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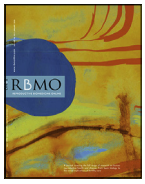

ARTICLE

‘There is only one thing that is truly important in an IVF laboratory: everything’ Cairo Consensus Guidelines on IVF Culture Conditions

BIOGRAPHY

Participants and speakers at the consensus meeting. Standing from left to right: Jacques Cohen, co-convener and corresponding author, USA; Don Rieger, Canada; Sebastiaan Mastenbroek, the Netherlands; Marius Meintjes, USA; Ronny Janssens, Belgium; James Catt, Australia; Dean Morbeck, New Zealand; David Mortimer, co-convener, Canada; Mohamed Fawzy, program organizer, Egypt. Seated from left to right: Mina Alikani, USA; Sharon Mortimer, writer, Canada; Alison Campbell, UK; Catherine Racowsky, USA; Ragaa Mansour, Egypt. Remote by webinar: Jason Swain, USA.

Cairo 2018 Consensus Group*

KEY MESSAGE

This report presents outcomes from an international expert meeting to establish consensus guidelines on IVF culture. Topics reviewed were: embryo culture; temperature; humidity; gas control, pH; workstations; incubators; micromanipulation; handling and assessment; stasis, composition, supplementation, type of culture and storage; equipment and monitoring. More than 50 consensus guideline points were established.

ABSTRACT

This proceedings report presents the outcomes from an international expert meeting to establish consensus guidelines on IVF culture conditions. Topics reviewed and discussed were: 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 physcochemical 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. More than 50 consensus guideline points were established under these general headings.

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KEYWORDS

Culture conditions
Incubators
IVF
Maintenance
Medium
Quality control

INTRODUCTION

Clinical IVF has had a relatively unrestricted development over the past 40 years, with the result that there is now a plethora of permutations of laboratory culture systems. Some laboratories have retained aspects from the mid-1980s, while others are more likely to embrace change and adopt novel aspects of IVF culture as they are introduced.

Against this background, it is a challenge to identify and define what might constitute 'best practice' in the IVF laboratory. However, there are key physcochemical factors that affect oocytes and embryos in every IVF laboratory: temperature control, maintaining osmolarity and pH, and protection from oxidative stress and toxic substances, such as volatile organic compounds (VOC) (*Mortimer et al., 2018*). The purpose of this consensus workshop, held at the UEARS 2018 conference (17–18 February 2018, Cairo, Egypt) was to define the technical and procedural requirements for an IVF laboratory's culture system while taking these factors into account. The overarching goal of the workshop was to identify how best to operate within a framework of quality and excellence to achieve best practice and to optimize the developmental competency of all gametes received and embryos obtained. The aim was to develop an international expert (TABLE 1) consensus opinion regarding the strengths and weaknesses of various options currently available for equipment and procedures and the criteria by which users can determine fitness for purpose within their own laboratories and operating environments, and to identify areas for priority research to fill knowledge gaps or resolve unknown optimization parameters. It was not the goal to define exactly what should – or should not – be done in the IVF laboratory, as these decisions must be taken in connection with local regulatory and licensing requirements, as well as local availability of products and services.

The structure of the meeting was presentations on the current state-of-the-art aspects of the IVF laboratory culture system, followed by facilitated discussions and the development of a set of guidelines and recommendations for each topic.

WORKSHOP REPORTS

Embryo culture – basic principles and interactions

It is often said that the goal of embryo culture is to replicate the conditions of the maternal reproductive tract. While it is true that the oviduct and uterus provide the best possible culture environment, the reproductive system is extremely complex and is the result of aeons of evolution. Given this complexity, it is unrealistic to suppose that all the diverse interactions between the embryo and the maternal tract will be understood in the near future, and even less realistic to suppose that they can be replicated perfectly *in vitro*. The goal of in-vitro embryo culture is to provide conditions that will lead to the production of embryos that have the same developmental potential as the embryos that develop *in vivo*.

That said, there are at least two facets of the maternal tract that are well recognized and can – and should – be replicated *in vitro*. The first is oxygen tension. Although the exact oxygen tension in the normal human female reproductive tract is not known, it is certain to be well below the ~21% found in ambient air. This has been borne out by the results of perhaps hundreds of studies of the effect of low (5–10%) versus high (ambient) oxygen tension on the development of embryos of every mammalian species studied, including humans. To date, no studies have shown a positive effect of high O_2 on the preimplantation development of human embryos, compared with many that have shown a deleterious effect (see *Gardner, 2016*). Consequently, placing human embryos in a high O_2 environment constitutes exposure to a known embryo toxin, which may be considered unethical.

The second area of concern is the exposure of the embryo to in-vitro conditions with the potential for adverse effects. For instance, it is possible that environmental pollutants, particularly VOCs, may play a role. Although the embryo is not completely protected from VOCs in the maternal tract, the mother's lungs, liver and kidneys do provide considerable filtration and detoxification of VOCs, thus reducing the exposure of the embryo. Conversely, the embryo *in vitro* has no such protective mechanisms, and therefore steps must be taken to actively reduce VOCs in the general laboratory air and within the incubator in particular (discussed in *Mortimer et al., 2018*).

Early approaches to culturing human embryos involved the use of culture media designed for the culture of somatic cells. Many of the components of those media were inappropriate and/or at the wrong concentrations for

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embryo culture. Since then, large strides have been made in the formulations of embryo culture media, beginning with the development of Quinn's Advantage Fertilization (HTF) Medium for culture to the cleavage stages, and then media for culture through to the blastocyst stage. The inclusion of all 20 amino acids has proved to be particularly important, with glutamine being provided via dipeptides with alanine or glycine. Commercial mass-produced and distributed media are manufactured under much more stringent and controlled conditions than could ever be possible in an IVF laboratory, and are subject to rigorous testing and regulatory oversight.

It is, however, important to recognize that the embryo culture medium is but one among perhaps hundreds of factors in the IVF laboratory that might affect the outcome of any given cycle (*Poole et al., 2012*). This includes every pipette tip, holding tube and culture dish that is in contact with any of several media. Laboratory apparatus, gases and procedures could all have effects independent of the culture medium: the fact that a developmental anomaly or failure appears while the embryo is in a culture medium does not mean that the medium is responsible for it. For example, the presence of vacuoles in the sperm head has been shown to have no effect on development through the cleavage stages, but to significantly decrease the rate of development to the blastocyst stage (*Gardner and Kelley, 2017*; *Vanderzwalmen et al., 2008*). There is only one thing that is truly important in an IVF laboratory: everything.

Some consideration must also be given to the measures of success of embryo culture. Although in-vitro evaluation of development can be informative, the evaluation must ultimately include measures of in-vivo development after transfer. Traditionally, this has centred on pregnancy, clinical pregnancy, ongoing pregnancy, miscarriage, birth and live birth rates. None of these are valid measures of embryo viability, because none of them account for the number of embryos transferred. For example, if two embryos are transferred, and one embryo fails to implant in each recipient, it would have absolutely no effect on the clinical pregnancy rate. Clearly, any measure that cannot distinguish between 0% and 50% embryonic loss is of no use in evaluating embryo viability. The use of cumulative pregnancy rate is also inappropriate, because it does not account for the number of transfers. In effect, transfers of viable embryos could be interspersed with the transfer of degenerate embryos, aneuploid embryos, grains of sand, air bubbles, or nothing at all, and it would have absolutely no effect on the cumulative pregnancy rate. Conversely, implantation rate, sustained implantation rate, fetal loss rate, and babies born per embryo transferred all account for the number of embryos transferred. It has been suggested that implantation rate is statistically biased (*Griesinger, 2016*), but this is easily circumvented by doing only single embryo transfer.

Without question, the trend in ART is towards embryo transfer at the blastocyst stage (Days 5–7), and rapidly diminishing transfer at the cleavage stage (Days 2–3). The transfer of cleavage-stage embryos to the uterus is non-physiological, particularly in the presence of elevated oestrogen levels following ovarian stimulation. In addition, culture to the blastocyst stage provides the opportunity for more and better evaluations of development, including morphological, morphokinetic, metabolic and cytogenetic aspects. As a result, the implantation rate for blastocyst transfer is significantly greater than for cleavage-stage transfer for all maternal age groups (*SART National Summary, 2015*–2017). Blastocyst transfer provides better synchrony between the embryo and uterus than does cleavage-stage transfer, so it would seem difficult to justify the transfer of cleavage-stage embryos. In clinics where the current blastocyst development rate might not support this approach, there should be a focus on improving this outcome measure.

However, even with blastocyst transfer, the high concentrations of oestrogen and progesterone resulting from ovarian stimulation produce a less than optimal uterine environment. The logical approach to eliminating this problem is to freeze all of the blastocysts and then transfer them (singly) in one or more subsequent unstimulated cycles (1985c). The implantation rate for the transfer of frozen–thawed blastocysts is significantly greater than for the transfer of fresh blastocysts for all maternal age groups (*SART National Summary, 2016 and 2017*). Moreover, the incidence of small for gestational age (SGA) babies is significantly decreased with frozen embryo transfer, while the incidence of large for gestational age (LGA) babies is significantly increased (*Luke et al., 2017*; *Wennerholm et al., 2013*). It is important to note that SGA is associated with increased overall rates of neonatal, infant and later death but there is no association between LGA and overall rates of neonatal, infant or later death (*Wennerström et al., 2015*; *Xu et al., 2010*).

