have moved into warmer areas. Thermotactic responses were observed in 5.7%–10.6% of the motile sperm over four temperature ranges. While this is an indication that sperms have undergone (induced) capacitation and are thermally responsive, it is not a direct indicator of sperm fecundity or DNA integrity. Therefore, coupled with other techniques, such as passive wall guidance or chemotaxis, sperm fecundity may become more apparent. It is still not fully understood whether thermally responsive sperm alone are able to improve reproductive outcomes. While few studies have investigated the combinational effects of sperm selection mechanisms

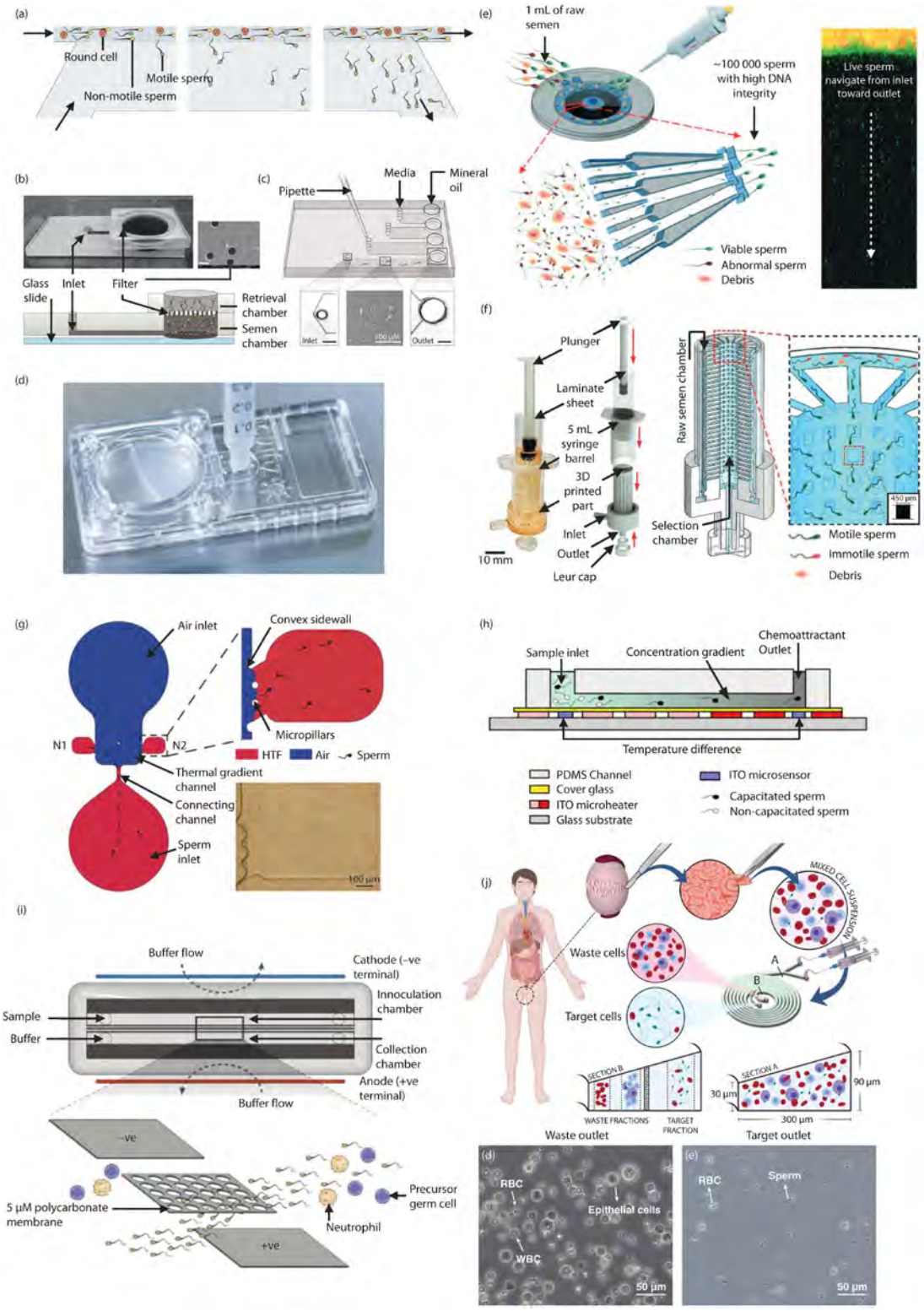

FIGURE 28.3 Microfluidic sperm selection platforms for use in treatment. (a) Simple channel for motile sperm selection against laminar flow streams; (b) polycarbonate membrane integrated with a microchannel to select motile sperm; (c) space-constrained model for sperm racing; (d) Zymōt sperm selection device using a membrane barrier to select motile sperm; (e) radial channel with viscous media to select sperm with boundary-following ability; (f) sperm syringe for motile sperm collected; (g) thermotactic intervalve sperm selection platform; (h) chemotaxis and thermotaxis platform to select motile sperm; (i) electric field selection of sperm across a polycarbonate membrane; (j) a microchannel spiral using inertial microfluidics to isolate sperm from surgical testicular tissue collections . ([a] From [18] with permission; [b] from [75] with permission; [c] from [81] with permission; [d] courtesy of ZymōtR Fertility; [e] from [21] with permission; [f] from [83] with permission; [g] from [87] with permission; [h] from [24] with permission; [i] from [18] with permission.)

such as motility, chemotaxis, and thermotaxis, there are emerging trends in more recent studies that take this into consideration. Ko et al. designed a microfluidic device for the chemotaxis and thermotaxis assays of mouse sperm [24]. Their study demonstrates that the combination of these two selection mechanisms elicited a greater response in mouse sperm than when each method was used individually (Figure 28.3h). An alternative to chemotaxis that has made the transition into industry (with mixed success), is the use of electrophoresis, which has been used to select healthy sperm based on a combination of their size and charge [86]. This method uses an applied electric field to attract sperm across a polycarbonate membrane. The transition from one side of the membrane to another facilitates a media exchange from semen to fresh sperm media (Figure 28.3i). The use of this membrane excluded debris, leukocytes, and immature germ cells. This study reports that electrophoretic separation resulted in significantly improved vitality, motility, and DNA integrity compared with the initial sample, with more negatively charged sperm possessing high DNA integrity, although the exact reason why is not fully understood [90]. Using a fundamentally different approach to sort sperm may prove effective, but to date, active methods such as electrophoresis have yet to be validated clinically [91, 92].

Another niche within clinical sperm selection that often goes overlooked is the isolation and selection of surgically retrieved sperm. Microdissection and aspiration of testicular tissues typically entails several hours of manual sperm recovery [93]. Sperm recovery rates are low, and processing is prone to human error. Microfluidics (such as spirals) can preserve the vitality and DNA of the sperm by limiting their time *in vitro* and their exposure to ROS, digestive enzymes, cellular debris, and other contaminants [94]. Previously discussed forms of microfluidic sperm selection which leverage sperm motility are ineffective for non-motile surgically recovered sperm. As a result, several studies have appropriated various forms of inertial microfluidics, which makes use of fluid pumps, to leverage the unique morphology and size of sperm to filter out debris and concentrate sperm within a dramatically improved timeframe [22, 95, 96]. Across multiple studies, Son and colleagues demonstrated the novel application of inertial microfluidics to separate non-motile sperm from microbeads, red blood cells (RBCs), and white blood cells (WBCs) [97–99]. While promising, these studies worked with idealized cell suspensions largely unrepresentative of the triturated tissue and performed sperm separation from each cell type in isolation rather than a complex mixture. Recently, Vasilescu et al. used a similar spiral microchannel device fabricated by 3D printing that recovers sperm from heterogeneous cell suspensions of sperm, WBCs, RBCs, muscle epithelial cells, microparticles, and leukemic cancer cells (Figure 28.3j) [22]. Sperm were isolated within five minutes and, very importantly, were shown to have no detrimental impact on sperm viability, morphology, or DNA integrity.

Thus, microfluidic sperm selection has the potential to select higher-quality sperm with less error and greater standardization than conventional methods. While studies are limited, there is some preliminary evidence that suggests microfluidic sperm sorting does improve reproductive outcomes, including ongoing pregnancy rates in IUI, higher quality embryo generation, and improved chances of euploid conceptus [100–102]. Continued clinical application of microfluidic sperm selection will determine if this technology results in repeatable improvements in ART outcomes and for which aetiologies these new devices provide the most benefit.

Modelling reproductive organs

Developing and using translatable models of human reproduction has served as a consistent barrier to implementing safe molecular and pharmaceutical interventions for treating infertility. Microfabrication and microfluidics enable development of complex, 3D culture platforms with many benefits over conventional 2D culture [29]. These micro-engineered physiological models, appropriately termed organ-on-a-chip systems (OOCs), are more effective at mimicking the *in vivo* 3D multicellular architecture and micro-environment of the specific organ or tissue [103, 104]. OOCs can simulate blood flow with pump-controlled physiological fluid flow, enabling nutrient and gas perfusion along with mechanical stress cues [16]. OOCs technology has been applied to modelling both the female [28] and male reproductive systems with promising results.

Testes-on-a-chip (seminiferous tubules)

Multiple testes-on-a-chip models have been developed with varying goals in humans [105, 106], primates [106], and mice [107, 108]. A multi-organ human model was developed to study the interaction between a testicular organoid system and liver equivalent to observe natural and drug-induced tissue interactions (Figure 28.4a) [105]. This group observed testosterone and inhibin B production by the testicular organoids as well as observable steroid metabolism by the liver spheroids and germ cell loss when adding a chemotherapeutic drug (cyclophosphamide). A simple perfusion device enabled culturing, and studying of prepubertal primate seminiferous tubules was developed; tissue integrity, cell morphology, and viability was assessed under both hormonal stimulation and non-stimulation conditions [106]. Dulbecco's modified Eagles medium (DMEM) (Thermo Fisher, Waltham, MA, US) supplemented with 10% fetal bovine serum (FBS) was perfused into the device with FSH (0.5 IU mL^{-1}), hCG (0.5 IU mL^{-1}), and marmoset serum (0.4%) for non-stimulation conditions, and FSH (5 IU mL^{-1}), hCG (5 IU mL^{-1}), and marmoset serum (4%) for stimulation conditions. In mice, a bioreactor model for culturing testicular tissue in a purpose-built OOCs simulated in vivo-like conditions by creating a device consisting of a porous membrane separating cultured tissue from slowly flowed medium at 0.05 µL/min (Figure 28.4b) [107]. This device could maintain spermatogenesis and testosterone production in response to luteinizing hormone for six months, and produced functional sperm to generate healthy offspring using round spermatid injection (ROSI) and ICSI [107]. This same device was made pumpless using hydrostatic pressure for continuous infusion of α-minimum essential medium (α-MEM) supplemented with 40 mg mL^{-1} bovine serum albumin (BSA) media [109] and altered further for improved visualization and monitoring of testes tissue during culture (Figure 28.4c) [108].

Such devices have considerable potential in both research and clinical applications, particularly as bioreactors to create usable sperm for use in ART. Creating robust and personalized platforms for culturing stem cells into usable sperm can be used to treat non-obstructive azoospermic patients and enable autologous gamete treatment options.

Oviduct-on-a-chip and uterus-on-a-chip

Monolayer culture of oviduct epithelial cells is notoriously difficult due to the rapid transformation of cuboidal columnar oviduct epithelial cells into flattened cells along with loss of beating cilia and reduced secretory function of these cells [110–112].

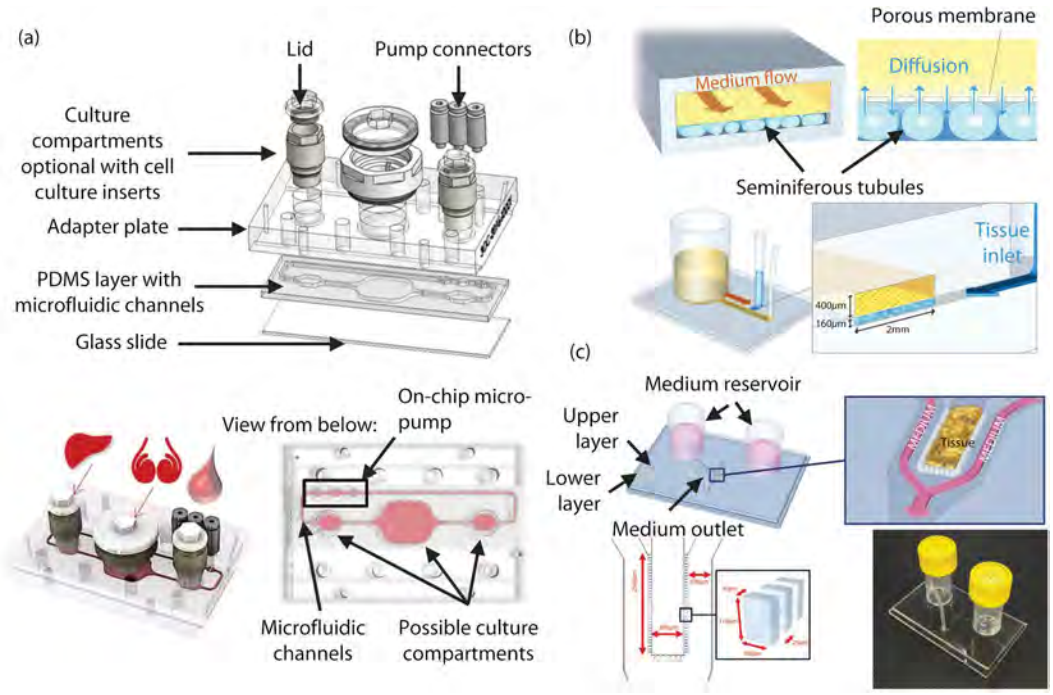

FIGURE 28.4 Male reproduction models showing (a) testicular tissue and liver equivalent model; (b) testicular tissue bioreactor to grow sperm from seminiferous tubules; (c) a pumpless diffusive perfusion version of this seminiferous tubule platform. ([a] From [105] with permission; [b] from [107] with permission; [c] from [108] with permission.)

Thus a bovine oviduct-on-a-chip was developed from a primary culture of bovine oviduct epithelium and was successful in maintaining beating cilia and secretory function of these cells (Figure 28.5a) [110]. This device was then used to facilitate sperm capacitation and IVF of bovine oocytes with reduced polyspermy when compared to conventional bovine IVF. Subsequently, this system was proposed as a mediator of improved fertilization in bovine models.

Recreating the uterine lining within a microfluidic OOC has been attempted using surgically excised human perivascular stroma and endothelial cells (Figure 28.5b) [113]. This system simulated temporal hormone changes during an idealized 28-day menstrual cycle and an enabled differentiation of stroma into functional decidual cells based on both morphology and prolactin production. Another "uterus-on-a-chip" model was developed using mice tissue which replicated some *in vivo* uterine functions to a greater extent, including implantation and embryo development within a physiologically favourable micro-environment [114]. Their device consisted of a co-culture of embryos and endometrial cells on either side of the porous membrane, allowing the diffusion of soluble factors as well as interaction of the embryo with the underlying cells through the membrane. When comparing embryo development in conventional petri dish culture versus their model, the uterus-on-a-chip model provided significantly higher morula and blastocyst rates [114]. This study provides an innovative approach to embryo culture and studying embryo development in an *in vivo*-like system. Mizuno et al. fabricated a uterus-on-a-chip platform mimicking the physicochemical features by co-culturing human endometrial cells with human zygotes [115]. Their microfluidic device improved blastocyst rates and overall embryo quality when compared to conventional microdrop culture. Chang et al. developed a perfused 3D-uterus-on-a-chip to facilitate the co-culture of embryos with endometrial stromal cells in a dynamic manner to provide mild mechanical simulation (Figure 28.5c) [116]. The device also improved blastocyst rate by providing uterus-like conditions for the timely development of embryos and is thus proposed as an *in vivo*-like option for *in vitro* embryo culture. Another device was developed integrating both human uterine endometrial cells and ovarian follicular cells to simulate bidirectional endocrine crosstalk between the uterus and ovaries [117]. This device, in concert with a reliable reproductive toxicity marker, SERPINB2, was used to predict reproductive toxicity of specific chemicals introduced to the system and proposed as a substitute for animal models in testing these responses.