The incidence of aneuploidy is high, even among morphologically high-quality blastocysts. Such aneuploid embryos have a greatly reduced chance of implanting or of resulting in a live birth. The implantation rate for the transfer of blastocysts identified as euploid by preimplantation genetic testing for aneuploidy (PGT-A) is significantly greater than that for untested blastocysts, for all maternal age groups (*SART National Summary, 2016*). Clinically, the use of PGT-A may be a better standard of care, although accepted evidence is still missing. In terms of experimental design, the presence of aneuploid embryos is a significant source of experimental noise and markedly increases the number of transfers, time to pregnancy, patient distress and cost of hypothesis testing for other aspects of ART.

In conclusion, the basic requirements for embryo culture seem to be:

- complete culture medium (all 20 amino acids);
- attention to all laboratory and clinical factors;
- low O_2 environment;
- low/no VOC environment;
- culture to Day 5(+);
- blastocyst vitrification (freeze-all); and
- optional transfer of single frozen–thawed (euploid) blastocysts.

Temperature in the IVF laboratory

Using and maintaining the appropriate temperature during cell handling and culture is a critical component of optimizing an IVF culture system because improper temperatures can compromise cell function and development and hence reduce IVF outcomes. However, the ideal temperature to use *in vitro* remains an area of debate.

While recreating *in vitro* the conditions that exist *in vivo* to accommodate the embryo's physiological needs is a logical

approach (*Gardner and Leese, 1990*; *Gardner et al., 1996*, *2002*; *Lane and Gardner, 2000a*), there is a risk of errors in the in-vivo measurements (*Ng et al., 2018*). Additionally, the in-vitro environment might necessitate unique considerations and require departure from in-vivo parameters to ensure optimal gamete handling and embryo development *in vitro*.

A core body temperature in humans of 37°C is widely accepted but this value might not be entirely accurate because body temperature is actually a range (*Dewdney, 1993*; *Elert, 2015*; *Mackowiak et al., 1992*; *Sund-Levander et al., 2002*), and can be affected by how the measurement is taken (oral versus axillary versus rectal versus tympanic), the time of day, the individual being measured, and their sex (*Elert, 2015*; *Sund-Levander et al., 2002*). Hence this could bring into question whether the current IVF laboratory standard of 37.0°C is optimal.

Testicular temperature of most mammals, including man, is 2–4°C lower than core body temperature, and is necessary for normal spermatogenesis and spermiogenesis (*Durairajanayagam et al., 2015*). Similarly, the temperature of the female reproductive tract might also be lower than core body temperature: a temperature gradient exists in the oviduct of pigs and rabbits, with the isthmus being 0.2–1.6°C cooler than the ampulla (*Hunter, 2012*; *Hunter and Nichol, 1986*; *Hunter et al., 2006*). Mature follicles in rabbit, pigs and cows are 1.3–1.7°C cooler than the ovarian stroma (*Grinsted et al., 1980*; *Hunter, 2012*; *Hunter and Einer-Jensen, 2005*; *Hunter et al., 1997*, *2000*, *2006*) and ~2.3°C cooler than stroma in human ovaries (*Grinsted et al., 1985*). Such observations, as well as a lack of reliable information on the uterine temperature at the time of implantation, have led to the proposition that human embryo development might benefit from a temperature lower than core body temperature (*Leese et al., 2008*).

Interestingly, apparent detrimental effects of short periods of reduced temperature on the oocyte has led to most practices in the IVF laboratory being performed at a strict 37°C. Prior studies, often using compromised starting material, have shown that: (i) cooling mouse oocytes to near 4°C resulted in zona hardening, probably as a result of premature cortical granule exocytosis; (ii) transient cooling of human oocytes to room temperature caused disorganized spindles, with some oocytes showing displaced chromosomes, and only some of the oocytes showing reorganization of the meiotic spindle upon rewarming; and (iii) the severity of defects increased with lower temperature and longer time (*Johnson et al., 1988*; *Pickering and Johnson, 1987*; *Pickering et al., 1990*). Reassembly of the human oocyte meiotic spindle observed using polarized light microscopy was retarded at room temperatures but was not different between 33°C or 37°C (*Wang et al., 2001*). Additionally, performing intracytoplasmic sperm injection (ICSI) at 37°C, rather than at 33°C or 34°C, resulted in a significantly higher number of oocytes with a visible meiotic spindle, as well as significantly higher fertilization and pregnancy rates (*Wang et al., 2002*).

It should be noted, however, that not all temperature excursions below 37°C are necessarily harmful to the oocyte. One report indicated that it was not necessary to perform ICSI at 37°C for successful outcomes to be achieved (*Atiee et al., 1995*), but did not address the achievement of optimum clinical outcomes. Many successful cryopreservation protocols require equilibration in cryoprotectant solutions at room temperature for several minutes – but these are special circumstances and are associated with careful thawing and warming protocols.

Temperature sensitivity of preimplantation embryos is less well studied. While one study on incubator performance reported an increased pregnancy rate from incubators with a temperature of 36.96 ± 0.13°C compared with those with a temperature of 37.03 ± 0.13°C (*Higdon et al., 2008*), another that reported slight temperature gradients of up to 0.3°C between shelves in culture incubators found no significant impact on clinical outcomes (*Walker et al., 2013*). Of importance, accuracy of these minor temperature variances is less than the uncertainty of measurement of most thermometers. However, a prospective randomized controlled trial (RCT) using patient zygotes cultured for 5–6 days at either 37°C or 36°C demonstrated that culture at 37°C yielded higher average cell numbers on Day 3 and higher blastocyst formation and more useable blastocysts compared with 36°C (*Hong et al., 2014*). Similarly, another prospective study also suggested 37°C was superior for extended embryo culture compared with 36.5°C (*Fawzy et al., 2018*). Thus, prolonged differences in culture temperature appear to impact embryo development.

Current IVF culture media have been formulated for use at ~37°C and it is perceived that alteration of culture temperature could affect cell metabolism as well as medium pH, especially in media containing HEPES or MOPS (*Jeyendran and Graham, 1982*; *Swain, 2010*, *2011*, *2012a*,*b*; *Will et al., 2011*). However, it should be noted that ΔpH/Δ°C is only –0.0096 for 25 mmol/l bicarbonate, hence medium pH barely differs between 36°C and 38°C. Nonetheless, a general change in culture temperature could necessitate medium formulation changes and adjustments to the culture atmosphere.

Although the optimal temperature for culturing embryos might not be established unequivocally, it is critical that a narrow acceptable temperature range be strictly adhered to in order to reduce culture system variability. Therefore, the laboratory should be kept at a temperature that is comfortable for the staff, and equipment set-points should meet the temperature needs of gametes and embryos. This requires the use of calibrated thermometers and proper measurements, i.e. of the culture medium surrounding the oocytes or embryos rather than the surface temperature of equipment (*Cooke et al., 2002*; *Lane et al., 2008*). Regardless, the embryo may experience temperature variations during location changes. Measurements should be performed on a daily basis at a minimum, usually taken in the morning prior to equipment use. Temperature dataloggers are useful in identifying temperature fluctuations during the day and during equipment use. In practical terms, a tightly controlled temperature range is required for each piece of warmed laboratory equipment, although a slightly lower temperature might be safer than a higher temperature.

Humidity in culture

Preimplantation embryos require consistent culture conditions to reduce environmental stress and optimize their development. These conditions include the culture medium and its osmolality and pH (which also depends on the

gas phase composition), temperature, humidity and air quality, all of which are crucial (*Pool et al., 2012*). While there are recommended values or ranges for most of these conditions (*Wale and Gardner, 2016*), the optimum level of incubator humidity has yet to be determined.

Originally, CO_2 regulation in incubators was mediated using thermal conductivity sensors that relied on a humidified atmosphere for correct operation, but the more recent shift to infrared sensors has removed this requirement (*Swain, 2014*). Further, concerns have been expressed that humidity could increase the likelihood of microorganism overgrowth in the incubator, negatively impacting embryo development (*Geraghty et al., 2014*), and it has been postulated that the use of an oil overlay in the culture dishes would prevent evaporation from the culture medium in dry culture, thereby protecting against the risk of increased osmolality. Together, these points have encouraged a shift towards embryo culture in a non-humidified environment.

Two recent studies have demonstrated that an oil overlay is not sufficient to protect against osmolality changes during dry incubation. *Fawzy et al. (2017a)* reported that introducing humidity into a normally dry incubator resulted in improved embryo development and ongoing pregnancy rates. *Albert et al. (2018)* used time-lapse imaging to evaluate the effect of removing humidification from a normally humidified incubator on embryo development, and also reported better outcomes with humidification. It must also be noted that 'humid' versus 'dry' is not a binary condition, and aspects of relative volumes and both surface and interface areas are likely to influence the precise relationship between a humidified incubator and the actual culture environment experienced by the embryos, and *vice versa*. Effects on osmolarity in non-humidified culture may be less deleterious in culture systems with regular medium renewal (every 48 h) as the shift in osmolality accumulates with time (*Fawzy et al., 2017a*; *Swain, 2018*, *Swain et al., 2016*). The central question is why might evaporation occur and osmolarity increase during dry incubation in oil-based culture? Room humidity may also play a role. Considering satisfactory development and outcomes reported in a host of papers after culture in the EmbryoScope™, which is used in a dry environment without medium changeover, one difference may be the large surface area of microdroplets under oil in flat dishes compared with the vertical tube-like culture in first-generation EmbryoScope™ slides. Mouse embryos cultured in non-humidified EmbryoScope™ slides developed as well or better than those cultured in microdroplets under oil in a humidified incubator using the same media (*Kelley and Gardner, 2017*). Until there is clarity regarding the reasons involved in promoting or inhibiting evaporation through oil, embryologists should consider humidification as an obvious choice for culture, but only if incubator manufacturers recommend the use of humidification.

Carbon dioxide control and medium pH

Carbon dioxide control is paramount for culture media to maintain a physiological pH, but it is not as simple as just adding sodium bicarbonate. The pH of a medium is complex, with interactions between amino acids and other zwitterions and proteins (because of their amino acid composition and bound caprylate). Generally, concentrations of CO_2 between 5 and 6% (at sea level) should result in the correct physiological pHe of 7.2–7.4 (*Swain, 2012b*), where pHe is the extracellular pH of the culture medium (*Bavister, 1995*).

But pHe is not the parameter of real biological significance; that is pHi, the intracellular pH of the cytoplasm, which should be about 0.1 pH units below the pHe because the bovine embryo utilizes this gradient to drive transport mechanisms and offset internal acidification from metabolic processes (*Lane et al., 1999*). The pHi of human oocytes and embryos varies slightly during development but is usually accepted to be 7.12, which means the pHe should be 7.2–7.3 (*Phillips et al., 2000*). This pH is achieved by culturing in an environment where the partial pressure of (pCO_2) is titrated to give the correct pH.

In principle, medium pH should be easy to measure, but in practice it is not. There are three main ways to measure the pH of culture media, each with their own pros and cons. A pH meter must have a three-point calibration, ideally inside the incubator. The glass electrode must be kept clean because protein in media will adsorb onto the glass affecting measurement; despite regular cleaning the electrode will still need to be replaced frequently. Blood gas analysers, whether static or portable, can measure pH as well as pCO_2 and pO_2. The results can be accurate but one must be very careful to ensure that the concentration of CO_2 and O_2 is maintained in the gas phase before the measurement is made. But pH meters and blood gas analysers only give a 'snapshot' of the pH, which might change over time.

A new technology uses fluoroscopy to measure pH continuously. It employs immobilized fluorescent dyes that change colour or intensity with the pH. The sensors with standard solutions are placed inside an incubator and give a continual pH readout. The problem with these sensors is that they rely on a standard that might not reflect the pH of the culture medium.

Control of pH is complex, and CO_2 measurement alone is not a good proxy for pHe in a complex culture medium, where pH should be measured directly. Different types and makes of incubators should be thoroughly validated before use, to ensure that the culture medium is maintained at the desired pHe.

Oxygen tension for embryo culture

Another variable important for embryo development and assisted reproductive outcomes is oxygen tension used within the culture incubators. Two alternate levels of O_2 are commonly used *in vitro*: ambient atmospheric O_2 (nearly 21% at sea level) and 5%, the proposed physiological level (review: *Nielsen and Ali, 2010*). Although O_2 levels in the reproductive tract are reported to be between 2 and 8% (*Fischer and Bavister, 1993*; *Kaufman and Mitchell, 1994*; *Kigawa, 1981*; *Mastroianni and Jones, 1965*; *Yedwab et al., 1976*), questions have been raised about the methods used in these studies, prompting a suggestion that they be re-evaluated (*Ng et al., 2018*).

Numerous studies have demonstrated superiority of 5% O_2 compared with ambient O_2 for human embryo culture. For example, better embryo cleavage and blastocyst development *in vitro* under a 5% O_2 atmosphere have been reported, and these culture conditions led to the first IVF live birth (*Edwards*

et al., 1970; Steptoe et al., 1971; Steptoe and Edwards, 1978). Other studies of human embryo culture have reported that 20% O_2 was inferior to 5% O_2 in terms of clinical pregnancy, live birth and implantation (Bontekoe et al., 2012; Meintjes et al., 2009a; Nastri et al., 2016), but recommended further studies with more stringent design and culture protocols. Time-lapse studies of mouse and human embryo culture have shown a detrimental effect of short exposure to atmospheric O_2 (Kirkegaard et al., 2013; Wale and Gardner, 2010).

Oxygen is considered a secondary stressor in embryo culture as it works with ammonium to disrupt the pathway of glutamine and alanine transamination, leading to abnormal fetal development (Wale and Gardner, 2016). Embryo culture under ambient O_2 has also been reported to increase the production of reactive oxygen species (ROS), which can affect embryonic growth and the resulting offspring (Bedaiwy et al., 2004, 2010; Yang et al., 1998). It can also disturb the transcriptome, proteome, carbohydrate and amino acid metabolism, embryo homeostasis, and the epigenome, including inducing premature X-chromosome inactivation, and differentially affects male and female embryos (Gardner and Lane, 2005; Katz-Jaffe et al., 2005; Lengner et al., 2010; Li et al., 2016; Rinaudo et al., 2006; Wale and Gardner, 2012, 2013).

It has been suggested that culture under an even lower O_2 level (2%) from the morula stage onwards might result in the development of healthier embryos, as the uterine O_2 tension is speculated to be lower than that of the oviduct (Ng et al., 2018), and aerobic glycolysis is more important after genomic activation, increasing the risk of ROS production in an O_2-rich environment (Morin, 2017). Studies directly addressing the question of using lower O_2 levels for human embryo culture, by Kaser et al. (2016) and Yang et al. (2016), have been underpowered, although Fawzy et al. (2017b) cultured human embryos continuously from Day 0 to Day 5 or 6 in either 3.5% or 5.0% O_2 concentration and found that while 3.5% O_2 was associated with significantly higher fertilization and cleavage rates it gave significantly lower blastocyst formation and clinical outcomes parameters.

The optimum O_2 level(s) for embryo culture remains to be determined, with the question of whether embryos need stage-specific O_2 levels remaining unclear. For the present, 5% remains the recommended O_2 tension for human embryo culture *in vitro*. One of the surprising facts has been the reluctance of some laboratories to reinvest in reduced O_2 incubators and to add the extra nitrogen gas phase, also associated with increased costs. Financial considerations are a central issue when suggesting guidelines, but the evidence for improved success after introducing reduced O_2 is apparent and must be the prime consideration.

Workstations – design and engineering

The entire IVF process is governed by the biology of the gametes and embryos and it is expected that the laboratory, its design, equipment and operational systems will provide optimal conditions for them. It is incumbent on the laboratory to protect the gametes and embryos from physiological stress (while embryos are highly adaptable, adaptation costs energy and hence equates to metabolic stress) as well as from adverse external factors. Hence, we must select equipment that has been designed and engineered to maintain the correct biophysical and biochemical conditions for each step of the IVF process, and then calibrate and maintain the equipment to ensure it is fit for purpose. Various types of workstation are required in the IVF laboratory so that key tasks can be performed under the appropriate physcochemical conditions: handling and assessing oocytes and embryos; micromanipulation workstations (see next section); processing oocytes and embryos during cryopreservation and thawing/warming; and preparing dishes.

Handling and assessing oocytes and embryos

Although many IVF laboratories use vertical laminar air flow (VLAF) cabinet-based workstations for many or all aspects of oocyte and embryo handling (horizontal LAF cabinets do not provide any protection to the operator), a critical analysis of their ability to support and maintain the key physcochemical factors that affect oocytes and embryos compared with 'IVF chamber' type workstations reveals substantial weaknesses (see Mortimer and Mortimer, 2015).

Optimizing the use of VLAF cabinets as IVF workstations includes accommodating the following challenges:

- While a VLAF-based workstation has very clean air (up to ISO Class 5), it operates at ambient temperature and the high air flow across the work surface causes a substantial cooling effect. The air flow may also enhance evaporation and hence increase osmolality.
- Integral stereozoom microscopes are set into a heated working surface, which must be calibrated to maintain dish contents or drops at 37°C. This can be tricky because although the observation area (glass plate) is surrounded by a heated metal work surface the glass plate is usually heated only by conduction from the sides, and so is cooler.
- Work surface temperature needs to be calibrated for each type of dish that is to be used because different sized dishes have different air gaps that introduce differential insulation.
- When performing dish temperature calibration should the dish lid be on or off, and should the LAF cabinet be running or not? Many laboratories still turn off the air flow when handling open dishes (especially during an egg search procedure), but if the air flow is off then there is no advantage to using a LAF cabinet. Also note that with the lid off (and no oil, e.g. during egg search) the evaporative cooling effect increases as a function of the square of the dish diameter.

Some IVF laboratories use Class II biohazard hoods for oocyte and embryo handling and assessment. While the rationale for this is clear when working with infectious cases (e.g. HIV and hepatitis), the vast majority of laboratories continue to use VLAF cabinets because they work on pre-screened patients.