The biomimetic nature of reproductive organ-on-a-chip models provides a unique opportunity for simulating *in vivo* conditions for optimal fertilization and pre-implantation embryo development. Simulating these conditions by creating autologous oviduct epithelium and endometrial co-culture can support these important events with molecular and micro-environment interactions which could improve outcomes of fertilization and culture when compared to conventional culture systems. The clinical translation of these concepts is not a reality currently, however these models can be used to improve lab approaches to closer resemble the *in vivo* micro-environment of the oviduct and receptive uterus.

Entire female reproductive system on a chip

A female reproductive system-on-a-chip was developed using a single modular system with multiple docks for selected tissue and cell types to be cultured [28]. This chip provides a model capable of functional simulation of the 28-day menstrual cycle

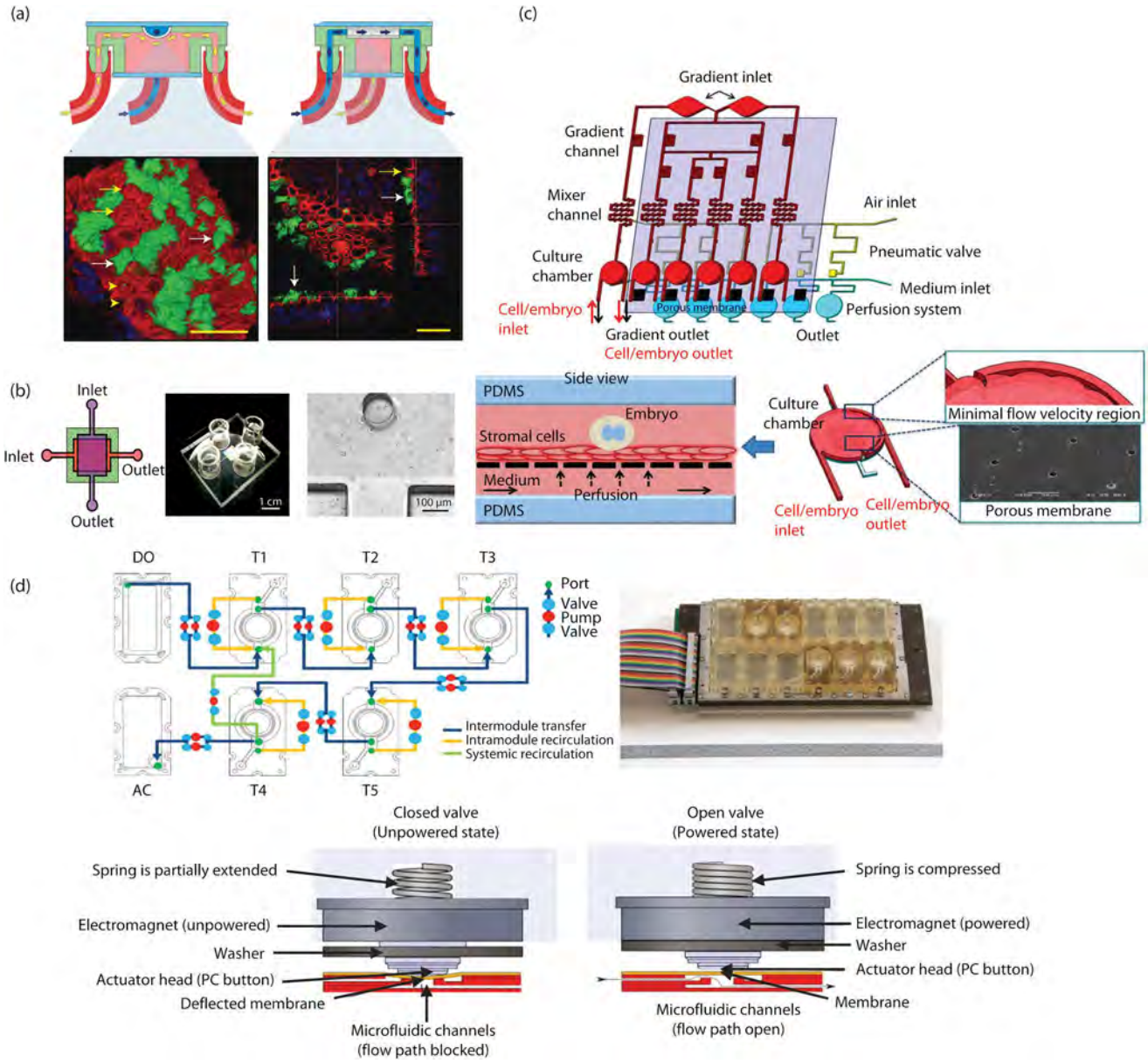

FIGURE 28.5 Female reproductive modelling: (a) bovine oviduct model; (b) human uterine lining model; (c) 3D-uterus-on-a-chip for embryo culture; (d) entire female reproductive system model. ([a] From [110] with permission; [b] from [113] under Creative Commons licence; [c] from [116] with permission; [d] from [28] under Creative Commons licence.)

including the culture, maturation, and differentiation of murine ovarian follicles (Figure 28.5d) [28]. Follicles were first perfused with growth medium containing 50% αMEM Glutamax and 50% F-12 Glutamax supplemented with 3 mg mL^{-1} bovine serum albumin, 0.5 mg mL^{-1} bovine fetuin, 5 mg mL^{-1} insulin, 5 mg mL^{-1} transferrin, and 5 mg mL^{-1} selenium then on day 0, stimulated with maturation media 1.5 IU mL^{-1} hCG, 10 ng mL^{-1} epidermal growth factor, and 10 mIU mL^{-1} FSH. These follicles successfully produced mature oocytes suitable for fertilization. This device was later integrated with organ modules for oviducts, uterus, cervix, and liver under continuous flow of media. This work presents a tool capable of mimicking micro-physiological interactions between the female reproductive tract and peripheral organs, providing a unique method of studying pharmacodynamics and biological processes.

Although this field is in its infancy, further development in microfabrication technology and cell culture scaffolds can broaden the applications of OOCs in medicine and biology. OOCs are cost-effective, reproducible, scalable, and provide high throughput and precise information, which can reduce the dependency on conventional *in vivo* models [118]. With further advancements and incorporation of multiple organ-specific models with each other, OOCs represent novel platforms for screening drugs and toxins which may affect reproductive health, and help to identify new therapeutics by addressing a wide range of biological problems [119, 120]. Furthermore, incorporating

human-induced pluripotent stem cells (hiPSCs) could enable the development of patient-specific organ models, leading to individualized body-on-a-chip models to foster personalized medicine approaches to infertility.

Microfluidics for embryo culture

Development of the human pre-implantation embryo *in vivo* is highly dynamic from several perspectives. The embryo itself undergoes changes in its morphology and structure as it differentiates. It undergoes changes in its gene expression profile, all while undergoing dramatic changes in its metabolic functions. In parallel, the female tract provides different environments (nutritional, gaseous, pH, and signalling molecules) as the embryo progresses through the oviduct to the uterus, with the uterine environment providing an increasingly more complex milieu to support the development of the embryo post-compaction [121]. Furthermore, the embryo is in constant movement due to both ciliary and muscular activity of the female tract. All of this is in stark contrast to the way in which we have attempted to culture the pre-implantation embryo in the laboratory, i.e. in a drop of medium on a polystyrene culture dish/microwell (although sequential media were developed to accommodate physiological nutrient gradients). Gardner proposed in 1994 that in order to optimize embryo development and viability in the laboratory a dynamic/perfusion methodology could be applied [122] (see chapter by Gardner). In order to achieve these aims, several groups embarked on developing perfusion systems, initially using conventional peristaltic pumps with relatively high flow rates of 30 to 38 μL per minute [123] and subsequently using microfluidics which provided for greatly reduced flow rates [124, 125]. Fluid flow through such early devices tended to be passive, established through the movement of fluid from large to small reservoirs, or active through peristaltic pumps (though volume control was limited in the latter approach). In order to facilitate greater control over fluid movement, Takayama and Smith established a computer-controlled, integrated fluid-control system utilizing up to hundreds of on-chip pumps and valves, driven by individually actuated Braille pins, which effectively squeezed fluid through individual channels. Using such an approach to establish a dynamic flow environment around the embryo (facilitated by the movement of media, but without the exchange of media) it was observed that mouse blastocyst cell number and subsequent implantation rate were significantly increased [126]. However, it is potentially through novel microfabrication approaches that such perfusion systems will be able to be evaluated in a clinical setting.

Microfabrication

As previously described, microfabrication of microfluidic devices for ART has been typically moulded from polydimethylsiloxane (PDMS) [38]. This material has been preferred due to its transparency, biocompatibility, and gas permeability. Nevertheless, the drive for sub-micron fluidic structures that have precise geometries and scalability for manufacturing is constantly being pursued; alternatives to PDMS are being sought. A major limitation from a cell and embryo culture perspective is the absorption and evaporation of media within microchannels of PDMS microfluidic devices [127, 128]. Furthermore, PDMS-moulded features do not have the resolution to create interchanging parts. An alternative is micron 3D-printable glass, now provided commercially from sources such as Glassomer Gmbh (Germany). A technology also capable of sub-micron feature 3D printing is two-photon polymerization (2PP). 2PP printing is a high-resolution micro additive manufacturing technique using photosensitive polymers. Similar in principle to 2-photon fluorescence microscopy, two-photon absorption (2PA) creates a nonlinear energy distribution centred at the laser focal point of two long-wavelength laser sources [129]. At that point, 2PA excitation induces UV-sensitive monomer crosslinking of the polymer. Consequently, 2PP can fabricate precise structures with high-resolution features, smaller than the wavelength of the laser, thereby creating devices with feature sizes in the sub-micron range [130]. Further, 2PP supports the use of photopolymers that are biocompatible and non-cytotoxic for cell culture applications following appropriate post-printing treatment, which has been demonstrated with a comparable printing technique called digital light processing [131].

Two recent publications reveal how versatile 2PP fabrication is for creating devices for use within IVF laboratories. The first describes a device for intracytoplasmic sperm injection (ICSI) [132]. The design of this device removed the need for a holding pipette, thereby removing at least one micromanipulator and pressure controller from an ICSI workstation. The oocyte is held within a cavity of a two-piece device created by 2PP, with dimensions and geometries unachievable with other manufacturing techniques. The linear array of chambers provides traceability of oocytes before and after injection, reducing the risk of failure to inject or double injection under conventional ICSI systems. The second publication [133] reports that 2PP devices are entirely suitable as cryopreservation devices. Similar cryo-survival and subsequent development rates were obtained following vitrification of mouse oocytes and embryos. An advantage of 2PP for this application is the minimal exposure volumes of cryo-protectants that such structures can impart.

Application of 2PP in microfluidics has been hampered by limitations in the time taken for printing and the size of print achievable. This is being rapidly resolved with further development of commercial 3D 2PP printers from companies such as Nanoscribe Gmbh (Germany) and UpNano (Austria). For example, the NanoOne (UpNano) has a horizontal print capacity of 1 cm^2. This can support several microfluidic channels and has now been developed for microfluidic culture of oocytes and embryos [134]. This publication demonstrated how 2PP can print two disparate fluidic parts ("nest" and "cradle"; Figure 28.6) that interlock through printed features such as a nozzle barb connector. The device supports oocyte–cumulus expansion and embryo development (Figure 28.7) under dynamic flow. As such, a new era of microfluidic devices centred on the high resolution and manufacturing capacity of 2PP micro 3D printing in various polymers and glass is rapidly emerging (Figures 28.6 and 28.7).

Not only does this breakthrough in microfabrication offer exciting new possibilities for performing *in vitro* maturation and embryo culture but also could lead to the development of an automated means of performing vitrification, as initially proposed by [135], whereby the cryopreservation solutions are introduced in a gradient fashion made feasible through built-in valving in the microfluidic device itself.

Summary

Microfluidics has held great promise for assisted human conception for more than 20 years. However, recent developments in this field, including novel microfabrication approaches, indicate that

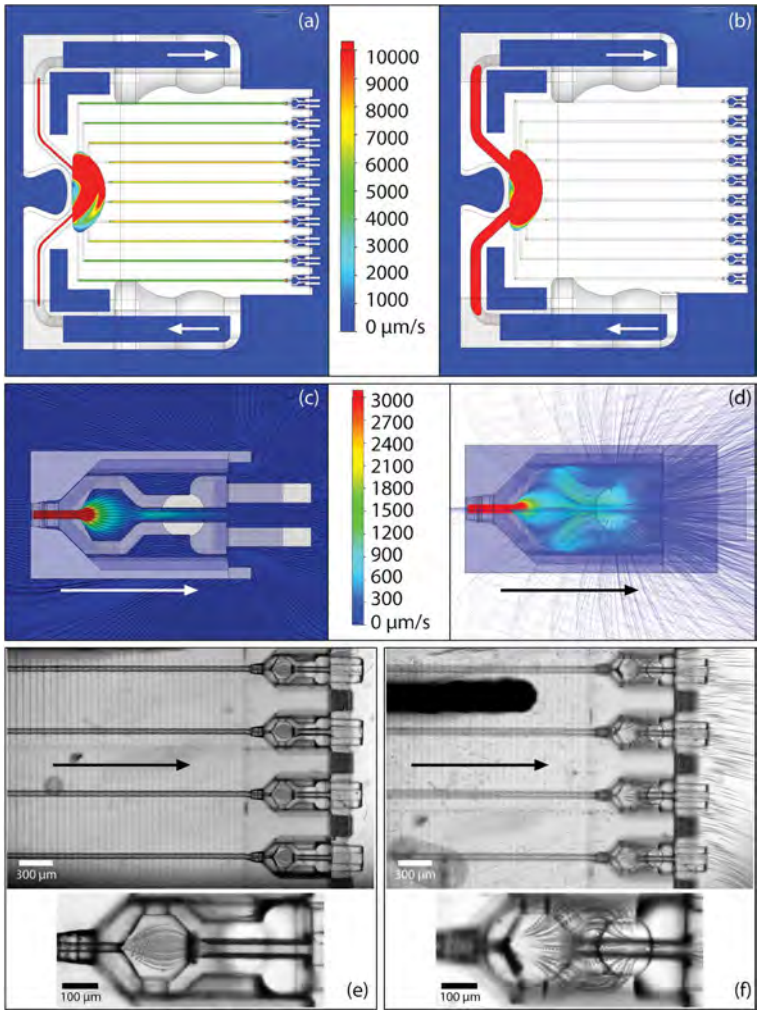

FIGURE 28.6 Predicted and actual flow patterns through the "nest"–"cradle" interlocking 2PP printed device: (a) computational fluid dynamic (CFD) modelling showing distribution of flow across the nest channels; (b) CFD modelling at nest surface showing flow through the inlet, back reservoir, and outlet located 250 µm above the centre of the channels; (c) CFD modelling showing the smoothing effect of the nozzle within an individual cradle; (d) CFD modelling of the flow trajectories within an individual cradle from below; (e) projection of bead tracks from above with the focal plane set at the centre of the channels; (f) projection of bead tracks from above with the focal plane set to nest top surface. (Scale bars (a)–(b) = 1 mm; (c)–(d) cradle length from the nozzle to opposite end = 1 mm; (e)–(f) = as labelled; arrows indicate the direction of fluid flow.)