It has been established that for culture dishes comprising 50 µl droplets of medium under oil, re-gassing (pH re-equilibration) takes ~20 × longer than de-gassing, which is very fast even with oil in that the pH will be >7.4 in <2 min (Debbie Blake, 1999 unpublished data, see Mortimer and Mortimer, 2016). Hence it is recommended that a culture dish be exposed to air for <2 min. Based on these timings, the use of a gas 'hood' (e.g. a glass funnel through which pre-mixed gas is delivered around the dish) can only minimally extend this working time: medium pH will rise, and embryos may be stressed, when the 'out' time exceeds ~2 min, even with rigorous use of a gas hood.

'IVF chamber' workstations have been used since the earliest days of IVF, when they were built using converted neonatal isolettes with a built-in microscope (*Testart et al., 1982*). The internal atmosphere is room air with temperature, CO_2 and humidity control, with contemporary custom-built models including HEPA filtration and even photocatalytic VOC removal. Some manufacturer's chambers have variants that can enclose an entire ICSI rig, and a very recent model has a dual optical system that combines both a stereozoom microscope and a digital inverted microscope with Hoffman-type optics.

How stable could the culture and handling systems be in an ergonomically designed laboratory based on 'IVF chamber' workstations with humidified bench-top incubators? The process would operate as:

- dish removed from bench-top incubator with lid on, so gas environment is maintained;
- dish transferred immediately (5 s) to the workstation;
- lid removed, same pCO_2 inside the workstation;
- lid replaced when finished, enclosing the controlled pCO_2 atmosphere;
- dish transferred (5 s) back into bench-top incubator; and
- bench-top incubator re-equilibrates its internal gas in 30 s.

In all practical terms this would be effectively 'undisturbed culture'. Achieving this using VLAF cabinets will require high operator skills and speed as well as enhanced workstation calibration and quality control (QC). Such cabinets may be recommended as a backup when central HVAC units are out of service.

Processing oocytes and embryos during cryopreservation

Cryopreservation media for both slow freezing and vitrification techniques are usually zwitterion buffered, and hence will be pH stable under an air atmosphere. Processing usually takes place at ambient temperature, although many protocols start or end at 37°C, which can be easily achieved using a heated surface or stage, and by having a 'holding' bench-top incubator nearby (with pre-mixed gas if necessary).

Typically slow freezing and thawing processing was performed in VLAF cabinet-type workstations, but because of the complications of needing liquid nitrogen immediately adjacent to the microscope, and avoiding cold nitrogen vapour accidentally cooling the solutions in the processing dishes, many embryologists perform vitrification and warming procedures on the open bench using a combination of large area (heated) stages on the stereozoom microscope, warming plates, and having a 'holding' bench-top incubator nearby (with pre-mixed gas if necessary).

Dish preparation

This needs to be performed in a VLAF cabinet to maintain sterility of the dishes, culture media and oil during handling, labelling and preparation of the culture dishes. Even though these tasks are all performed at ambient temperature, speed is of the essence to avoid evaporation from microdrops between them being dispensed and overlaid with oil (*Swain et al., 2012*).

Incubators – maintaining the culture environment

The maintenance of incubator performance for each variable within predetermined acceptable limits is, perhaps, the single most important aspect of running an IVF laboratory. Acceptable performance is established through identification of set-points and tolerances. These benchmarks are met after an incubator is turned on for the first time and stabilized, and then are maintained by constant surveillance of performance through a quality management programme. For all variables impacting incubator operation, including temperature as discussed above, gas phase, pH, humidity and air quality, any correction after disturbance in the environment entails an equilibration phase prior to reaching the set-point, and then stabilization within an acceptable range of tolerance (*Swain, 2010*).

Factors influencing stability of the culture environment

There is a variety of factors that influence stability of the culture environment, not the least of which is the frequency of opening/closing the incubator door (if box incubator) or lid (in the case of bench-top incubators). These openings/closings can be kept to a minimum by ensuring there is an adequate number of culture incubators for a programme's caseload, and by having 'holding' incubators dedicated to non-culture activities such as dish equilibrations, sperm preparations, etc. Importantly, only one patient's gametes/embryos should be cultured in any one incubator compartment (whether this is a box incubator with a split door per shelf, or a bench-top incubator). Such a principle not only reduces door opening/closing but also reduces the likelihood of sample mix-up. Beyond physical perturbation of the incubator environment, other factors influencing stability of the culture environment include the type of culture dish, whether a lid is used over the dish, the type and volume of medium used and the protein supplementation, as well as the presence/absence of an oil overlay. Multiple dishes should be prepared in series and not all at once.

Box versus bench-top/top-load incubators offer varying advantages and disadvantages regarding temperature control and measurement and control of humidity (reviewed in *Swain, 2014*). Moreover, the critical requirement for a stable and pure gaseous environment requires consideration of both the supply and control of gases. There are both advantages and disadvantages to using either pre-mixed gas or a gas that is regulated by the incubator. Regardless of which gas supply is used, in-line filters are recommended for removal of various known toxins including VOC, dust, bacteria, etc., and backup gas supplies should be available for use. Individual incubator outcomes should be monitored using key performance indicators such as blastocyst development and pregnancy rates.

Principles for incubator management

There are two broad principles for incubator management:

1. Preventative maintenance, which should be performed at least annually. The machine should be shut down and, if not an incubator that is sterilizable by elevated dry heat, the chamber must be disassembled according to the manufacturer's recommendations. All components, as well as the interior chamber, should be thoroughly cleaned with a diluted mild soap from IVF-specialized manufacturers, although trace residues may be found even after thorough rinsing with deionized water and sterilization with hydrogen peroxide (Fawzy, unpublished data). Hydrogen peroxide can be used

instead of mild soap as it removes protein and other residues. Successful sterilization can be achieved using both diluted mild soap cleaning and sterilization cycles or hydrogen peroxide by itself. Cleaning of time-lapse chambers needs to be performed with great care and manufacturer's instructions must be followed. Chamber ventilation is recommended before the machine is turned back on for recalibration. Aside from cleaning, the gas sensors should be checked against certified standards and the temperature calibrated against a certified NIST thermometer (USA) or performed by an ISO17025 accredited company. A NIST thermometer should be certified within 1 year of use.

2. Daily operation, which should entail implementation of a robust and verified QC programme (see below), minimization of door/lid openings and electrical stability. The latter is particularly important for time-lapse incubators. All incubators should be connected to generator-protected electrical outlets and/or battery operated backup units as well as an alarm system that is regularly tested for functionality and that sends alarms through a telephone tree of personnel to enable quick response.

General rules for incubator QC

Optimum set-points and tolerances should be established for each culture system because different systems probably have different system-specific requirements. These values should be determined using independent calibrated external NIST devices and not be obtained from digital readouts. Each laboratory should determine the minimum frequency for measuring each variable although it is recommended that critical variables discussed previously, such as temperature, CO_2 and O_2, are measured daily, with pH measurements read a minimum of weekly. Recordings should be captured on P-charts (performance charts) or electronically real-time with dataloggers and performance reviewed daily to verify acceptable performance with corrections made as indicated.

Micromanipulation – maintaining a steady physcochemical environment

Micromanipulation includes all procedures requiring technology-mediated distance-controlled robotic manipulation of gametes or embryos. In short, the systems are designed to eliminate tremor and allow precise manipulation of cells, even at high magnification. Micromanipulation rigs are routinely used in all IVF laboratories during ICSI to improve the chances of fertilization, zona opening for enhancing or facilitating hatching and for biopsy of the trophectoderm, and for blastocoel collapse prior to vitrification. Maintaining stable environmental conditions for gametes and embryos during micromanipulation requires the following considerations:

- maintenance of a steady, optimal temperature, similar to culture conditions as described above;
- limitation of exposure to light;
- vibration reduction to reduce damage to cells and enhance ease of handling;
- maintenance of osmolality similar to controlled culture conditions; and
- maintenance of pH similar to controlled culture conditions.

Despite hundreds of papers on work involving micromanipulation, literature on the rationale and technical requirements for achieving steady conditions during micromanipulation is sparse. Consequently, our understanding of optimization, and even the necessity of steady-state conditions, is rudimentary.

Among the most important criteria for maintaining a steady state during excursions of gametes and embryos outside the incubator is the need to avoid potential problems such as unexpected temperature changes or drifts in osmolality. Diligence and risk management should always remain a central focus of an embryology laboratory (see *Mortimer and Mortimer, 2015*, Chapter 9), even when optimization of conditions is relatively unknown.

Intracytoplasmic sperm injection (ICSI)

Sperm preparation methodologies dramatically changed during the first 15 years of clinical IVF. The basic purpose is to remove all traces of seminal plasma and debris and select a subpopulation of highly motile spermatozoa, all in an affordable manner that is fast and with the least trauma to the viable cells. It is known that maintaining physiological pH, temperature and buffering is less critical for spermatozoa than for unfertilized oocytes (*Fleming and King, 2003*), but there are time constraints in terms of exposure to seminal plasma and body temperature (*Cohen et al., 1985a*). Handling techniques should follow general tissue culture techniques and include an appreciation of potential materials toxicity, sperm survival tests, protein supplementation, use of antibiotics, optimal osmolality (250–290 mOsm/kg) and aseptic technique.

Sperm preparation aspects are covered under 'Handling practices' (below). Commercial products include density gradient kits, motility enhancers and hypo-osmotic swelling solutions, and are subject to sterility, pH, endotoxin and osmolarity quality testing. Product QC by the mouse embryo assay (MEA) test is considered essential by some, but its relevance is debatable and some holding products are not MEA tested.

Oocytes are much more sensitive to changes in pH and temperature, and possibly osmolality, than spermatozoa. A deviation from acceptable standards might not affect fertilization but might influence later development. Denudation by hyaluronidase and narrow-bored pipettes or strippers might have long-term effects, but clinical studies are lacking. In 1998, Van de Velde concluded that cytoplasmic maturation must be complete during or shortly after egg retrieval based on an RCT that showed that eggs denuded after 1–2 h had similar outcomes to those denuded at a 4–6 h interval (*Van de Velde et al., 1998*), and this information is used by many practitioners as the basis for ICSI timing – although this will also depend on the interval between ovulation trigger and egg collection. Recently, *Pujol et al. (2018)*, in a large retrospective multivariate analysis, reported that increasing oocyte retrieval to ICSI time increased the fertilization rate, and that each 1 h increase in this interval reduced the likelihood of biochemical pregnancy by 7.3%, and of clinical pregnancy by 7.7% after fresh embryo transfer, although there was no effect on either the ongoing pregnancy or live birth rates. Another large retrospective analysis by *Naji et al. (2018)* reported that retrieval to denudation time intervals of <2 h and 2–5 h did not affect outcomes, but the analysis did not consider the actual trigger-to-stripping interval, and the overall fertilization, implantation and pregnancy rates were modest (68%, 26–27% and 37–38%, respectively).

However, these recent papers contrast with a number of studies published over the last 20 years that generally support the principle that a delay of more than 2 h before oocyte denudation gives improved oocyte maturity, fertilization rate, embryo quality and/or ICSI outcome (*Dozortsev et al., 2004*; *Ho et al., 2003*; *Isiklar et al., 2004*; *Patrat et al., 2012*; *Rienzi et al., 1998*). In addition, another study reported better outcomes by delaying stripping until 4 h post-oocyte retrieval and then performing ICSI promptly, compared with either stripping and injecting immediately after oocyte retrieval or stripping immediately and delaying injection for 4 h (*Hassan, 2001*).

Although clinical information is sparse, prolonged exposure to hyaluronidase could affect oocytes. In the mouse, the duration of hyaluronidase exposure might reduce fertilization and adversely affect subsequent developmental steps (*Ishizuka et al., 2014*). Short co-incubation of spermatozoa and eggs during standard IVF seems to be beneficial according to a Cochrane Review on eight RCT (*Huang et al., 2013*) but the quality of evidence was considered low.

Oocyte handling during micromanipulation

Oocyte handling during regular observations does not involve a change from the standard bicarbonate-buffered conditions, but manipulations such as preparation for ICSI or vitrification could require a change to the external conditions outside the incubator. There is a wide variation in commercially available products for handling oocytes outside incubators: the choice of handling medium is usually HEPES-buffered, less frequently phosphate-buffered saline, while some laboratories still use regular bicarbonate-buffered culture media. MOPS is also used in a number of handling media now. There are heparin-free media as well as antibiotic-free variants for use when patients have allergies. Hyaluronidase is occasionally replaced by other enzymatic products such as recombinant coronase. Sourcing and testing of these products rarely involve studies on fertilization effects or long-term outcomes.

Mechanical stress during micromanipulation

It has been shown that shear stress, similar to the force that occurs during pipetting, has a negative effect on mouse embryos, up-regulating the stress gene for phosphorylated MAPK8/9, formerly known as stress-activated protein kinase/jun kinase/SAPK/JNK (*Xie et al., 2007*); the effects were similar at nearly all stages and enhanced in zona-free embryos. This work is under-appreciated among practitioners and relatively rough handling of gametes and embryos is common in IVF laboratories.

Culture system configurations during micromanipulation

The preferred incubation system during micromanipulation is microdroplets under oil (closed system). Advantages of an oil overlay include: reduced microbial contamination; providing a higher heat capacity and stability than air; controlled separation of fluid droplets; limiting evaporation; and providing a limited VOC sink. ICSI results are probably enhanced when the procedure is performed quickly with a limited number of eggs per dish. There is no recommended maximum egg range per dish, whereas the number of spermatozoa in a pipette should probably be limited. Before starting the procedure, it is recommended to optimize visualization of spermatozoa, egg, pipette tips, alignment of all objects, and perform a quick check of suction devices. The literature is not helpful when defining laser alignment and validation. The use of the blade or laser knife is probably permissible for rapid zona opening, but probably less advantageous when cutting the trophectoderm for biopsy as it might increase cell lysis.

In the mouse, the optimal handling temperature is room temperature and mouse zygotes and embryos are more tolerant to a reduction in temperature during incubation than an increase over 38°C. For human material 37°C is recommended for unfertilized eggs and embryos, and any time at room temperature is a possible confounder. QC assessment of the stage warmer or heating system should be performed periodically, watching for cold and hot spots, drifts and fluctuations.

Polyvinylpyrrolidone (PVP) has been used for half a century in cell culture. For ICSI, PVP (average 10 kDa molecular weight) or an alternative like hyaluronate is used to increase the medium's viscosity so as to decrease sperm motility and reduce fluid movement in micro-pipettes. PVP has been associated with ultrastructural sperm damage and sperm membrane effects, and can delay calcium oscillations and decondensation and remain in the oocyte for an extended time (*Kato and Nagao, 2012*). Half-life time for PVP differs between brands of media (*Kato and Nagao, 2009*).

Handling practices

The use of time-lapse incubators can negate the need to remove embryos from incubators from Day 1 (or Day 0 for ICSI) until the time of embryo transfer. This enables more consistent and possibly better outcomes (*Chen et al., 2017*; *Racowsky et al., 2015*). Unfortunately, there are up to six procedures that must take place during IVF (five for ICSI) before zygotes are put into culture, and up to three procedures at the termination of culture. Care must be taken during these procedures to avoid stressing both gametes and embryos.

General practices

Procedures outside of the incubator usually involve pipetting and microscopic observation. Unless one uses a controlled environmental chamber to conduct these procedures then great care must be exercised to control both temperature and pH. The central importance of these environmental aspects has been described above.

Mathematical modelling and actual measurements of temperature changes during pipetting have shown that a pipette tip quickly loses heat – up to 5°C in 5 s in a glass pipette (*Blomfield, 2011*). Preheating the pipette makes no difference: even though the pipette body retains heat the tip does not. Similarly, keeping embryo transfer catheters in a warm oven does nothing and may increase the VOC concentration inside the catheter, and the catheter tip will be at ambient temperature within seconds. Realization of this rapid loss of heat should govern how embryologists work outside of a controlled environment, and any procedure that is conducted at ambient temperature (e.g. oocyte recovery) should be simulated and a datalogging probe used to determine where any heat losses occur, so they can be minimized.

Most embryologists have been using warm stages for years without fully understanding the basic principles by

which they work. Again, mathematical modelling and actual measurement have shown that, depending on the culture dish and contents, a warm stage takes at least 3 min to start to warm a dish because of the air trapped between the dish bottom and the warm stage. This is exacerbated using metal warm stages with a hole in them.

When one considers that pH changes will show similarities to the temperature changes, then the argument for controlled environment 'IVF chambers' becomes compelling.

Oocyte recovery

A heated tube holder is essential, but precautions should also be taken to minimize heat loss that could occur in either the oocyte retrieval needle or the tubing from the needle to the collection tube (*Yeung et al., 2004*). Simulation measurements (Mortimer and Mortimer, unpublished data) show that the main heat loss is in the oocyte retrieval needle itself and not in the Teflon tubing from the needle to the tube, probably due to Teflon being a much better insulator than stainless steel. The heat loss is variable, being dependent on the flow rate of the fluid, and is minimized when the flow rate is maximal – although flow rate should be restricted to approximately 20 ml/min to avoid damage to the cumulus–oocyte complex (COC). While aspiration of the follicles as quickly as possible is recommended to minimize heat loss, even in a continuous flow model the temperature of the initial part of the aspirate still falls by about 2°C as it enters the collection tube. A possible solution to this problem is to prime the collection tubes with 2 ml of warm buffer to restore the aspirate to 37°C as quickly as possible.

If an environmental chamber is not used, then pipetting during the egg search should be performed as quickly as possible and putative COC should not be held in the pipette while searching for others. Trimming of COC is recommended if blood has infiltrated as this may affect embryo development (*Ebner et al., 2008*). An effect of reducing cumulus size in the absence of blood clots on development has not been shown *Ebner et al., 2017*.