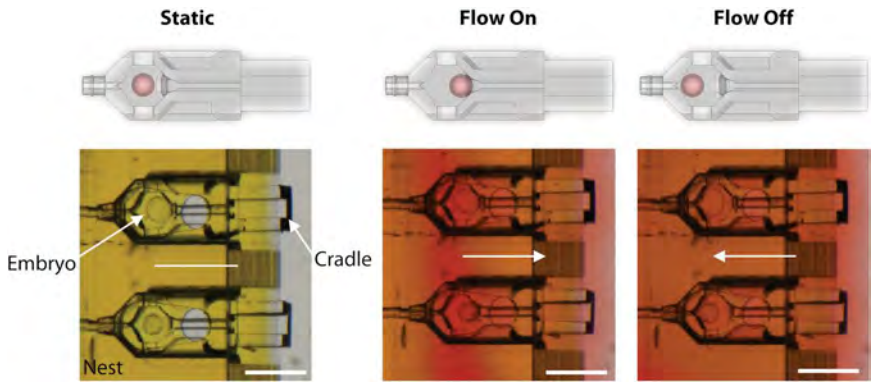

FIGURE 28.7 Mouse embryo movement in a changing dynamic flow environment with the introduction of red stained medium: (a) static conditions show the expanded blastocysts housed inside the cradles and nest after 48 hours of culture; (b) Flow On shows the embryos move to the right as the flow pushed them to the back of the cell chamber; (c) Flow Off shows the embryos move to the left back towards the nozzle. (Scale bars = 300 µm.)

we are finally on the verge of seeing these technologies being clinically validated for a wide range of tasks in infertility treatment, from sperm preparation and diagnosis, ICSI, embryo culture, and finally vitrification. Furthermore, all of these approaches lend themselves towards automation (in total or in part) of several key procedures in the IVF laboratory [136], thereby greatly reducing operator variability inherent in these technically demanding tasks. This in turn will lead to improved efficiencies and efficacies in the IVF laboratory and improve standardization in lab processes, culminating in reduced time to pregnancy and increased pregnancy rates. Ultimately, this will increase the accessibility of IVF to more patients worldwide [137].

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GENOME EDITING IN HUMAN REPRODUCTION

Helen C. O'Neill

Clustered regularly interspaced short palindromic repeats (CRISPR)-based genome editing is a revolutionary technology that allows for precise and efficient manipulation of DNA sequences. It has rapidly become one of the most powerful tools in molecular biology, with a wide range of applications in areas such as agriculture, medicine, and biotechnology and with expanding editing capabilities. One of the most promising areas of research for CRISPR is human health.

The CRISPR-Cas system is a prokaryotic adaptive immune system that utilizes small RNAs, called guide RNAs (gRNAs), to target and cleave specific DNA sequences. The gRNA guides a nuclease, usually Cas9, to a specific location on the genome, where it can make a double-stranded break (DSB) in the DNA. The DSB can then be repaired by the cell's own repair machinery, leading to changes in the DNA sequence. These changes can include insertions, deletions, or substitutions of nucleotides, which can be used to correct genetic mutations or disrupt the function of specific genes. CRISPR knockout, CRISPR activation, and CRISPR interference-based genetic screens also offer the opportunity to assess functions of thousands of genes in massively parallel assays [1].

Human genome editing holds tremendous potential for the treatment and prevention of disease. Beyond clinical applications, human germline genome editing would permit unprecedented investigation into gene function and cell fate in human embryogenesis; allowing resolution of the elusive mechanisms that underpin pre-implantation human development [2]. Genome editing systems permit targeted gene disruption or modification in the living cells of almost all organisms [3], the molecular tools for human genome editing are now readily available [4, 5]. These technologies are amenable to both somatic and germline cells. CRISPR/Cas editing systems have already been successfully trialled in human somatic cell editing, including improving anti-tumour immunity in cancer patient T cells [6, 7]. However, human germline genome editing has only been conducted in a handful of experiments [8]. The insights from many of these studies have been limited, as many original studies used non-viable tripronuclear (3PN) embryos to circumvent ethical challenges.

The advancement of human germline genome editing remains unequivocally controversial and evokes several long-standing, significant social and bioethical objections, including regulatory considerations regarding the intent of use, specifically in the use of genome editing for enhancement; concerns surrounding ethnic representation in samples; and ensuring equity of access [9]. Yet, debates surrounding the ethical ramifications of human germline editing are reduced if safe and efficacious protocols for practice can be established. Currently, germline genome editing experiments may risk the introduction of potentially dangerous, heritable changes to the human genome, but also have the potential to correct devastating familial mutations. Most regard previous attempts at human genome editing as premature and irresponsible [10]. Off-target editing, unintentional chromosomal rearrangements, and mosaicism persist as adverse, but not infrequent, outcomes of CRISPR/Cas9 editing experiments [11, 12], but these are overcome with newer methods of genome editing. The timing and delivery methods through which editing components are introduced to target cells are key determinants of experimental outcomes. However, there is a paucity of information regarding best practice for introduction of CRISPR/Cas systems to large animal and human cells and embryos and therefore little information about what different editing methods could mean in terms of success.

CRISPR/Cas genome editing mechanisms

CRISPR/Cas editing experiments rely on two components: a single guide RNA (sgRNA) and a Cas endonuclease [13, 14]. The sgRNA consists of a "scaffold" trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA) (Figure 29.1a) [3]. The "spacer" region of crRNA shares 17–20 nt homology with the target sequence, which is located a proximal to the protospacer adjacent motif (PAM) [15]. The PAM, which can be as short as a trinucleotide sequence, functions as the Cas binding site and signals for target site-specific DNA cleavage (Figure 29.1b) [16]. The resulting double-strand break (DSB) can be repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Figure 29.1c) [17, 18]. Predominantly used for gene knockouts, NHEJ utilizes native, error-prone DNA repair machinery to resolve the DSB; here, small insertions or deletions (indels) result in gene disruption [17, 19]. Alternatively, a single-stranded oligodeoxynucleotide (ssODN) template with homology to the target region can be delivered with the Cas enzyme to initiate HDR and introduce specific changes to the target sequence, including gene knock-ins or point mutations. However, HDR typically occurs at a lower propensity than NHEJ-mediated repair [20].

Streptococcus pyogenes Cas9 ("Cas9") remains the most widely used Cas enzyme [3, 21]. However, the early success of Cas9 propelled efforts to diversify the potential applications of CRISPR-based editing systems. Subsequently, several novel endonuclease-directed systems for gene targeting have been identified, including Cas12a, Cas13a, and LbCpf1 [22]. Each endonuclease recognizes a distinct PAM, has a divergent target sequence length, and harbours different cutting characteristics. In tandem with the ease with which sgRNAs can be programmed, this extensive arsenal of CRISPR-based technologies now permits remarkable flexibility in gene editing experiments. Simultaneously, the simplicity and versatility of CRISPR/Cas editing systems has encouraged widespread use and displaced the need for less efficient, yet more arduous, systems, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and meganucleases [4, 5].

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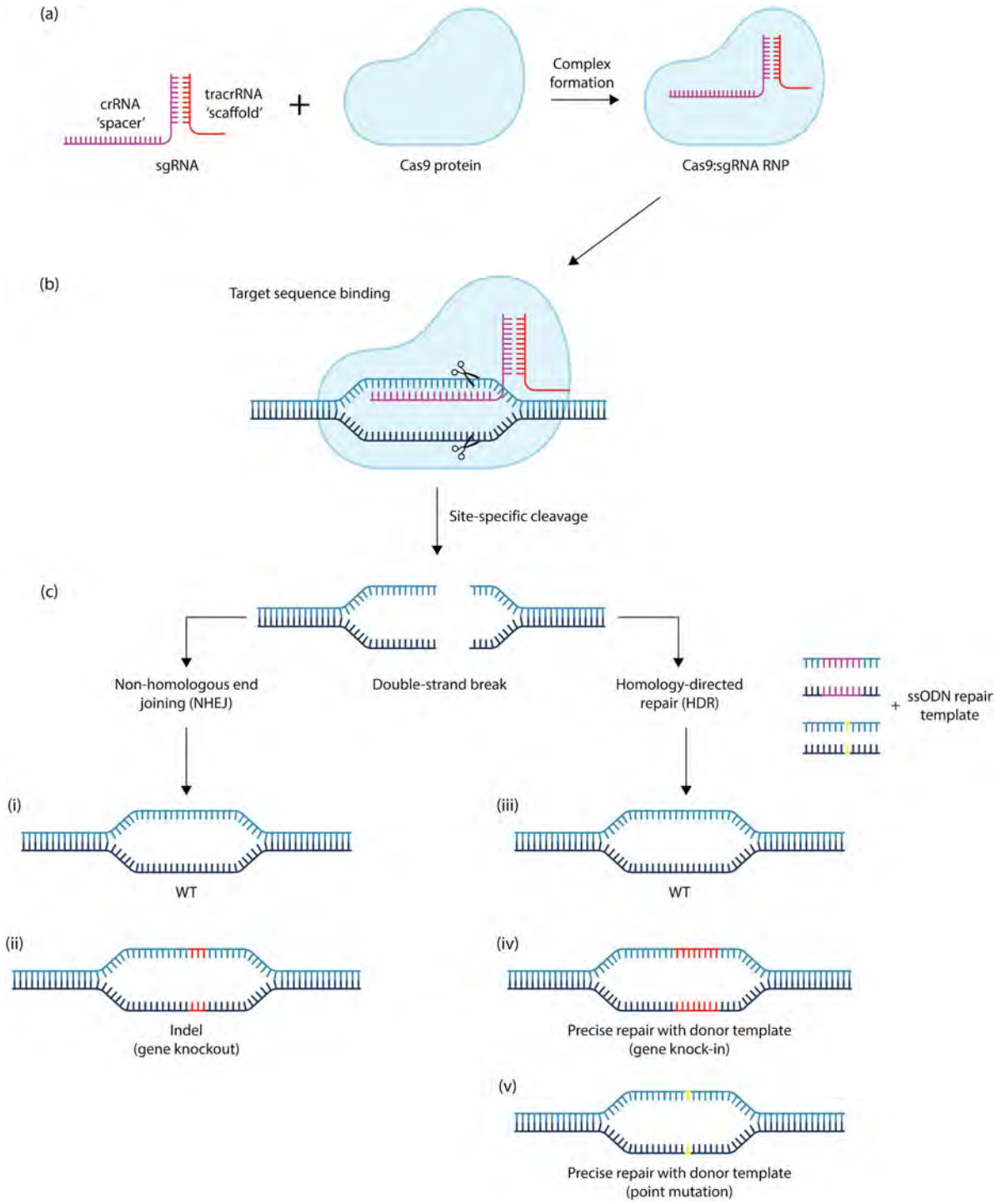

FIGURE 29.1 CRISPR/Cas-mediated DSB repair mechanisms. (a) The sgRNA consists of a "scaffold" trans-activating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA), which associates with a Cas protein to form an editing complex. (b) The crRNA "spacer" region directs the endonuclease to the target site. (c) Following the formation of a double-stranded break (DSB), endogenous DNA repair can occur by non-homologous end joining (NHEJ) resulting in (i) restoration of the Wt sequence, (ii) random indels that cause gene disruption or by homology-directed repair (HDR) which uses a template DNA strand for precise repair, resulting in (iii) restoration of WT sequence, (iv) precise repair with gene insertion, or (v) precise repair with point mutation. Figure created using BioRender (www.biorender.com).

Assessing editing outcomes in CRISPR/Cas genome editing

Off-target editing and mosaicism are an ongoing challenge for CRISPR/Cas-mediated gene editing. Site-directed Cas endonucleases can unintentionally cleave host DNA at off-target sites, which can produce unwanted effects [23, 24]. Conversely, if editing complexes remain active throughout rounds of DNA replication, this can result in mosaicism: a phenomenon in which cells of the same organism carry distinct genotypes [12, 25]. Two studies attempting gene editing of 3PN human embryos both reported the occurrence of unintentional editing and mosaic mutants [26, 27]. Large chromosomal rearrangements and loss of heterozygosity have been highlighted as a further complications of CRISPR/Cas editing experiments [28–31].

Genome editing delivery strategies

There are three forms in which CRISPR/Cas gene editing systems can be introduced to target cells (Figure 29.2a) [21, 32]. The first strategy is to introduce a plasmid vector encoding both the Cas endonuclease and sgRNA [33]. Upon delivery into the cell, the *Cas* gene and sgRNA sequence are transcribed and the editing system is expressed until the plasmid is cleared from the cell (Figure 29.2b). However, failure of the host cell to efficiently clear plasmids can result in prolonged *Cas*/sgRNA expression and increase propensity for off-target editing [34]. Alternatively, editing components can be delivered as *Cas* mRNA and sgRNA, thus circumventing the use of host transcription. Yet, this delivery strategy invokes a "lag" time between *Cas* mRNA translation and sgRNA binding, which can increase incidence of mosaicism [35]. Finally, CRISPR/Cas systems can be introduced as ribonucleoproteins (RNPs). Here, the inherently functional Cas protein:sgRNA complex can begin genome editing instantaneously [27, 36]. In recent years, RNPs have become the dominant delivery strategy in CRISPR/Cas editing experiments. RNPs negate translation waittime thus reducing mosaicism, whilst the shorter half-life of Cas proteins, compared to mRNA, diminishes off-target editing [27].

Methods for embryo transfection

Introduction of sufficient sgRNA and Cas endonuclease to target cells is fundamental to achieving high-impact editing outcomes. Delivery methods can be broadly classified into three groups: viral-based transduction, and chemical or physical transfection [37]. Transduction protocols are not compatible with germline editing due to high cytotoxicity, risk of viral infection, and potential integration of viral DNA into the host genome [38]. Conversely, chemical transfection methods, including liposomal and cationic polymer-based, have been trialled with limited success [39]. As such physical transfection techniques present the prevailing opportunity for introduction of CRISPR/Cas systems to zygotes. Microinjection persist as the predominant method for delivery of gene-editing components into the nucleus or cytoplasm of zygotes [39, 40]. Cytoplasmic injection is associated with higher embryo survival rates, as pronuclear injection can induce chromosomal breaks [41]. Yet, both types of microinjection are invasive and can pose serious harm to embryos, including post-transfection mortality [40]. Microinjection is a conceptually straightforward delivery method; however, necessitating a skilled technician to inject embryos individually, micromanipulation techniques are accompanied by several practical challenges [42]. The manual requirement leaves the technique liable to major inter-operator variability, which can confound comparisons between experimental outcomes. To combat these limitations, the development of computer-assisted microinjection has permitted high-throughput micromanipulation of non-mammalian embryos with reproducible results [43]. So-called "autoinjection" has only recently been attempted in mammalian embryos and exhibited low mutational capacity [44, 45]. As such, the need for a safe, reproducible delivery method remains unabating.

Electroporation of embryos

Electroporation has emerged as a promising alternative to microinjection for zygotic transfection. The technique may be a uniquely beneficial transfection method for clinical applications: negating operator-specific variability, whilst simultaneously avoiding the risks associated with viral transduction [46]. Here, pulsed electric fields are employed to transiently increase cell membrane permeability, permitting entry of otherwise impermeant gene editing components into up to 100 embryos synchronously [47]. The capacity to transfect multiple zygotes simultaneously has significant advantages, allowing tight temporal regulation of delivery and augmenting consistency among samples. Conversely, sequential microinjection of embryos can result in sizable divergence in the time point at which CRISPR/Cas components are introduced to each embryo.

Electroporation is now routinely used to create transgenic mice. In these murine models, the technique has been shown to outperform microinjection: yielding higher mutation rates and bolstering offspring survival rates [48, 49]. Electroporation has also been successfully leveraged for CRISPR/Cas delivery to zygotes in larger mammals, with high-impact editing outcomes [50]. To our knowledge, electroporation-mediated delivery of CRISPR/Cas editing components is yet to be attempted on human embryos. Given the ease and reported successes of electroporation in animals, the application of electroporation in human germline editing experiments seems sensible. In preparation for this transition, it would prove worthwhile to investigate how electroporation could be deployed to enable more precise editing of human embryos.