Sperm preparation

Seminal plasma contains one or more factors, prolonged exposure to which adversely affects sperm function, including the ability to penetrate cervical mucus, undergo the acrosome reaction *in vitro* and the fertilization process generally (*Björndahl et al., 2010*; *Mortimer, 2000*; *Yanagimachi, 1994*). Exposure to seminal plasma for more than 30 min after ejaculation permanently diminishes the fertilizing capacity of human spermatozoa *in vitro* (*Rogers et al., 1983*), and contamination of prepared sperm populations with only trace amounts of seminal plasma (0.01% v/v = 1 in 10,000) can decrease their fertilizing capacity (*Kanwar et al., 1979*).

Variables to control during sperm preparation are the choice of method, type of medium and temperature. Currently there are three main methods of sperm preparation: swim-up, microfluidics and density gradients. The first two methods are usually conducted in bicarbonate-buffered 'medium' inside an incubator. Density gradients usually employ a HEPES- or MOPS-based medium ('buffer') for use under air in the centrifuge. The advantage of using 'medium' is that spermatozoa have a requirement for bicarbonate for normal functionality (*Boatman and Robbins, 1991*) and the bicarbonate concentration is reduced in buffer formulations. A 'buffer' is easier to use than a 'medium' because pH control is not a problem.

Temperature control is relatively easy to achieve, but there is some question about what temperature to use. Swim-ups and microfluidic devices are usually inside an incubator so 37°C is the norm, but density gradients are variable depending on the time in the centrifuge, although heated centrifuges are available. Consideration has to be given to these variables and each laboratory will have to define its own protocols to ensure a consistent approach is used. The most common sperm separation techniques have been well described with detailed standardized protocols (e.g. *Björndahl et al., 2010*).

IVF insemination, hyaluronidase use, ICSI and embryo transfer are procedures where pH and temperature can be controlled with an environmental chamber or judicious use of a warm stage and speed. The importance of these aspects was discussed above. To predict the range of temperatures that will occur, each procedure should be simulated, and temperatures checked at key stages using calibrated datalogging temperature probes. Although there will always be a temperature drop during embryo transfer, this can be minimized with care and speed.

Vitrification/warming

There is a plethora of methods and associated commercial media and devices for effective embryo cryopreservation. The most important steps are the initial movement of the embryo from the incubator to the holding buffer, and from the final warming solution to the incubator, because the embryo is more susceptible to temperature changes at these points than in the vitrification solutions, when embryonic metabolism is dramatically slowed. It is suggested that at the first step in vitrification the embryo(s) be removed from culture into the holding buffer at 37°C and allowed to cool to ambient temperature before vitrifying. It is also suggested that if the final warming solution is at ambient temperature then it should be warmed to 37°C before returning the embryos to culture.

Assessment practices

The need to assess

Assessment of gametes and embryos is a necessary, routine aspect of the IVF process, which is primarily morphology based. Gamete assessment aids decision-making, alongside patient clinical factors, regarding the type of fertility treatment required. The frequency and timing of assessment must be specified within a laboratory's SOP and allow sufficient and timely observations to aid effective embryo assessment and selection practice.

Assessment of semen should include, as a minimum, volume, motility, count and morphology of a liquefied sample, following standardized timely procedures and ensuring appropriate temperature control (*Björndahl et al., 2010*; *World Health Organization, 2010*).

Assessment of oocytes can be subjective due to variable morphologies, and the appearance of the cumulus–corona complex or denuded oocyte can be discordant with oocyte maturity or viability. The literature relating oocyte quality and clinical outcome is inconsistent (*Rienzi et al., 2011*). Cumulus removal and oocyte assessment is necessary prior to ICSI, although care

should be taken to ensure this is not performed too soon after oocyte retrieval (as already noted), and the presence of a polar body is the standard requirement to proceed with sperm injection.

Embryo assessment is performed to select the embryo with the highest potential for implantation (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*) and is considered in more detail below. Gamete and embryo assessments allow preferential selection to optimize clinical outcome and are used by embryologists to inform and set expectations for patients, and for monitoring the quality of laboratory performance against relevant key performance indicators (KPI). Laboratory KPI such as fertilization, cleavage, blastulation and embryo utilization rates are considered broad and beneficial indicators of IVF laboratory performance (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*).

Optimizing assessment conditions

Control and maintenance of physcochemical conditions are essential to minimize stress to the gametes and embryos during assessment. During assessments the laboratory environment, equipment and consumables used for handling, as well as the actual manipulations, should be reduced to essential steps only and risks and benefits must be considered when defining related practices. All consumables should be traceable and, where possible, designed and certified for IVF use. Equipment should be preventatively maintained and specified for use. High magnification microscopy should be available (generally 200–400 ×) with Hoffman modulation contrast, or similar, optics being recommended for the detailed examination in plasticware of low-contrast specimens such as oocytes and embryos (*ESHRE Guideline Group on Good Practice in IVF Laboratories, 2016*).

Without time-lapse or video monitoring, assessment of embryos necessitates their removal from the optimized and stable incubator environment. Dedicated or compartmentalized incubators, and models with rapid CO_2 recovery (infrared CO_2 sensors) reduce the possible detrimental impact of inevitable fluctuations in conditions caused by door openings. Oil overlays are recommended to minimize evaporation, gaseous exchange and temperature-induced changes in culture conditions during assessment. As described above, temperature, pH, osmolarity and air quality should be optimized, validated, monitored and standardized across devices and work areas to protect gametes and embryos. Optimal working ranges for these variables should be established and validated for the culture system being utilized. Regular tracking and monitoring of these limits is required for optimized IVF laboratory practice.

The impact of atmospheric or ambient conditions on culture media, and hence the gametes and embryos, must be considered if humidity and gas levels differ between the incubator and the heated assessment area or workstation. General aspects of temperature and humidity during IVF and embryo development were discussed above. With an appropriate heated workstation, the use of zwitterion-buffered media for pH maintenance during very brief visual assessments (<2 min) is not considered justified, although when denuding oocytes and assessing their maturity prior to ICSI such media can be used to provide a stable environment (*Koustas and Sjoblom, 2011*). Medical-grade compressed gases with in-line VOC filters help to ensure high air quality in incubators and workstations, and protect against particulates, VOC and other contaminants. Profiling of pH changes to assess culture media stability limits in the assessment environment is advisable if an isolette/humidicrib-style or similar controlled environment workstation is not available. Exposure to ambient pCO_2 must be controlled and minimized so as to avoid significant changes in medium pH (*Lane et al., 2000*). If sequential culture media are used, then assessments should be aligned with the medium changeover to minimize time outside of stable incubation. For cumulative or sequential embryo assessments over a number of days, single embryo culture or the use of specified microwell culture plates is required to allow robust tracking of individual embryos over time.

The wavelength of light from all sources within the laboratory should be considered if exposure can occur during assessment, and adverse, shorter wavelengths avoided (*Ottosen et al., 2007*). Literature on the relative impact of different wavelengths on human gametes and embryos is sparse, but many IVF laboratories take the precautionary approach of minimizing light exposure and use ultraviolet light filters. The positioning of critical equipment used for assessments is an important consideration during laboratory design: close proximity between the incubators and microscopes helps reduce exposure to ambient laboratory conditions.

Assessment practice and guidelines

Assessment of oocytes and embryos must be performed by properly trained and qualified personnel. Embryologists should be competency-assessed and participate in internal and external quality assurance schemes (*ESHRE Guideline Group on Good Practice in IVF Laboratories, 2016*). Assessments should be performed as swiftly and accurately as possible, for maintenance of intracellular homeostasis, and records of each embryo assessment collected, ideally using live data entry or contemporaneous scribing rather than retrospective recollection. If time-lapse image capture is available, more time is available for the study and assessment of images because the embryos remain within the incubation environment.

Consensus guidelines derived from evidence and experience, and standardized by time of insemination, still provide the most robust foundation for non-invasive gamete and embryo assessment. There are several published grading schemes, offering either formulated or cumulative scoring, or ranking, as well as assessment guidelines from professional organizations (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*; *Cutting et al., 2008*; *Machtinger and Racowsky, 2013*; *Racowsky et al., 2010*, *2011*). However, despite the availability of several guidelines the assessment and selection of embryos is not standardized across the IVF field. Practice varies from single to sequential assessments, to computer-assisted and time-lapse evaluation (*Paternot et al., 2011*; *Racowsky et al., 2009*). Most of the guidelines recommend that cleavage-stage embryo assessment include cell number, size and symmetry, percentage of fragmentation, granulation, vacuoles and nuclear status. Blastocyst stage assessment recommendations include

assessment of expansion, blastocoel cavity size and morphology of the inner cell mass and trophectoderm. Blastocyst grading is most commonly performed using criteria adapted from the scheme published by *Gardner and Schoolcraft (1999a,b)*. Laboratories performing blastocyst culture may use sequential or one-step culture media, the latter with or without continuous culture. Some laboratories use time-lapse observations, others static observations each day or only once or twice. All of these approaches seem to be compatible with acceptable outcomes. The advantages and disadvantages of each of these alternatives are complex and the optimal approach is unknown.

Time-lapse multi-plane imaging provides the opportunity for more detailed and protected assessment, and is generally considered safe practice (*Nakahara et al., 2010*), although oocytes or embryos might need to be removed from this environment to change their orientation or to observe at higher magnification. Time-lapse assessment permits precise logging of cleavage events and developmental milestones; standardized annotation of images is recommended (*Ciray et al., 2014*). Although several time-lapse embryo selection models have been proposed, and while there are similarities between them, they have not been demonstrated to be widely transferable between laboratories (*Barrie et al., 2017*; *Pedersen et al., 2016*).

A recent meta-analysis of five RCT reported an association between morphokinetic embryo selection and significantly higher pregnancy and birth rates compared with standard methodological assessment of embryos cultured in 'big box' incubators, as well as significantly lower early pregnancy loss (*Pribenszky et al., 2017*), while another reported no benefit (*Racowsky and Martins, 2017*). Clearly, further RCT are still needed, as the meta-analyses are of necessity limited by the heterogeneity of the trials included.

Culture media – buffering and pH

Stabilization of pH during IVF is critical to avoid environmental stress that can compromise embryo development. The pH of culture medium, or the external pH (pHe), is normally determined by the balance between CO_2 levels provided in the incubator and, primarily, the sodium bicarbonate concentration in the culture medium, although other factors can impact the final pHe. Periodic fluctuations in culture conditions, such as pHe, can be harmful as these can be transduced into deleterious intracellular perturbations (*Phillips et al., 2000*). Improper intracellular pH (pHi) can impede sperm function (*Babcock and Pfeiffer, 1987*; *Babcock et al., 1983*; *Hamamah and Gatti, 1998*; *Marquez and Suarez, 2007*), impairs embryo metabolism (*Edwards et al., 1998*; *Lane et al., 2000*), alters organelle localization (*Squirrell et al., 2001*), and is detrimental to embryo development (*Lane and Bavister, 1999*; *Lane et al., 1999*; *Leclerc et al., 1994*; *Zhao and Baltz, 1996*; *Zhao et al., 1995*), and even retards subsequent fetal growth (*Zander-Fox et al., 2008*). This is more apparent in cell types like the denuded mature oocyte (*FitzHarris and Baltz, 2006*; *FitzHarris et al., 2007*; *Lane et al., 1999*; *Phillips and Baltz, 1999*), or cryopreserved/thawed embryos (*Lane et al., 2000*), which lack robust pHi regulatory mechanisms, making them especially susceptible to deviations in pHe. Optimizing embryo handling and culture systems must therefore include careful selection of buffers to stabilize pHe.

Buffers are selected based on their optimal pH buffering capacity (the ability to resist pH change) or pKa value, which needs to be matched to the application because many biological processes only function over a narrow range of pHe. Buffers can impact cell development and function independently of pH, and different cell types display different growth rates dependent on the type, as well as concentration, of buffer used (*Eagle, 1971*; *Ferguson et al., 1980*; *Good and Izawa, 1972*; *Good et al., 1966*). Different buffers can also differentially impact various cellular processes, including electron transport, photophosphorylation and mitochondrial oxidation (*Ferguson et al., 1980*; *Good et al., 1966*). Unfortunately, very few comparative studies have been performed to assess the impact of various biological buffers on oocyte and embryo development, but evidence reviewed elsewhere has shown that many concerns regarding the use of buffers other than bicarbonate in human ART are unfounded, being based on misinterpretation of studies with faulty experimental design and invalid conclusions (*Swain and Pool, 2009*; *Will et al., 2011*). From these extensive reviews it can be concluded that:

- Concern over the use of buffers during ICSI on the oocyte's pHi is unwarranted.
- Concerns regarding zwitterionic buffers and IVF are based on studies that were confounded by simultaneous alterations in other media components that impacted embryo development, such as the reduction in bicarbonate levels and the consequent need for less CO_2.
- HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], a modified taurine molecule, is an extensively studied zwitterionic organic buffer, the efficacy of which with spermatozoa is widely accepted; yet its safety with oocytes and embryos remains controversial. This is in spite of numerous studies actually indicating HEPES can efficiently support oocyte maturation (*Byrd et al., 1997*; *Downs and Mastropolo, 1997*), fertilization (*Bhattacharyya and Yanagimachi, 1988*; *Behr et al., 1990*; *Hagen et al., 1991*) and embryo development (*Ali et al., 1993*; *Hagen et al., 1991*; *Mahadevan et al., 1986*; *Ozawa et al., 2006*). In particular, HEPES supports embryo development in the presence of bicarbonate, but not when bicarbonate is absent (*Mahadevan et al., 1986*). Bicarbonate levels influence blastocyst development, possibly through activity of various HCO_3^--dependent transporters, and the CO_2 of the incubator atmosphere is utilized by embryos as a carbon source (*Graves and Biggers, 1970*; *Quinn and Wales, 1971*, *1974*). Indeed, after accounting for CO_2 and bicarbonate levels as co-variables, medium with up to 50 mmol/l HEPES gave similar rates of blastocyst development and cell number compared with medium without HEPES (*Swain and Pool, 2009*).
- MOPS buffer can be used at 25 or even 50 mmol/l to culture mouse zygotes to blastocysts with no significant differences in rates of development or cell number compared with control medium (*Swain and Pool, 2009*).
- Gene expression profiling of environmentally sensitive genes in bovine embryos indicated that expression levels of embryos handled in MOPS or HEPES-buffered media were most similar to embryos derived *in vivo* (*Palasz et al., 2008*).
- MOPS is now included in commercial handling media and used successfully for human ART procedures, ranging from sperm washing to oocyte and embryo handling and vitrification.

MOPS has been reported to be superior to HEPES for vitrification, although the exact reason for this remains unclear (*El-Danasouri et al., 2004*).
- Phosphate buffers tend to precipitate polyvalent cations (e.g. Ca^{2+}), while also acting as a metabolite or inhibitor in various systems. Although phosphate-buffered saline (PBS) has an appropriate pKa, elevated levels of phosphate can damage cellular function, compromise gamete and embryo metabolic activity (perhaps via the Crabtree effect), disrupt organelle distribution, and interfere with intracellular ionic homeostasis, including pHi.

The simultaneous use of multiple different buffers allows for adjustment of pKa values to the desired range, while also permitting a reduction in individual buffer concentrations and potentially alleviating concerns for toxicity. Recent attention has focused on use of combination buffers in handling media for IVF to further refine the current mono-buffered systems (*Swain and Pool, 2009*).

Culture media – general composition and protein supplementation

Little has changed since the statement 'It remains true in 2011 that there is no culture medium available that is truly optimized for human embryo development' (*Harper et al., 2012*). Consequently, attempting to define consensus is limited by our relative uncertainty of what is optimal for human embryo development.

There are only a few concepts that have reached consensus status for medium composition. Because most culture media contain over 40 components, at different concentrations and ratios relative to each other, the number of possible combinations is limitless. As osmolality and pH are reviewed elsewhere in this document, the discussion here will be limited to the presence, form and concentration of nutrients. The following areas of general consensus are based on years, often decades, of evidence supporting their central role in metabolism and development of preimplantation mammalian embryos.

Antibiotics, mostly in the form of gentamicin, are a ubiquitous component of human embryo culture media. Although not required, the benefits outweigh the risks, making inclusion of antibiotics a standard practice in the industry (*Kastrop et al., 2007*; *Swain, 2015*).

Carbohydrate needs are met via pyruvate, lactate and glucose, with pre-compaction embryos preferring pyruvate followed by a shift to glucose for compaction and blastocyst formation (*Leese, 2012*). Most culture media provide pyruvate at concentrations between 0.2 and 0.5 mmol/l (*Morbeck et al., 2014a*, *2017*), reflecting both the concentration as measured in fallopian tubes (*Dickens et al., 1995*; *Gardner et al., 1996*; *Lopata et al., 1976*; *Tay et al., 1997*), and the concentrations derived via simplex optimization (*Lawitts and Biggers, 1991*). Results from the latter study suggest that pyruvate concentrations higher than 0.5 mmol/l may be detrimental. Perhaps not surprising, all but one now-discontinued medium provided pyruvate at ≤0.5 mmol/l (*Morbeck et al., 2017*).

Glucose, while not required for pre-compaction embryo development, and at one point considered detrimental to early cleavage divisions (*Quinn et al., 1995*), is now included as a standard component of all complex culture media on the market. Amino acids and EDTA are important contributors to the metabolic environment that limits the utilization of glucose during the pre-compaction stage (*Biggers and McGinnis, 2001*; *Houghton, 2012*; *Lane and Gardner, 1997*). Post-compaction, glucose becomes the preferred energy source and is supplied at ostensibly physiological levels of ~3 mmol/l in blastocyst-stage media (*Gardner et al., 1996*; *Morbeck et al., 2014a*) versus 0.2–1.0 mmol/l for 'single-step' or cleavage-stage media (*Morbeck et al., 2017*). The lack of consensus on glucose concentration probably reflects the embryo's ability to obtain its energy and carbon from diverse sources, including pyruvate, lactate and amino acids. It is worth noting that EDTA, an additive that was believed to be necessary to chelate potentially toxic metals, is used in all media at varying concentrations as it may be involved with glucose metabolism, limiting the availability of magnesium as a cofactor for glucose metabolism (*Lane and Gardner, 2001*).