Mammalian embryos—practical considerations

To date, research carried out on non-human embryos yields interesting but insufficient comparative evidence for understanding genome editing in human embryos. There are several practical considerations to evaluate regarding when introducing CRISPR/Cas systems to zygotes. Zygote size has been shown to affect gene editing outcome, as cell diameter is positively correlated with membrane potential [51]. As such, smaller embryos generally require a higher voltage to become permeabilized than larger embryos.

Mammalian oocytes are encapsulated by the zona pellucida: a thick, acellular, glycoprotein matrix that functions to support communication between oocytes and follicular cells during oogenesis and to protect oocytes and eggs during development [52, 53]. Bovine and porcine zonae pellucida are both constituted of three glycoproteins (ZP1-3), whilst the human zona pellucida contains four distinct glycoproteins (ZP1-4) [54]. The human zona pellucida "hardens" upon fertilization to prevent

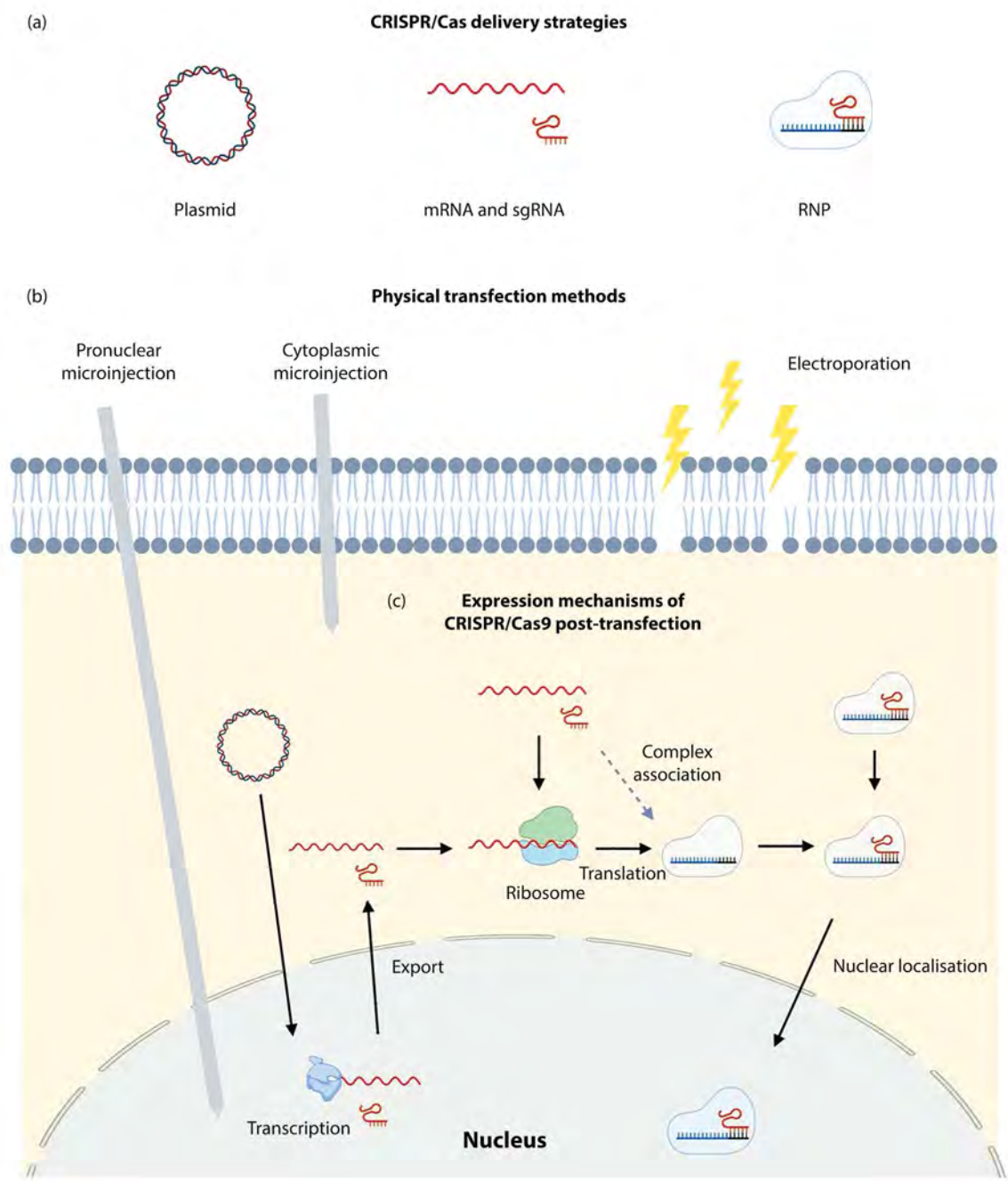

FIGURE 29.2 Strategies to deliver and edit genes using CRISPR/Cas9. (a) CRISPR/Cas systems can be introduced to cells in three forms: plasmid, mRNA, or RNP. (b) Pronuclear and cytoplasmic microinjection, as well as electroporation, are available physical transfection methods. (c) Plasmid delivery requires migration of the plasmid into the nucleus, transcription of the DNA, and exportation of the mRNA into the cytoplasm. The mRNA is then translated to produce Cas9 protein. The second strategy is to deliver a combination of the Cas 9 mRNA and the sgRNA. The Cas9 mRNA must be translated to Cas9 protein in cells from the Cas9/sgRNA complex. Finally, Cas9/sgRNA complexes can be delivered directly to cells. Figure created using BioRender (www.biorender.com).

polyspermy, conversely there is evidence to suggest bovine and porcine zonae pellucida harden before fertilization [55, 56]. This "shell" may present a barrier to the delivery of editing components to zygotes and thus the zona pellucida warrants consideration in CRISPR/Cas editing experimental design. Early protocols for ZFN and TALEN-mediated editing typically reported zona pellucida weakening by Tyrode's solution prior to electroporation to improve editing efficiency [57]. Yet, loss of the zona pellucida may in turn affect embryo viability following electroporation [58].

Perhaps the most valuable use of genome editing will be in furthering our understanding of early human development, and the first licence was given to Niakan et al. to apply CRISPR/Cas9

to human embryos to interrogate the role of OCT4 in human embryo development [59].

Research has increasingly highlighted the marked mechanistic differences in embryonic genome activation (EGA), the initiation of gene expression following fertilization, between mice and humans [60, 61]. As such, whilst mice are undoubtedly an excellent model organism, they provide limited insights into human embryogenesis and present a poor candidate for elucidating how genome editing techniques can be best applied to human zygotes. In contrast, porcine, as well as bovine, embryos have been shown to better resemble human embryos (Figure 29.3) [62, 63]. Pigs also more closely model humans in terms of size, physiology, and genetics [64, 65]. As such, in a bid to advance safe and effective electroporation-mediated genome editing protocols for humans, efforts are perhaps better focused on larger mammalian embryos.

CRISPR/Cas gene editing has immense potential; however, several obstacles must be overcome before the technology can be widely deployed on human embryos. Our failure to establish delivery protocols that guarantee safe and efficient introduction of editing components to cells continues to present a significant challenge to widespread use.

Advancing tools in genome editing: Base editing and beyond

While genome editing strategies using nucleases hold great promise for the treatment of disorders, a major drawback of these traditional approaches is the generation of double-strand breaks (DSBs), which can have unpredictable and potentially harmful effects. Base editing is a novel CRISPR-Cas9-based genome editing technology that allows the introduction of point mutations in the DNA without generating DSBs. This is achieved by using a specific type of enzymes, called Base Editors, which can change a single base by cutting one strand of the DNA and then using the cell's repair machinery to introduce a new base. Three major classes of base editors have been developed: Adenine Base Editors or ABEs, allowing A>G conversions, and Cytidine Base Editors or CBEs allowing C>T conversions [16]. The applicability and use of base editing tools has been extensively broadened to include Prime Editing (PE) [66], which can make more complex changes in the genome. This newer class of nucleases allows for greater specificity, higher efficiency, and increased accessibility to previously inaccessible genetic loci while maintaining a low rate of off-target effects as well as unwanted insertions and deletions. PE has expanded the CRISPR-base-edit toolkit to all 12 possible transition and transversion mutations, as well as small insertion or deletion mutations [67].

Base editing is a type of genome editing that allows for precise changes to a single base in the genome without making a double-stranded break (DSB) in the DNA. Base editing is a new and rapidly evolving field, which is becoming increasingly popular for its high precision and reduced off-target effects compared to traditional DSB-based genome editing methods such as CRISPR-Cas9. The high precision and reduced off-target effects of base editing make it a powerful tool for genetic research and have the potential to lead to new treatments and therapies for a wide range of genetic disorders.

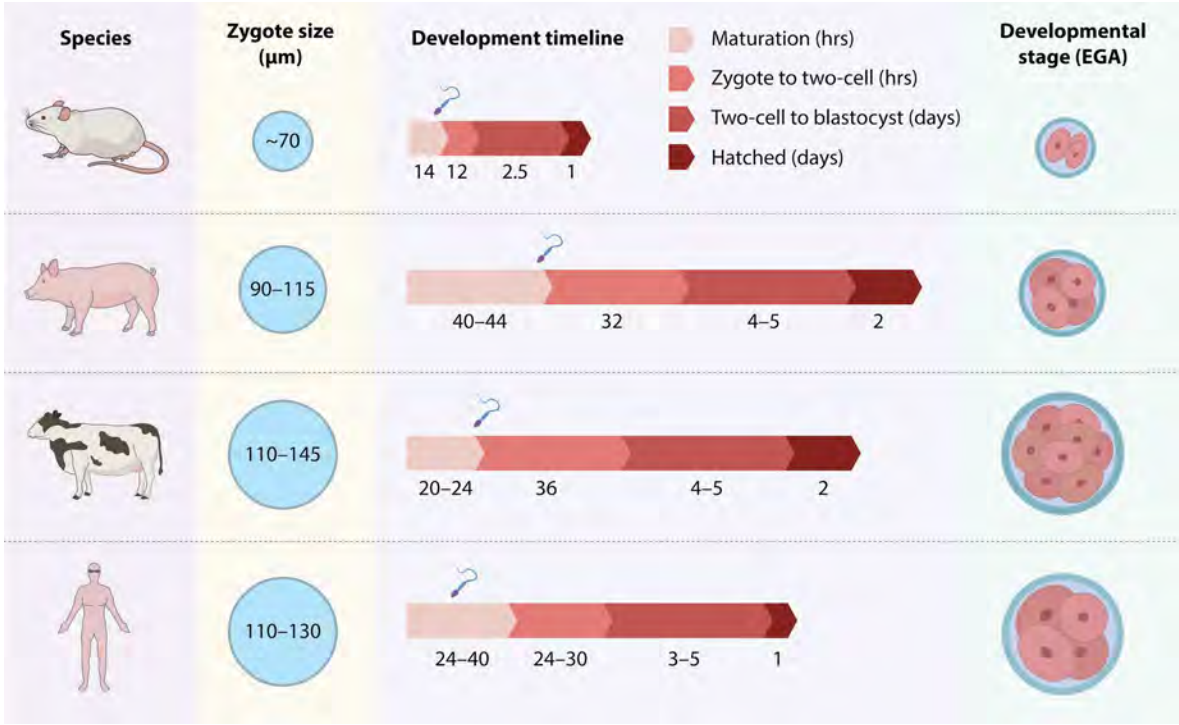

FIGURE 29.3 Oocyte size and timeline of early embryo development in mice, pigs, cattle, and humans. (From left to right): species; oocyte size (shown to scale for comparison); relative timeline of embryo development, including maturation, zygote to two-cell to blastocyst, then finally to hatching; and developmental stage at which EGA occurs. (Figure adapted under Creative Commons Attribution Licence from Santos RR, Schoevers EJ, Roelen BAJ, Usefulness of bovine and porcine IVM/IVF models for Reproductive toxicology, Reprod Biol Endocrinol. 2014; 12: 117; created using BioRender (www.biorender.com).)

Genome editing in human reproduction—past, present, and future

The 2018 International Summit on Human Genome Editing was a major international meeting held in Hong Kong in November 2018. The summit brought together leading experts from around the world to discuss the latest developments in genome editing and to consider the ethical, legal, and social implications of the technology. On the eve of the summit, a press release was made that Chinese scientist He Jiankui had used CRISPR-Cas9 genome editing to modify the genes of two human embryos.

The announcement by He Jiankui was met with widespread criticism from the scientific community, and the summit provided an opportunity for experts to address the concerns raised by his experiment [68, 69]. Many attendees expressed their disappointment with the lack of transparency and ethical considerations in He Jiankui's work, and they called for greater international collaboration and oversight in the development and use of genome editing technology.

The summit also served as an opportunity to discuss the potential benefits of genome editing, particularly in the area of human health. Attendees emphasized the need for responsible and ethical use of the technology, and they stressed the importance of continued research and development to ensure that the benefits of genome editing are realized in a safe and responsible manner.

In 2019, the World Health Organization (WHO) published a report on heritable genome editing, which called for a global ban on the use of CRISPR in human embryos [70]. The report emphasized the need for caution and transparency in the development and use of the technology, and called for international cooperation in developing ethical and regulatory frameworks to govern its use.

The Nuffield Council on Bioethics, a UK-based independent organization, also released a report on genome editing in the same year [71]. The report concluded that while the potential benefits of genome editing are significant, there are also significant ethical concerns, including the risk of creating new inequalities and the potential for unintended consequences. The report recommended that the use of CRISPR in human embryos be limited to cases where there is a serious medical need and that the technology be subject to rigorous ethical review.

In 2020, the National Academies of Sciences, Engineering, and Medicine in the United States released a consensus study report on genome editing [72]. The report agreed with the conclusions of WHO and Nuffield Council reports, and emphasized the need for caution in the development and use of the technology. The report called for rigorous ethical review of all proposals to use CRISPR in human embryos, and for the development of international guidelines to ensure that the technology is used responsibly.

Overall, the reports by WHO, Nuffield Council, and National Academies of Sciences, Engineering, and Medicine reflect the need for caution and transparency in the development and use of CRISPR technology, particularly in the area of human embryology. The reports emphasize the importance of rigorous ethical review and the development of international guidelines to ensure that the technology is used responsibly and in accordance with ethical standards.

In summary, genome editing is a revolutionary technology that has the potential to revolutionize medicine and biology by enabling the precise modification of genes. However, much work is still needed to fully understand the breadth of its function, particularly in the context of human embryology and the ethical implications of editing the human germline. Despite the tremendous advances that have been made in the field, there are still many unknowns and uncertainties, and much research is needed to better understand the potential risks and benefits of this powerful technology. As the field of CRISPR genome editing continues to evolve, it is important that research is conducted in a responsible and ethical manner, taking into account the potential implications of these technologies for human health and well-being.

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DESIGNING DISASTER PLANS FOR IVF LABORATORIES

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Not all disasters are as dramatic as a hurricane ravaging an entire city, a forest fire threatening a clinic, or an earthquake tearing apart a hospital. There are many unsuspecting disasters that come upon us without warning. A laboratory flooded in Boston when a water line broke on the fifth floor and 28,000 gallons of water flooded the downstairs. An IVF clinic in San Diego, although not threatened by nearby fires, had toxic air around the clinic for several weeks potentially affecting outcomes in IVF cycles. In Arizona, a toilet valve stuck, flooding the laboratory with two inches of water. In New Zealand, an IVF clinic below a birthing facility was inundated with water from a tub left on above them. After trying many methods to remediate the resulting mould growing everywhere in the laboratory, they finally had success in using hydrogen peroxide mist to fumigate the building. Some, like those located in Florida and southern Texas are accustomed to activating disaster plans every year due to the common occurrence of hurricanes in their areas. We can learn from these examples and be better prepared.

Dr Richard Dickey, who was involved with hurricane Katrina's impact on the Fertility Institute of New Orleans reported, "Our experience with Katrina proved that it is not necessary to be at ground zero to be affected by a natural disaster. An event miles away may disrupt electrical power and accessibility for an extended period of time; therefore, having to rely on generators until power is restored and delaying transfer to the fifth day may not be sufficient. All IVF programs need plans to protect fresh and cryopreserved embryos in the event of a natural or human-made disaster" [1].

No matter where a laboratory is located, even if it is not in a hurricane or earthquake zone, there are many catastrophes that can ruin a clinic and its patients' chances of a healthy birth. The recent Covid-19 pandemic has emphasized how important disaster plans are. These plans should be made in a time of relative safety when one has time to thoroughly think through how to best get through an emergency. In many cases, resources may already be, or will become, scarce and the first person, if prepared, can react to these disasters to secure these resources. Better yet, these resources can be secured before the disaster occurs.

In the beginning of the Covid-19 pandemic, most people were left on their own to react while waiting for the experts to release responses. Nobody in the fertility industry foresaw a pandemic as a cause for concern, certainly not enough concern to enact a pandemic preparedness plan, despite the warnings from HIV and ZIKA.

> "The primary objective of an emergency action plan (the 'Emergency Plan') should be to provide for the safety of program personnel and patients, fresh and cryopreserved human specimens, and critical equipment and records" [2].

Types of disasters

Disasters can be divided into two major categories, natural and man-made. Natural disasters include hurricanes, tornados, forest fires, floods, earthquakes, blizzards, and high winds. Man-made threats include terrorism, arsonists, and strikes.

The consequences of these disasters must be considered for each potential disaster. Flooding may occur from a backed-up toilet or from a river that overran its banks. Electrical outages can occur from too much demand on the power system or from a hurricane. Shortages of materials can occur from a workforce stoppage, strike, or from a pandemic.

In developing a plan, all potential interruptions and sources of damage to the laboratory and clinic should be considered. Each type of disaster may necessitate a different solution to the specific type of damage, depending on available resources. Flooding from a backed-up toilet can be overseen by a flood recovery company, but these companies most likely will be overburdened after a hurricane; and so a separate solution must be found—maybe purchasing your own clean-up equipment including vacuums and pumps prior to an emergency.

Regulations pertaining to disaster plans

The laboratory and clinic have both a regulatory and an ethical responsibility to its patients and its staff during a disaster situation. These responsibilities include protection (people and tissue); continuation of services when possible; and a rapid, safe, and responsible recovery from the dangers imposed by the disaster. A comprehensive disaster plan, if done correctly, can make sure these responsibilities are taken care of.

Laboratories and clinics are required by regulatory bodies such as the College of American Pathologists (CAP), the American Society of Reproductive Medicine, and the European Society of Human Reproduction and Embrology to have disaster preparedness plans to help them in handling emergencies [3–6]. The CAP checklist item Gen.73.800 addresses disaster plans and states, "The specific elements to be included in the emergency preparedness plan must be based on a risk assessment using an 'all-hazards' approach to evaluate the types of hazards most likely to occur that would potentially disrupt services" [3]. The Joint Commission standard EM.09.09.01 requires an "all-hazard" approach to the development of a comprehensive disaster preparedness plan [7]. It addresses leadership roles, communication during the emergency, staffing during the emergency, a disaster recovery plan, and an emergency education plan. These plans must ensure the safety of staff, patients, and tissue.

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Software to aid in making a disaster plan

There are several software programs (Noggin Emergency Management, for example) that advertise management solutions, disaster planning, incident reporting, prediction algorithms, and so forth, but be aware that many are focused on large geographic disaster scenarios. Most ART programs have specific needs, and you may spend time and money trying to modify or force a program to meet your needs. If you are a member of a larger group or corporate institution, a software option may already be available to you, so make sure to check with a member of your institution's safety committee. Also, many of these programs are aimed at data recovery, and are less useful for general planning purposes. If you are not part of a larger institution, a colleague may be willing to share their policies and procedures and could be adapted as needed.

How to design a disaster plan

Designing a disaster plan is not easy. There are several tools available to aid in their development. One of the best resources is from the Federal Emergency Management Agency (FEMA). They provide numerous "kits" that will walk you through the development of plans for several types of disasters. These kits are available free at their website http://www.ready.gov/business. There are kits to help design plans for power outages, flooding, high winds, earthquakes, and hurricanes.

The American Red Cross has a free, self-guided program to help businesses, organizations, and schools become prepared for emergencies (https://www.readyrating.org/How-It-Works) [8]. This program, Ready Rating, consists of five sections: participation, emergency planning, facility and equipment, training and exercises, and extended community. These programs will guide one through best industry practices in making and implementing a preparedness plan. A group's preparedness is rated by answering questions in either the ReadyGo or the ReadyAdvance module. The ReadyAdvance program will probably fit the needs of most fertility practices. It will measure an emergency preparedness plan's maturity and completeness. The Red Cross also has several free phone apps that address many types of emergencies. These applications can generate alerts for areas that are of interest to you—your home, the clinic, your loved ones—when an emergency is approaching. They also have information on how to prepare and what to do and not do during a particular emergency.

Getting started

The best way to get started, if you do not already have a plan to work from, is to outline and address topics, then revisit areas of concern with other team members. Initially you will no doubt generate more questions than answers. See Box 30.1.

Create a safety team if you have not already done so. Include physicians or anaesthetists participating in anaesthesia for egg retrievals or other office surgical procedures, as evacuations may require ambulatory measures. Also include nurses, medical assistants, and clerical staff in the design of a disaster plan. Assign specific tasks, and make sure to follow up to be sure that all tasks are being addressed. Chemical, biological, and environmental safety are all areas to consider.

The initial key part of any disaster plan is the investigation phase. The investigation phase should include all departments involved—the laboratory, clinical staff, and office staff. In this phase, one should brainstorm and write down the most common emergencies that one can foresee. Examine the last 10 years and include these emergencies first. Ideally, one may want to start by listing specific mechanical emergencies and use these plans to work into more general emergencies. These mechanical emergencies might include loss of power, loss of water, loss of HVAC, general flooding, stoppage of supplies, evacuation of a building, inability to enter the laboratory, etc. The general emergencies might include flooding, smoke, earthquake, hurricane, and pandemic, for example. Focus on one plan at a time. Even if it is not possible to think of every potential emergency, these mechanical emergencies can form the basis for assisting in any emergency. It might help in the next steps, and especially in the recovery phase, to take a wide-angle photo of each room in the clinic and make a list of equipment and key supplies. Not only will these photos help in planning, but they will also assist if insurance is needed to corroborate recovery costs. A full inventory of any assets in the clinic should be done. This can also be used for insurance purposes during the recovery phase.

Good preparation for a disaster includes the design of informed consents so that patients will have been told prior to an emergency what will potentially happen to their treatment and their tissues. Included in this could be an alternative method of communication with the clinic that clinic employees can monitor. Ideally, this should be a site where the patient can leave a question and the staff can monitor daily.

When making a plan, set aside time, without distractions, for a walk-through of all spaces. Have copies of the floorplans available for your own space and areas outside of your space. Make sure to take notes on all ingress and egress points and have team members open and close doors. Also, not all doors open in the same direction. Some may be one-way doors that lock when they close behind you, or have magnetic or powered properties, and

BOX 30.1 STEPS TO DISASTER PLANNING

1. Take photos of the entire laboratory and clinic.
2. Identify key equipment.
3. Build a disaster committee that includes staff members from every department.
4. Start with addressing disasters/emergencies that have occurred in the past and then look at the rarer ones in order of probability of occurring.
5. List important individual functions that might be disrupted.
6. Design protocols for how to manage important individual functions and at varying levels of interruption.
7. Visualize the disaster and what items need to be cared for.
8. Itemize protocols to mitigate these items of concern prior to the disaster.
9. Itemize protocols that will be implemented during the disaster. Be sure to include when they will be implemented, how they will be implemented, and who will implement them.
10. Address communication concerns for a major disaster.
11. Include in the protocols what will happen when the disaster is either minimized or is gone.
12. Design a method to train the staff on what their part will be in the disaster.

alarms. A walk-through will also ensure that the space on the other side of an egress door is not blocked or cluttered or used for storage—you don't want anything to block the exit and there may not be time to clear a path. If your facility has windows, inspect these as well—can they be used as an escape route, or are they a hazard in high winds? Are you in a facility with multiple floors? If so, make sure that you have ready access to stairwells—again, can the doors be opened?—and don't forget to walk the stairwells and see where they lead. You might be surprised, and you don't want to end up in a dead-end courtyard or parking garage with few or blocked exits.

One of the prominent components of an overall disaster plan is the evacuation route—fire, earthquakes, power outages, and many other scenarios involve the safe evacuation of staff and patients. This is the most likely part of the disaster plan that will be activated, as most emergencies on a larger or smaller scale will require that you move from your current location to a designated meeting site.

Consider how well your evacuation route will work for ambulatory situations. Can you move freely carrying someone, moving someone in a wheelchair or on a gurney? Look for obvious engineering controls, e.g. fire extinguishers (you may have to use them to clear a path through a fire), emergency lighting, emergency exit signs that come on during any power failure.

Purchase flashlights for the clinic if you do not already have them. A real example—the clinic was in the basement of a physicians' office building, with no windows or outside natural lighting. The power lines to the building were cut by a backhoe, including a nearby power line from the emergency backup generator. No light, no power, pitch black, with patients and staff still in the clinic. A few individuals had cell phones and were able to use them as flashlights while being evacuated.

Find a safe meeting point that everyone is familiar with—keep it simple and use obvious landmarks, visible in low light or darkness, and in low visibility conditions.

Once you have set up an evacuation route, assign specific duties to individuals to clear the workspace. Bathrooms, offices, break rooms, exam rooms, closets, and storage rooms—don't miss a single place. Make sure that everyone knows their areas of responsibility. Better yet, if you have enough staff, team up to help each other and patients.

Mitigation

The next step of the plan is mitigation [9]. In mitigation, one should focus on what can be done to either avoid the disaster or to decrease the harm from a disaster. For example, in case of minor flooding this might include making sure all electrical equipment is raised above the surface of the floor to avoid minor flooding and destruction of critical electrical equipment.

One fertility clinic had minor flooding from an overflowing toilet. Had the tower computer been placed on a small platform to raise it just two inches from the floor, a vital piece of equipment would not have had to be taken out of service until a new computer could be put into service. Another mitigation to minor flooding could be installation of drains in critical areas and water monitors/alarms to provide early warning of water encroachment. Approaches should be taken to not only reduce harm but also to avoid harm if possible.

For each type of potential disaster, examine what can be done to decrease the disaster's affect. How can electrical equipment be protected from electrical surges? How can windows be protected from damage due to high winds? Should storm shutters be installed? How can critical items be protected from high heat? If the HVAC goes down, how will incubators function? Will an alternative portable refrigeration unit be needed? Do you have enough liquid nitrogen for a lock out of a week? How will preparation for potential shortages in the supply chain of materials be managed? What quantity of important laboratory and clinical supplies will be kept on hand? Will a secondary source of information of clinical records or cryo-storage records be available if needed and local computers and the internet are down? One should sit down with the disaster planning team to come up with a brain-storming list of all potential problems that might occur.

Response phase

The response phase is next; and it is a major part of any disaster plan. It describes what will happen when a disaster hits and how the effects will be minimized with proper backups, contingencies, and sufficient supplies. In this phase, it is important to decide what items will be needed to handle the emergency when it occurs so that they can be purchased ahead of time. For example, if one needs to move cryo-storage tanks, are there wheeled carts or hand trucks available to move them? If the elevators are not functioning, is there a system to allow for the relocation of equipment and tissue that does not rely on the elevator? How will staff keep in contact if cellular phone systems are inoperable?

One needs to plan what the reactions should be to the emergency. Who will instigate the plan and under what circumstances? How will storage tanks be moved and where will they be moved to? If a fire in a nearby forest is close, what will the response be? What responses will occur when a hurricane watch is posted for your area? What about when a hurricane warning is posted? How will current or upcoming IVF cases be managed?

An IVF group in Miami bases their hurricane disaster plan on when a hurricane is several days out (Inea Collazo, personal communication). At this time, they sit down and chart out all of the patients that have embryos in the incubator and those that are in stimulation. When a watch is declared, which means the hurricane is about two days away, they begin vitrifying all embryos in the incubators and all patients that are in the middle of a stimulation are cancelled.

An effective way to approach preparing a disaster plan is to first design plans for each type/level and duration of interruption see Box 30.2. These can be called sub-disasters. For example, a hurricane disaster may include several sub-disasters such as flooding, power outages, and disruption of transportation. Identify these sub-disasters and design plans for differing levels of these sub-disasters which can then be plugged into a major disaster

BOX 30.2 SUB-DISASTERS TO ADDRESS IN PLANNING

1. Power outages
2. Flooding
3. Inability to access the clinic
4. High winds
5. Too hot or cold
6. Toxic air
7. Supply chain stoppage or slow down
8. Communication interruptions
9. Transportation interruptions

plan. Consider flooding. Design responses for minor flooding and major flooding, e.g. for floods lasting hours and for floods lasting days. Then look at power outages that are short-term versus long-term. Have a plan for no physical access to the facilities for a day versus many days. These plans for each type of interruption can then be added to each disaster plan depending on the type of disaster.

Even with practice, expect that someone will panic during a real disaster—routine drills will help alleviate this, but there is no guarantee that everyone will stay calm. Having to control a panicked person is difficult—emotionally and physically. Panic can be contagious. During a real evacuation, one may experience fire, smoke, loud alarms, flashing lights, loud voices, and sounds—it could feel dark, chaotic, frightening, and there is no way to predict how others could react. Some individuals may be terrified, afraid to move forward or backward until physically guided out of danger, whereas others might run without looking back. So be prepared to take charge of the situation. If you find yourself panicking, get help if you can, and try not to get separated from the group.

Recovery phase

The final phase of writing a disaster plan is the recovery phase. It examines what will happen after the disaster happens and how the laboratory or clinic will resume operation. The first priorities should include ensuring safety, providing essential needs, and restoring basic services. Have a contact list for local and state agencies, e.g. fire, police, state police, as they will be able to help determine if, and when, you can return. What criteria must be met to reopen and how will one ensure that the laboratory is safe and ready to culture and freeze embryos? How will debris be removed? How will clinical and laboratory areas be cleaned? What steps will be taken to ensure the incubators and other critical equipment are working correctly?

When the disaster has passed, and it has been deemed safe to enter the building, it is important to do a complete inventory of damage. It is at this time that professional disaster recovery companies can be invited in to help evaluate and offer suggestions regarding the building's structural integrity, safety as relates to utilities, and options to remove debris and restore the working space to its original. If flooding has occurred, special attention should be placed on how to mitigate contamination of the building from fungi.

First attempts to mitigate damage do not always work and so one must monitor the outcomes of recovery attempts over time until success is achieved. A clinic in Australia was inundated with water when a jacuzzi in another office space upstairs flooded the downstairs IVF clinic. At first, a simple vacuuming of the water and clean up was done, but after a few weeks, it became apparent that fungi from the walls was contaminating the IVF cultures. Several methods were tried to kill the fungi, but none were successful until hydrogen peroxide mist was used to fumigate the entire clinic.

A video camera and/or a camera can be used to record the aftermath. Notes should be taken on the damage to the facility and the equipment. The initial inventory of equipment may be useful for this, especially if some of the equipment is missing. Attention should be paid to functionality and safety.

Power outages

Power outages are common in many parts of the world. How you prepare for them depends on how long these outages typically last. The most common method used for short-term outages is a battery backup. Chains of batteries may work well for an hour or two (depending on the equipment one is backing up) but are not a solution for all but minor outages. Batteries often are heavy and so are placed on floors where inundating water may damage them. They can be protected from minor flooding by placing them on raised platforms or on shelves. It is important that only critical equipment, such as incubators and refrigerators are drawing current during an emergency. Providing enough batteries for powering all the equipment for an egg retrieval during an emergency may be excessive due to the current drawn from ultrasounds and warmers.

An on-site generator is the next level of protection. Generators can run on gasoline or natural gas. They can also be purchased and installed so that they will automatically come on should the power go out. There are several problems with generators though. One, is their cost. Two, is the amount of maintenance and testing required. Finally, if a big disaster hits, will there be fuel available to run these generators for several days? Storing of fuels and the generator with its toxic fumes is another problem. Natural gas is probably a better fuel source than gasoline, as natural gas sources are often operable even during major disasters while dependence on transportation may decrease the availability of gasoline during a major disaster. In some emergencies, one may be able to depend on the rental of large mobile generators. In most emergencies that last several days, most likely all procedures will be halted, and so long-term power may not be needed once embryos are cryopreserved. For this reason, cryo-storage methods that are static and require no power are ideal. Still, one must consider how cryo-storage alarms will be managed in each type of emergency. The inclusion of solar arrays for disaster recovery should also be considered.

During any power outage, concerns regarding the heating or cooling of the building, maintenance of refrigerators and freezers, and any current embryos in incubators need to be addressed. For example, all incubators will lose the ability to pump gasses, but depending on the make/model, temperature may be maintained for a longer, or brief, time. All power backup systems should be tested periodically for efficacy and how long they can provide backup. If batteries cannot provide adequate time for the maintenance of equipment, they need to be replaced.

Communication during a disaster

A system for communication during a disaster is one of the most often overlooked items. Communication is critical; how will you communicate during and after evacuation? What will you do if someone is not accounted for after an evacuation? Going back for someone may not be safe—so make sure to communicate effectively, and determine where everyone should be, where everyone is. If land line or cell phones are not working, how will the coordination of the disaster plan occur? How will you know proper steps have been taken to ensure the safety of tissues and equipment?

In 2011, a tornado struck the area around Tuscaloosa, Alabama. It caused 54 fatalities, 1500 injuries, and even more were left homeless. Druid City Hospital was the sole remaining building in the centre of destruction. As a result of problems during the disaster, the hospital began to evaluate push-to-talk devices as a method to improve communication [10].

It is important to have a central location (and possibly an alternate one) for communication during the disaster. One IVF clinic has a phone line with an answering machine that can be used as a

central messaging centre during a disaster. Some communication methods may not be available during distinct parts of a disaster. For example, initially, power may be out so that one must depend solely on methods that do not use the local electric grid. Also, during the initial part of any major disaster, local mobile services are often either unavailable or so clogged with traffic that communication using cell phones is impossible. Have an awareness of who is in the clinic, staff, and patients. Sign in boards for staff and patients can help and prevent leaving someone behind.

Forms of communication that should be considered are cell phones, texting, fax machines, email, ham, or citizen band radios. Instantaneous communication may not be available in some circumstances and so a passive system where one can leave a message for later retrieval may be best. Texting is a good method for communicating when cell service is overloaded or erratic.

Prior to any emergency, employees should all have access to phone numbers for employees, service staff, current patients, stakeholders, suppliers, and key incident response personnel (governmental and private disaster agencies, for example). The time to put together this extensive list is prior to the disaster. This list can be secured in a centralized system or location. In most major disasters it will be important to have access to patient medical charts to advise on patients undergoing treatment. Access to cryopreservation records will also be important as patients call with questions on their frozen specimens.

There should be a day-by-day plan prior, during, and after a disaster for all procedures in progress, either a whiteboard, an electronic or paper sheet for each incubator explaining what must be done each day. These worksheets can be used to make decisions—what procedures need to be done today, what will need to be done tomorrow, for example, retrievals, inseminations, fertilization checks, hatching, biopsy, freezing, and so on. These lists can be used by staff in the event of personnel shortages, immediate or anticipated, to bring additional staff into the laboratory the same or next day—the lists will help bring the auxiliary staff up to speed on what is required.

Transportation

Soon after hurricane Katrina hit the New Orleans area in 2005, the laboratory director of a local IVF lab, Roman Pyrzak, was able to obtain satellite images from NASA that showed him his hospital was surrounded by water but was intact. He reached out to several agencies to help him evacuate the cryo-storage tanks using boats [11]. These groups included the National Guard and local police. Lack of transportation can occur due to many causes, including fuel shortages, bridge outages, road closures, evacuations, traffic jams, and the stopping of public transportation. Disaster plans should address how to manage these circumstances. Consider using those that live closest for handling some of the critical steps of the plan if transportation becomes an issue.

Training

Development of a disaster plan is a futile exercise if nobody knows what their job will be should a disaster occur. Periodic training is key to the execution of a good plan. In this training, staff should know when to act and how to act. They should be assigned tasks for each disaster type. Because these plans may be full of what-ifs and can be complex, it is important that each staff member can easily access the plan from work and home so that they can remind themselves of what their role is in the disaster plan. Assign someone to document the drills, not just doing it, but every aspect—when the drill started, the people involved, and how they behave. Review the effectiveness of drills at least annually—if something is not working, then adapt the plan.

It may be important to have refresher courses just prior to a critical time when a disaster has the highest probability of occurring. For example, just prior to hurricane or fire season. After each training session, it is an excellent time to ask for input from the trainees on how the plan can be improved. Having periodic drills for specific disaster scenarios should be considered.

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EARLY HUMAN EMBRYO DEVELOPMENT REVEALED BY STATIC IMAGING

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Introduction

Our understanding of early human embryo development has for many decades derived from examining images of fixed specimens and, only more recently, cultured human embryos. Careful analysis of serial sections of human embryos retrieved during hysterectomies and stained with haematoxylin and eosin revealed the complex morphological changes the embryo must undergo as it transits through the fallopian tube and implants into the uterus [1]. For many years these scarce specimens provided nearly all our information about early human development. However, the subsequent development of *in vitro* systems that enable human embryo culture, combined with improvements in microscopy technologies constitutes a major advance that has allowed a glimpse into early embryogenesis and even the previously intractable process of implantation. Human embryos can develop through early to late implantation stages in 2D co-cultures with supporting cells, or in more complex 3D models respectively [2]. It is now also possible for embryos to undergo attachment *in vitro* without any exogenous cells or tissues present, enabling the investigation of embryo-autonomous peri-implantation organization [3–5]. Imaging *in vitro*-implanted embryos at various stages is revealing novel insights into processes directing embryo development and implantation. Innovative imaging technologies and new automated analysis approaches offer the potential to non-invasively select embryos with the best developmental capacity. Visualizing the molecular and morphological changes that occur as the preimplantation embryo develops is essential to drive new knowledge to improve the outcomes of assisted reproductive technologies (ART).

Human embryo development

Day 1: Zygote

Fertilization of an oocyte by a sperm to form a zygote triggers a series of morphological changes, including extrusion of the second polar body and the development and gradual migration of the male and female pronuclei. Once formed, the female pronucleus migrates towards the male pronucleus until they are in apposition and move together to the centre of the oocyte. Inside the migrating pronuclei, nuclear precursor bodies appear and coalesce into nucleoli. For development to proceed, these events—which depend on both maternal and paternal factors—must be executed in a timely and coordinated manner.

Various systems for grading zygotes have been developed based on several easily visible parameters including the number, size, and distribution of nucleoli and the size and alignment of pronuclei [6–8]. Scores assigned on these features likely reflect the fidelity of critical early processes such as chromosomal segregation, decondensation of chromatin, and activation of zygotic RNA synthesis, and have been associated with the developmental potential of the embryo [7, 9]. However, the highly dynamic nature of these features makes the scoring very sensitive to the timepoint at which they are measured, perhaps explaining why many studies do not support an association between zygotic scoring and pregnancy [10–13].

During the first few days of human development, the fertilized zygote undergoes a series of cleavage divisions in which each cell splits in half, producing two smaller cells (or blastomeres) without changing the total volume of the embryo. These divisions are prone to errors due to the high rate of chromosomal instability in human embryos [14]. Confocal microscopy of fixed human embryos donated from clinical *in vitro* fertilization (IVF) cycles showed that even good quality embryos have many nuclear abnormalities, including cells with small additional micronuclei, cells with two equally sized nuclei, and cells with many small nuclei (Figure 31.1) [15].

Cells with abnormal nuclei are likely to be aneuploid and show evidence of DNA damage. However, the frequency of nuclear abnormalities decreases as development progresses, suggesting a potential error-correcting mechanism may exist. Although the underlying causes of these errors in chromosome segregation are not known, it has recently been demonstrated that disruption of the mitotic spindle in human cleavage-stage embryos does not trigger cell death as would normally occur in somatic cells (16).

Day 2: Four-cell stage

The second cleavage division produces an embryo with four blastomeres. Depending on the cleavage orientation of each blastomere at the 2-cell stage, the 4-cell embryo may have either a tetrahedral or planar configuration (Figure 31.2).

Given that at least some proteins display a polarized localization in the oocyte, the orientation of the cleavage divisions can result in unequal distribution of these proteins between blastomeres from the 4-cell stage onwards [18]. It is currently unknown what determines the orientation of the cleavage divisions; however, tetrahedral 4-cell embryos are more likely to develop into high-quality embryos *in vitro* [17, 19].

Day 3–4: Morula

Compaction

The first obvious morphogenetic process to occur during preimplantation development is compaction. During compaction the cells of the embryo flatten against each other, increasing their contact areas and transforming the embryo from a loose cluster of spherical cells into a tightly packed mass [20]. Compaction may be initiated between the 4- to 16-cell stages, but most human embryos begin to compact at the 8-cell stage and the timing of compaction is associated with blastocyst quality and implantation [21, 22]. The cell adhesion molecule, E-CADHERIN accumulates at cell–cell contacts in compacting embryos and abnormal distribution of E-CADHERIN is associated with developmental defects including non-compaction, cell fragmentation, and embryo arrest [23].

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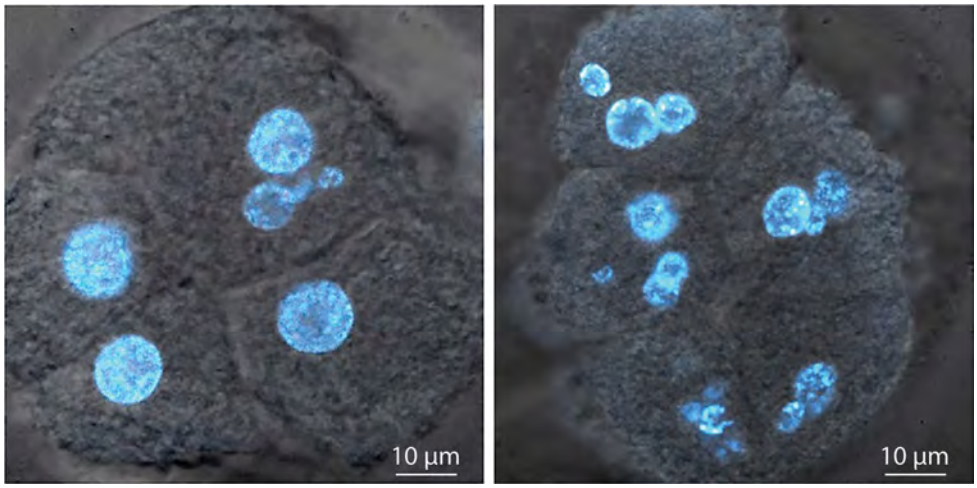

FIGURE 31.1 Nuclear abnormalities in human cleavage stage embryos. (Adapted with permission from [15].)

Polarization

Concurrent with compaction in human embryos is the establishment of apical-basal cell polarity. Immunofluorescence and confocal microscopy of human embryos fixed on day 4 of development revealed that the apical domain is generated in two steps [24]. First, activation of Phospholipase C (PLC) signalling triggers the enrichment of actin at the contact-free apical surface of the blastomeres. Next, the PAR polarity complex components PARD6 and aPKC are localized to the nascent apical domain [24, 25]. Establishing the apical domain promotes onset of the first cell lineage differentiation: a trophectoderm-associated transcriptional program characterized by expression of the GATA3 transcription factor and nuclear localization of YAP1 (Figure 31.3). The trophectoderm (TE) mediates implantation of the embryo into the uterine wall and will give rise to the placenta. Inhibiting PLC or aPKC signalling impairs the initiation of TE specification and the expansion of the first cavity to form a blastocyst [24, 25].

Day 5–6: Blastocyst

The human pre-implantation blastocyst is a hollow ball of cells consisting of a small cluster of tightly packed pluripotent cells (the inner cell mass, ICM) located at one side of the cavity, surrounded by larger TE cells forming the external surface. These first two cell lineages can be distinguished based on differential expression of transcription factors (Figure 31.4).

ICM cells express OCT4 and either NANOG or variable levels of GATA6 and will give rise to the embryonic tissues [4]. The extraembryonic TE cells express GATA3, variable levels of CDX2, and low levels of OCT4 and GATA6. Once the blastocyst has fully expanded, it will hatch from the zona pellucida and is ready for implantation. The recent development of culture systems that facilitate *in vitro* attachment of human embryos is enabling the first in-depth investigations of peri-implantation development and revealing critical processes driving this previously intractable developmental stage (Figure 31.5).

Day 7–8: Epiblast and hypoblast segregation

Failure of the embryo to implant into the uterus is a major cause of early pregnancy loss and a critical barrier that must be overcome for successful ART [26, 27]. Implantation requires the blastocyst to adhere to the epithelial layer of the endometrium and invade into the endometrial stroma under the epithelium. Here it

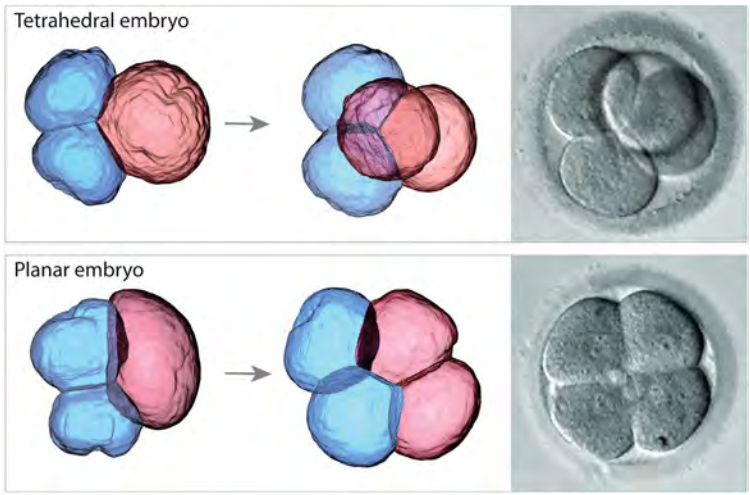

FIGURE 31.2 Different cleavage orientations produce tetrahedral or planar embryos. Images of human 4-cell stage embryos on day 2 of pre-implantation development. (Adapted with permission from [17].)

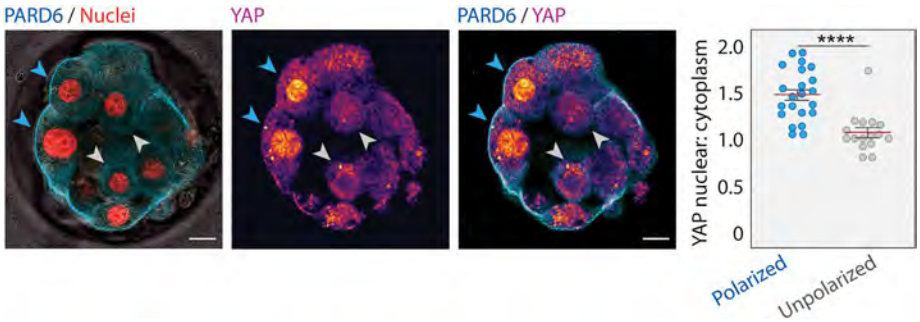

FIGURE 31.3 Human embryos establish apical-basal polarity on day 4. Embryos immunolabelled with PARD6 and YAP1 and stained with DAPI to label nuclei. Blue arrowheads indicate outer cells, white arrowheads indicate inner cells. Graph shows quantification of the nucleus to cytoplasm ratio of YAP1. ****$p < 0.0001$, Mann–Whitney test. (Adapted from [24] under Open Access.)

must penetrate and remodel maternal blood vessels to establish a blood flow for subsequent development.

Addition of blastocysts to a relatively simple 2D co-culture system comprised of a monolayer of Ishikawa cells derived from an endometrial adenocarcinoma demonstrated that most, if not all, good-quality embryos will initiate attachment with receptive luminal epithelial cells [28]. The majority of human embryos attach at the polar TE, which is the region of TE that surrounds the ICM [4, 28–30]. This initial attachment is likely mediated by cell adhesion molecules shown to be upregulated in the human blastocyst such as integrin αvβ3, thrombospondin 1, and laminin α3 [28, 31].

As the embryo undergoes attachment, it flattens and begins a cellular reorganization that segregates the embryonic and extraembryonic cells [3, 4]. The embryonic epiblast cells are characterized by OCT4 expression and will give rise to the fetus. On days 7–8, the epiblast consists of a cluster of OCT4-positive cells surrounded by GATA6-positive extra-embryonic hypoblast cells (primitive endoderm), which will form the yolk sac (Figure 31.5). *In vitro* culture of embryos in atmospheric air (21% O_2) conditions demonstrated improved preservation of epiblast cells and reduced cell death [3]. However, in IVF clinics, human pre-implantation embryos are increasingly cultured in hypoxic conditions (5% O_2) as this replicates the oxygen concentration in the oviduct and uterus and is proposed to favour embryo survival [32, 33]. Given these conflicting findings, it is of utmost importance that the molecular effects of oxygen concentration on epiblast development and embryo survival be carefully evaluated as this a parameter that may be easily controlled for the improvement of IVF outcomes.

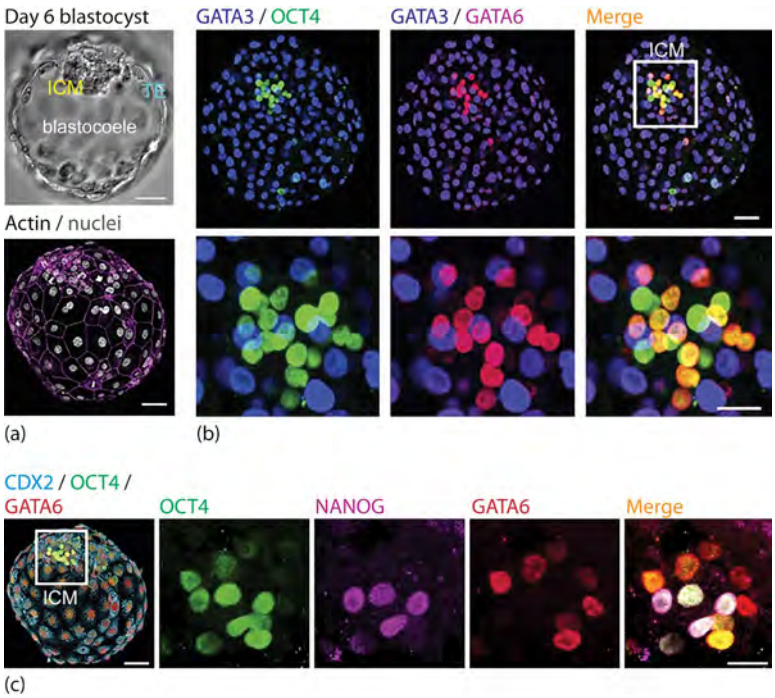

FIGURE 31.4 Establishment of the first two cell lineages in the day 6 human embryo. (a) DIC image (above) and 3D projection of day 6 blastocyst stained with Phalloidin to label actin and DAPI to label nuclei (below). Scale bar 100 µm. (b) and (c) 3D projections of day 6 blastocysts immunolabelled with markers for ICM and TE. (Adapted with permission from [4].)

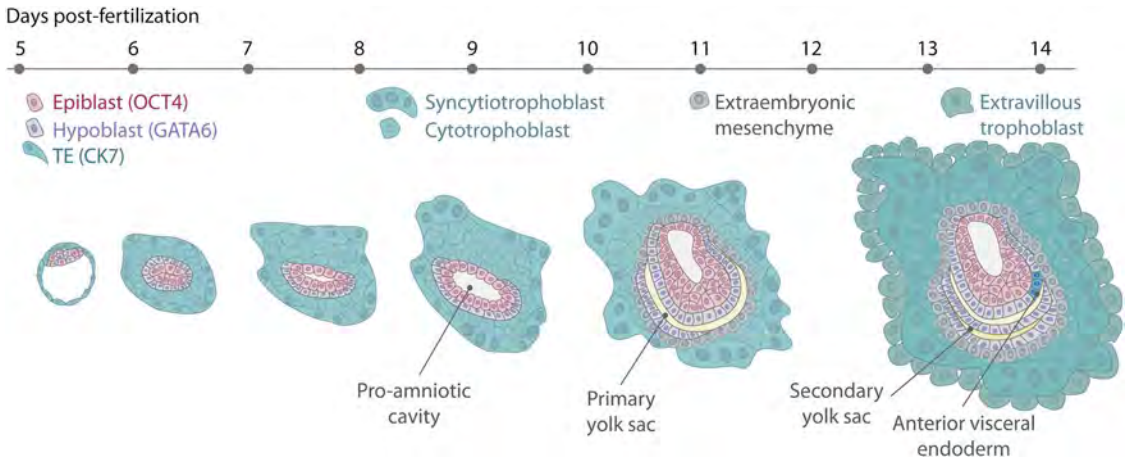

FIGURE 31.5 Schematic of *in vitro* implanted human embryo development.

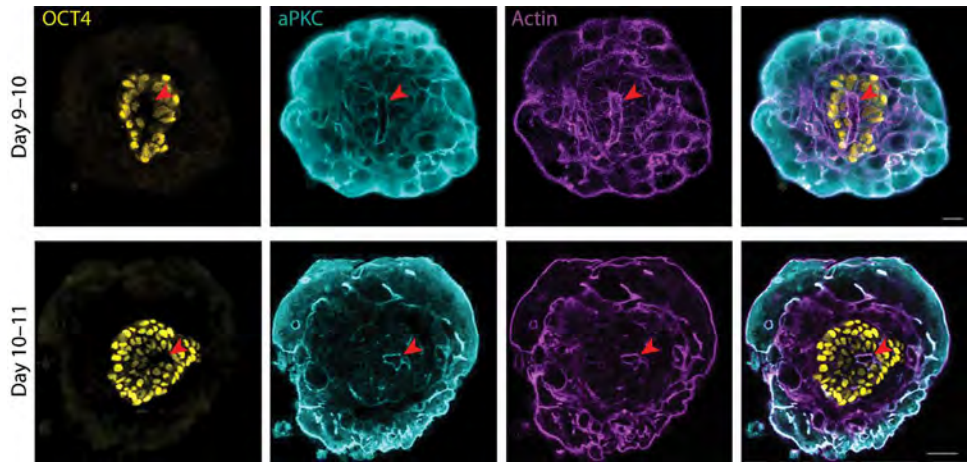

FIGURE 31.6 Formation of the pro-amniotic cavity in cultured human embryos. Confocal Z-sections through the centre of human embryos at different stages immunolabelled for OCT4 and aPKC and stained with Phalloidin to label actin. The arrowhead indicates the presence of a lumen. Scale bar, 20 µm. (Adapted with permission from [3].)

Day 8–10: Epiblast polarization and formation of pro-amniotic cavity and primary yolk sac

From days 8 to 10 *in vitro*, the OCT4-expressing epiblast cells become radially organized and establish apical-basal polarity [3, 5]. Actomyosin and the main kinase of the apical PAR polarity complex, aPKC, become progressively restricted to the apical domain of the cells, whereas integrin β1 is confined to the basolateral domain [3, 34]. A subset of the epiblast cells displays apical localization of the lumenogenesis component, PODXL, and a small lumen lined with actin cytoskeleton forms, indicating the onset of pro-amniotic cavity formation (Figure 31.6) [4, 35].

Concurrently, on days 8–9 the hypoblast cells become localized to one side of the epiblast and an increasingly restricted subset of cells express CER1 and LEFTY1, antagonists of WNT, BMP and NODAL signalling pathways [3, 5, 36]. This population of cells may serve as a signalling centre to initiate patterning of the anterior-posterior axis of the embryo before gastrulation. Adjacent to the hypoblast cells, the putative yolk sac cavity forms (Figure 31.7)

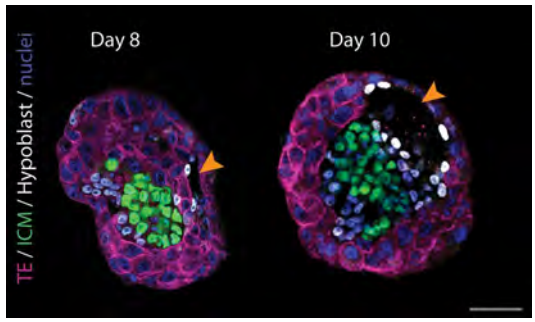

FIGURE 31.7 Development of the primary yolk sac from day 8–10 in a human blastocyst. Confocal Z-section through the middle of an embryo immunolabelled for ICM (OCT4, green), hypoblast (GATA6, grey) and TE (CK7, magenta). Arrowhead indicates developing primary yolk sac. Scale bar, 50 µm. (Adapted with permission from [5].)

and is lined by a novel cell type expressing CDX2 and low levels of GATA6 and OCT4, recently described as yolk sac TE [4].

Trophoblast differentiation

On the surface of the embryo, the TE cells are polarized with apical localization of PAR6, cytokeratin 7 (CK7), and actin [3, 5]. Beginning at day 8, two subpopulations of TE cells emerge (Figure 31.8). TE cells closest to the epiblast and hypoblast retain a single nucleus and likely correspond to the cytotrophoblast (CTB) lineage. However, TE cells in the periphery of the embryo begin to express human chorionic gonadotropin (HCG) and fuse to become multinucleated, a feature of the syncytiotrophoblast (STB) lineage (Figure 31.8a) [3, 4]. Expression of HCG is important for the formation of the STB, modulation of endometrial receptivity at the implantation site and subsequent development of umbilical circulation [37]. Low levels of HCG are associated with recurrent miscarriage and non-viable pregnancies following ART [38, 39], demonstrating the vital role of trophoblast differentiation and sufficient HCG expression in establishing and maintaining pregnancy.

Recent work studying whole chromosome aneuploidies in *in vitro* cultured human embryos revealed that embryos with trisomy 16 or monosomy 21 are smaller due to underdevelopment of their trophoblast cells (Figure 31.8c) [41]. In trisomy 16 embryos, the trophoblast hypo-proliferation was proposed to result from overexpression of the E-CADHERIN gene, which is located on chromosome 16 and promotes cell cycle arrest and trophoblast differentiation. Indeed, immunofluorescence and confocal

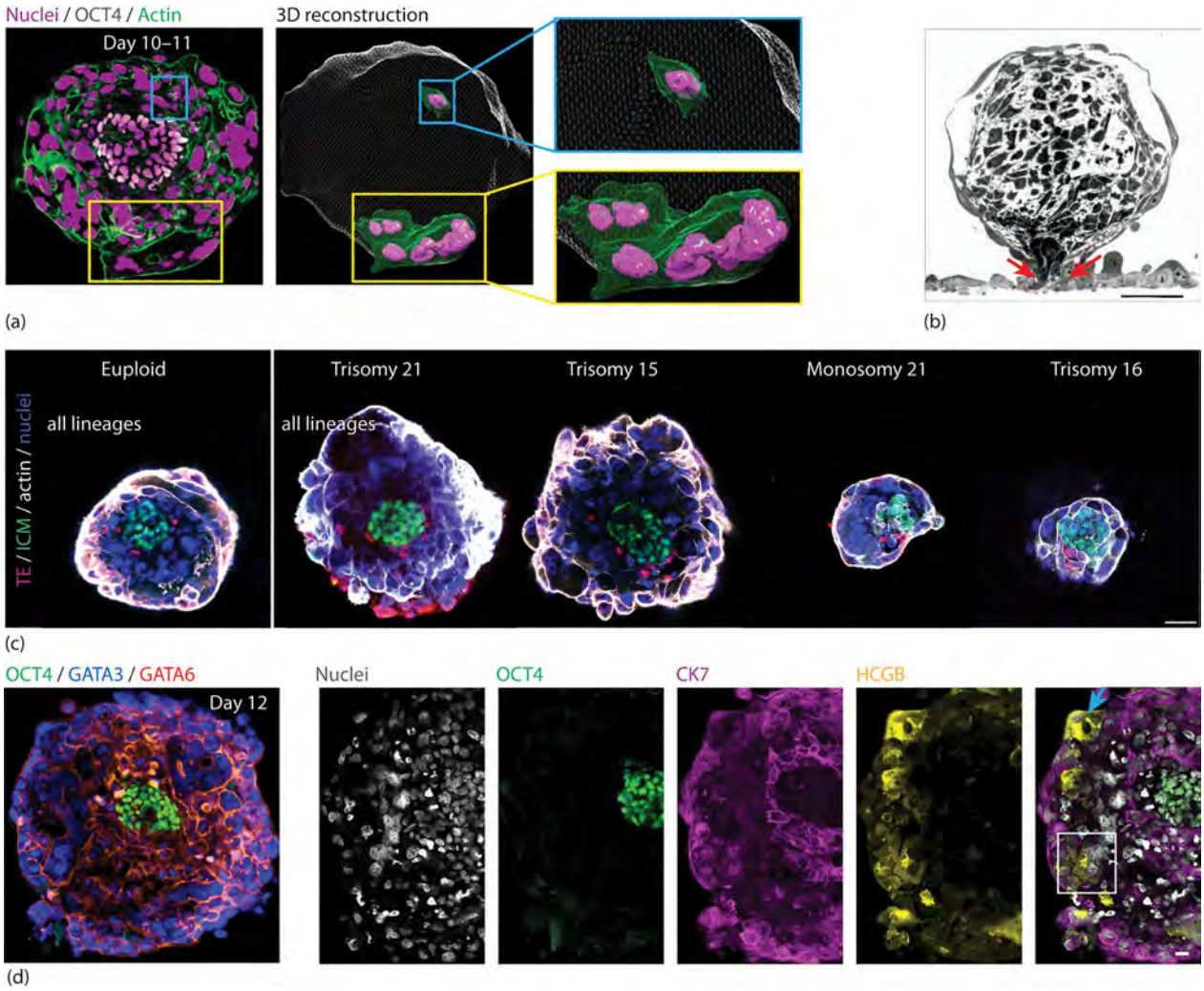

FIGURE 31.8 Trophoblast differentiation in implanting human embryos. (a) 3D reconstruction of the cellular and nuclear shape of representative trophectoderm cells. Note that cells near the epiblast have a single nucleus, whereas cells in the periphery of the embryo are multinucleated. (b) Light micrograph of human blastocyst adhering to cultured endometrial cells and forming a trophoblastic penetration cone (arrows). The penetration cone is occupied by cells from the inner cell mass that almost fill the blastocyst cavity. (c) Day 9 human embryos with whole chromosome aneuploidies. Embryos are immunolabelled to show TE (GATA6, magenta) and ICM (OCT4, green) and stained for actin (Phalloidin, grey) and nuclei (DAPI, blue). Monosomy 21 and trisomy 16 embryos are small due to a lack of trophoblast cells. (d) Day 12 human blastocyst showing differentiation of syncytiotrophoblast (CK7, HCGB) lineage. The blue arrow indicates an example of nascent lacuna, typical of STB cells; the box indicates an example of multinucleated cells characteristic of STB lineage progression. Scale bar, 20 µm. ([a] Adapted with permission from [3]; [b] Adapted with permission from [40]; [c] Adapted from [4] under Open Access; [d] Adapted with permission from [4].)

imaging of trisomy 16 embryos demonstrated increased levels of E-CADHERIN protein and the trophoblast differentiation marker SDC-1, combined with lower numbers of mitotic trophoblast cells. These findings suggest a potential explanation for the intrauterine growth restriction and pre-eclampsia often observed in cases of placental trisomy 16 [41].

At the implantation site, the STB cells are highly invasive, forming contacts with uterine endometrial cells and pushing in between them [1, 29, 40, 42]. As more STB cells penetrate the endometrial layer, they can form a cone which contains fully surrounded epiblast cells (Figure 31.8b) [40]. The endothelial cells are displaced and accumulate around the implantation site. Invasion of the trophoblast cells is proposed to exert pulling and stretching forces on the epiblast, which may contribute to its transformation into a bilaminar disc-like structure at this stage [43].

The STB is also characterized by the formation of lacunae: interconnected spaces that will contain maternal blood once the STB cells breach the maternal vessels (Figure 31.8d) [29, 44]. This lays the foundation for the maternal-utero circulatory system, which will eventually supply the fetus with sufficient blood flow to support development through the second and third trimesters. The CTB and STB cells continue to reorganize into concentric rings from days 9 to 12 until the STB forms the outer trophoblast layer and the CTB is restricted to the areas adjacent to the epiblast and developing yolk sac [3, 4, 44].

Day 10–14

Careful analysis of *in vitro*-attached blastocysts revealed that from day 10, the epiblast cells appear to segregate into two subpopulations [3]. Epiblast cells adjacent to the hypoblast acquire a columnar morphology, express E-CADHERIN adhesion proteins at the cell membrane, and become the bilaminar epiblast disc [3, 5]. However, the epiblast cells which are in contact with the CTB become flat and squamous, likely representing the prospective amniotic epithelium (AME). Expansion of the amniotic cavity separates the epiblast disc from the AME [5]. By day 11 of development, the primary yolk sac is fully formed [1, 3, 5]. *In vitro* 3D culture of human embryos up to the 14-day limit established by internationally recognized guidelines [45] allowed the development of the secondary yolk sac and the initiation of gene expression that defines the anterior-posterior axis of the embryo (LEFTY) and early hallmarks of the primitive streak (N-CADHERIN and T, Figure 31.9) [5].

Remarkably, the human embryo can direct all these lineage specification, cellular reorganization, and early tissue morphogenesis events autonomously without input from any maternal tissues [3, 4]. *In vitro* culture systems that support early human development are enabling an unprecedented view of these early processes, and the insights they yield offer hope for the improvement of embryo culture and selection for ART.

Alternative microscopy approaches for non-invasive embryo imaging

The clinical benefits to both mother and child of transferring a single embryo during IVF are now commonly accepted [46–48], placing increasing importance on the ability to select the embryo with the most developmental potential for transfer. Traditionally, embryos have been selected based on their morphological features, making microscopy an indispensable tool for ART [49]. Despite the recent advent of time-lapse incubation systems which take digital images at regular intervals to enable continuous monitoring of embryo development, static morphological assessment remains the standard approach for embryo selection worldwide. Typically, embryologists remove the embryo from the incubator at specific timepoints and examine it on a microscope. A score is assigned based on morphological features such as blastomere number, fragmentation and symmetry and the quality of the ICM and TE [50]. Although continuous embryo monitoring enables morphokinetic analysis, the higher cost of time-lapse incubation systems remains a significant barrier in many IVF clinics and it is not yet clear whether the technology improves implantation rates [51, 52].

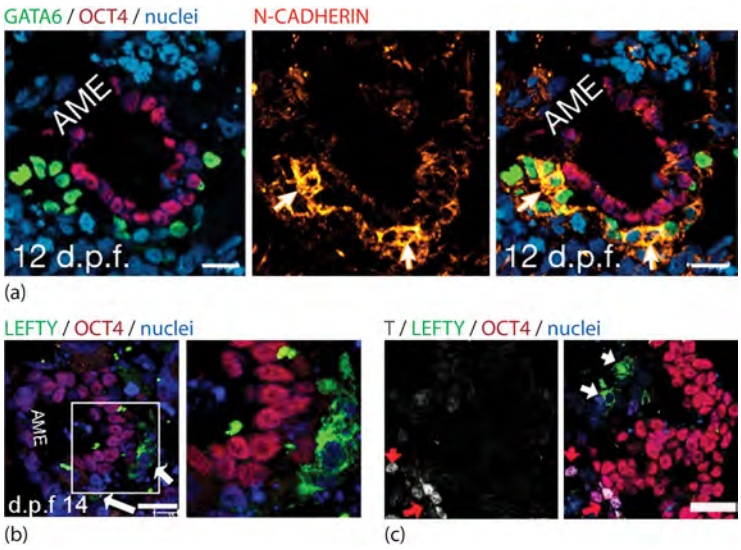

FIGURE 31.9 Expression of primitive streak markers and anterior-posterior polarity in human embryos from days 12–14. (a) Immunolabelling of day 12 embryo for ICM (OCT4, red), hypoblast (GATA6, green), and mesenchymal marker (N-CADHERIN, orange). (b) and (c) Anterior marker LEFTY (green) and an early primitive streak marker (T, grey) are expressed in a day-14 embryo. *Abbreviation*: AME, amniotic epithelium. Scale bar, 50 µm. (Adapted with permission from [5]).

Polarization microscopy

Standard laboratory microscopes are equipped with brightfield illumination which is excellent for visualizing stained specimens but resolves few details in unstained live cells. To improve imaging of live specimens, modern microscopes often have specialized objectives and condensers, or light polarizers to provide phase contrast that allows far greater resolution of subcellular features. Polarization microscopy has been of particular use in embryology due to its ability to significantly increase the image quality of thick unlabelled specimens (Figure 31.10) [53, 54]. This technique images macromolecular structures based on their birefringence; a unique optical property whereby light entering a sample containing highly ordered molecules is refracted as two light components with differing phase. Birefringence materials are characterized by having two orthogonal optical axes, with a different index of refraction along each axis. Light beams parallel to one of the optical axes travel at a different speed through the sample than does light polarized parallel to the orthogonal axis. As a result, these two light components, which were in phase before they entered the sample, are retarded, and exit the sample out of phase. Measuring this differential retardation quantifies the magnitude and orientation of molecular order in the specimen.

Polarizing microscopy made it possible to image the mitotic spindles in unlabelled living cells due to the array of aligned spindle microtubules (Figure 31.10) [56–58]. Subsequent improvements include adding electro-optical modulators, employing circularly polarized light, and exploiting the angle dependence of birefringence to visualize other cytoskeletal elements in living cells, including stress fibres and vesicular structures travelling along the cytoskeleton [60, 62, 65, 69].

Two structures in the mammalian egg that exhibit molecular order when imaged with polarized optics are the meiotic spindle [63] and zona pellucida [61]. Prior to the introduction of polarization microscopy, it was extremely difficult to visualize the spindle in live human oocytes. Using polarization microscopy, it became possible to examine spindle dynamics, detect spindle morphology, predict chromosome misalignment, monitor thermal control, and perform spindle transfer [70–73]. Numerous studies have investigated whether the presence of a spindle in human oocytes is associated with improved ART outcomes. Although the results of these studies are sometimes contradictory (reviewed in [74]), a meta-analysis of 10 trials determined that oocytes with a spindle detectable by polarization microscopy show higher rates of fertilization and faster rates of cleavage and embryo development up to the blastocyst stage [75].

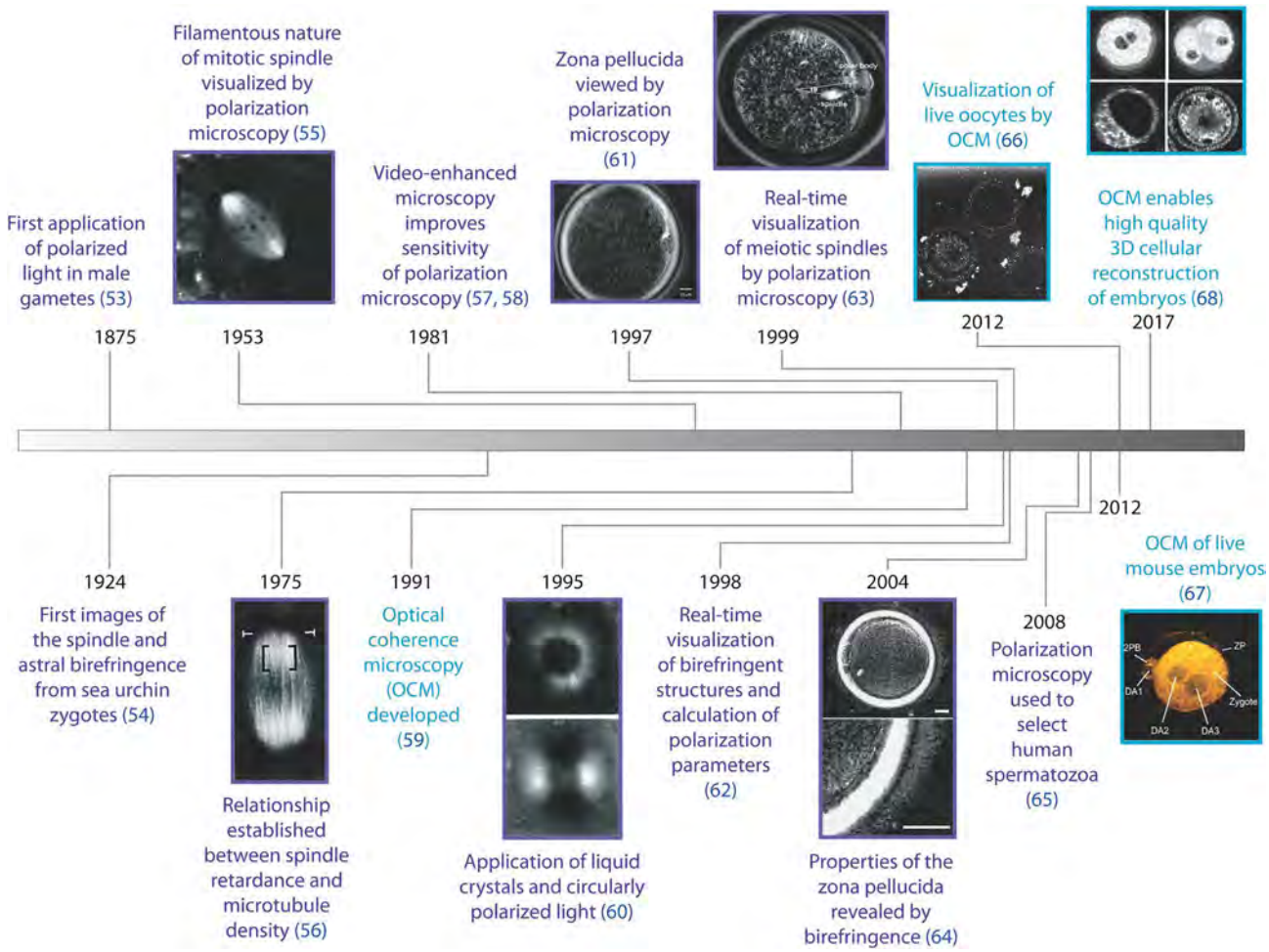

FIGURE 31.10 Timeline of alternative approaches for non-invasive label-free live imaging. (Images reproduced with permission from [55, 56, 60, 61, 63, 64, 66] (© The Optical Society), and [67, 68]).

Characterization of the architecture of the zona pellucida of human oocytes with polarization microscopy revealed an association between the birefringence of the inner layer and the developmental potential of an oocyte [76]. Subsequent studies confirmed an association between the birefringence of the zona pellucida and blastocyst formation, implantation, and pregnancy rates [77–79], although the underlying mechanisms remain to be determined.

Optical coherence microscopy (OCM)

OCM is a relatively new non-invasive technology for label-free imaging which generates 3D reconstructions based on intrinsic contrasting of back-scattered coherent light [59]. OCM has proven to be a very useful technique for embryonic developmental imaging particularly due to non-invasive depth-resolved imaging, rapid acquisition speed, and high spatial resolution. It has been named the "optical biopsy" due to the similarities between cross-sectional OCM images of different embryos and their histological sections [80]. Moreover, OCM can distinguish between normal and abnormal embryonic morphology [81].

In mammalian embryos, OCM can provide images of critical intracellular organelles like nuclei and nucleoli, metaphase spindles, networks of endoplasmic reticulum and mitochondria and, most importantly, may be used to monitor and quantitatively analyse their dynamic behaviour and evolution over time (Figure 31.10) [68]. Although OCM has yet to be translated into the IVF clinic, its capacity for high-resolution label-free imaging and its rapid uptake in other fields of medicine such as ophthalmology, suggest this technology may become an invaluable tool to both further our understanding of human embryo development and improve assisted reproductive outcomes.

Artificial intelligence for embryo selection

In recent years, there has been an increasing focus on using artificial intelligence (AI) to identify the best embryos for transfer. The advantage of this approach is that it can be non-invasive, requiring only a static image of the embryo and removes the potential variability introduced by different embryologists. Generally, this type of analysis requires computational segmentation of various features within an image of an embryo and the application of a model based on machine learning to predict the developmental potential. The models are trained by iterative learning from thousands of example images of embryos with known outcomes and do not depend on the specification of features by humans [82, 83].

Machine learning approaches can rank embryos based on quality at least as well as embryologists and can even outperform highly experienced embryologists in selecting between good quality blastocysts for implantation potential, biopsy, or cryopreservation [84–86]. Models are now being developed with the aim of improving each step of the ART process from fertilization to implantation and clinical pregnancy (reviewed in [87]). Whilst AI has the potential to improve ART outcomes, there are important limitations which must be considered. Machine learning is very sensitive to data quality and most AI systems do not adapt well to data acquired on different imaging systems or changes in imaging parameters. Indeed, embryo scores have been shown to be affected by the imaging magnification and the focal plane of the image capture [88]. This poses problems when attempting to use AI with data acquired on various systems at different clinics. Very few clinics have the same expensive imaging systems used to produce the training datasets. One potential solution is to retrain the algorithm using lower quality data acquired on in-house systems which may include inexpensive portable cameras or even smartphone-based systems [89]. In addition, training data sets often contain images of embryos that failed to implant. However, it is not possible to know if this failure is due to a problem with the embryo or adverse maternal factors, making the data less reliable.

Given the critical role of the maternal uterine environment in the establishment of a viable pregnancy, AI prediction of implantation will always be limited. Nevertheless, AI analysis of embryo images can improve consistency in embryo selection and while it is still a long way from replacing embryologists, AI is a useful tool to enhance the performance of trained embryologists [90].

Conclusion

Since the first staging of human embryos by Franklin P. Mall in 1914, static images have provided a wealth of information about early human development. Recent advances in *in vitro* culture and implantation of human embryos are providing an unprecedented view of the cellular and molecular events directing early embryogenesis. Coupling these approaches with new imaging technologies and advances in computational image analysis will yield new insights that could improve human embryo culture and selection for assisted reproduction.

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