Amino acids are ubiquitously present in culture media and in at least one protein supplement (*Morbeck et al., 2014a*, *2014b*, *2017*). Early iterations of culture media for human embryos were mostly amino acid free, e.g. Earle's medium (*Cohen et al., 1985b*) and Quinn's HTF (*Quinn et al., 1985*), although Ham's F-10 (*Leung et al., 1984*) and Menezo's B2 and B3 media (*Menezo et al., 1984*) contained amino acids based on Eagle's formulation (*Eagle, 1959*). While all amino acids were included in Gardner's blastocyst-stage media, only non-essential amino acids were included in cleavage-stage sequential media (*Gardner and Lane, 1998*) due to the possibility of reduced development of cleavage-stage embryos exposed to essential amino acids – at least when used at MEM (minimal essential medium) levels (*Lane and Gardner, 1997*). In contrast, the single-step media include most or all of the 20 amino acids (*Morbeck et al., 2017*). Amino acids play numerous roles in cellular function, including but not limited to acting as metabolites, antioxidants, osmolytes and buffers. Thus, there is a consensus that complex media should contain a robust complement of essential and non-essential amino acids. The selective efficiency of non-essential and essential amino acids in early stages needs further clinical research, but there seems to be consensus that both groups are needed for post-compaction development.

L-glutamine, an amino acid with many roles during embryo growth and thus considered an essential component of culture medium, breaks down spontaneously with ammonium as a by-product (*Kleijkers et al., 2016a*). There are concerns regarding the relative amount and impact of ammonium accumulation in culture media (*Biggers et al., 2004*), but the effect is magnified in 20% O_2 (*Wale and Gardner, 2016*). A simple solution has been the introduction of glutamine in a stable dipeptide form (*Lane and Gardner, 2003*). The use of dipeptide glutamine is now standard practice for all versions of complex ART culture media.

Protein is an important component of culture medium that is provided in several forms and, by consensus, should be included in all embryo culture media. Albumin is the most abundant protein in the oviduct (*Leese, 1988*) and thus the most common protein used in culture media. Albumin serves many functions, most notably as an antioxidant (*Bavister, 1995*; *Morbeck, 2015*). Lot-to-lot variations exist with human serum

albumin (HSA) (*Swain, 2015*), particularly with respect to the stabilizer octanoic acid (*Fredrickson et al., 2015*; *Leonard et al., 2013*). Human albumin is also available in a recombinant form (recHSA; *Bungum et al., 2002*), although exclusivity rights restrict its availability, mostly as a supplement for end-users to add to non-supplemented media. Even the sole supplier does not offer media pre-supplemented with recHSA, probably due to issues with cost of the product. So, while recHSA might offer theoretical advantages over HSA in terms of safety and product consistency, its routine use is unlikely while commercial exclusivity remains in place.

Complex protein supplements are common and have a strong market presence, particularly in the Americas. There are two classes of complex protein supplements: HSA solutions containing alpha and beta globulins or dextran. The latter is a surfactant and volume stabilizer, although there is little evidence for the benefit of using a polysaccharide along with albumin (*Campbell et al., 2013*). In contrast to dextran, alpha and beta globulins carry potential benefits that can yield improved live birth rates (*Meintjes et al., 2009b*). There are two formulations of protein supplements containing globulins: one where USP (US Pharmacopeial Convention) grade alpha and beta globulins are mixed with HSA and one that is a less pure fraction of human serum that contains both albumin and alpha and beta globulins. Both empirical observations and regulatory barriers limit the widespread use of these complex protein supplements globally. Globulin-containing products are poorly defined, can contain high levels of pro-oxidant metals (*Morbeck et al., 2014b*), and suffer from significant lot-to-lot variation (*Meintjes, 2012*). For these reasons, and with limited evidence for benefit, the proposed consensus is that industry should move to protein supplements that are the most defined, and, at some point, not a human product. The proposed consensus is that recHSA is theoretically safer and more consistent than HSA, which is safer and more consistent than globulin-containing formulations.

Human serum, the predominant form of protein supplement used during the early years of clinical IVF (*Edwards and Steptoe, 1983*), is inherently variable and can carry risks to the health of the offspring. Thus, the field reached consensus in the 1990s that serum was no longer suitable as a supplement for human embryo culture.

Phenol red is included in many culture media as a QC measure to monitor medium pH, rather than it having any role in embryo development (*Swain, 2015*). However, phenol red addition carries some hypothetical risks, based on its oestrogenic activity (*Moreno-Cuevas and Sirbasku, 2000*) or as a source of ROS (*Nakayama et al., 1994*), thus making it a component for which risks outweigh benefits. In this era of microdrop culture, where volumes of media are <100 µl, a colour indicator is neither necessary nor beneficial because visual assessment of colour changes in such small volumes is very hard to standardize.

However, in other areas, consensus is lacking, as described below.

Lactate concentrations vary widely. An important partner with pyruvate during the first few days of embryo development, lactate is intimately involved in pyruvate uptake and metabolism (*Lane and Gardner, 2000b*) and production of NADH and thus REDOX potential in blastomeres (*Dumollard et al., 2007*, *2008*). Nearly all studies of reproductive fluids report lactate concentrations of 5–10 mmol/l (*Dickens et al., 1995*; *Gardner et al., 1996*; *Lopata et al., 1976*; *Tay et al., 1997*) and the concentration chosen, at least for single-step media, is within this range to give a lactate:pyruvate ratio of approximately 20 (*Morbeck et al., 2017*). Lactate concentrations at 2 mmol/l, which result in lactate to pyruvate ratios of 2 to 5, have been introduced by some designers (*Morbeck et al., 2014a*), although these low lactate concentrations and lactate:pyruvate ratios have not been rigorously studied in animal models and thus lack consensus.

Calcium and magnesium, in terms of their absolute and relative concentrations, also vary widely with a nearly 10-fold difference in magnesium concentrations (0.2–1.8 mmol/l) that result in a 10-fold difference in the ratio of calcium to magnesium (0.6–7.1; *Morbeck et al., 2017*). The significance of the difference in Ca:Mg ratios is unknown, although calcium signalling after fertilization is a likely target, and differences in this ratio could affect developmental potential (*Lu et al., 2018*). Thus, absolute concentrations of Ca^{2+} and Mg^{2+} and their ratio lacks consensus.

Hyaluronan, a glycosaminoglycan, and taurine/hypotaurine, an aminosulfonic acid, are added to some but not all culture media. Benefits of hyaluronan have been described for embryo transfer media (*Bontekoe et al., 2010*), but there is a limited clinical evidence base for benefits in embryo culture (*Gardner et al., 1999*). Recent work in bovines and sheep, particularly after cryopreservation, has shown outcome improvement (*Dattena et al., 2007*; *Palasz et al., 2008*). In contrast, taurine is used in culture media for domestic animals with proposed antioxidant and osmolytic benefits (*Devreker and Hardy, 1997*; *Dumoulin et al., 1997*).

There are numerous micronutrients present in some culture media, components with minimal evidence basis and thus lack of consensus among media manufacturers. These factors include antioxidants, such as lipoic acid, essential nutrients like choline, and vitamins, including but not limited to the B vitamins, vitamin C and vitamin E.

Insulin has been included in a minority of embryo culture media, possibly as a reflection of the common inclusion of insulin, transferrin and selenium in cell culture media (*Shamsuddin et al., 1994*). A recent study showed that insulin added to a single-step human embryo culture medium improved embryo development and ongoing pregnancy rates (*Fawzy et al., 2017c*), although further studies are warranted to validate these findings.

Growth factor or cytokine supplementation of commercial human embryo culture media is currently limited to granulocyte-macrophage colony-stimulating factor (GM-CSF), a multifunctional cytokine present within the oviduct and uterine epithelium during the peri-implantation period (*Robertson, 2011*). A large RCT showed a benefit of GM-CSF in select patient populations (*Ziebe et al., 2013*), but only when the level of HSA was reduced. In the control group the lower level of HSA (2 mg/ml) had lower outcomes than the standard 5 mg/ml. Similar to recHSA, manufacturer exclusivity limits the availability of recGM-CSF to one culture medium. This commercial exclusivity limits the broad

application and clinical assessment of GM-CSF, making it premature to establish consensus on its use.

In conclusion, reaching a consensus on the composition of human embryo culture medium is difficult due to the fact that individual components have not – and cannot – be optimized in patients, notwithstanding the possibility that one culture medium might not be suitable for all patients. Hence the conclusions summarized here are actually an expert opinion based on industry practices. There are several components that can be considered standardized and several others that merit consideration for consensus. These two groups of components comprise a small minority of the total constituents of culture media on the market, making most of the composition not standard.

- Carbohydrates – pyruvate, lactate and glucose – are universally added to culture media, as are most if not all common amino acids.
- Glucose and pyruvate concentrations fall within a relatively narrow range.
- Amino acid concentrations vary considerably; glutamine is notable given its lability and hence its routine inclusion as a stable dipeptide.
- Inclusion of the antibiotic gentamicin is standard.
- Serum-free culture is standard.
- Protein supplementation has considerable variation and presents an opportunity for standardization. Complex protein supplements may bring benefits, yet are poorly defined and variable from lot to lot. Moving to the most defined protein available, and preferably not from human sources, is warranted. Ideally, protein is added to the culture medium by the manufacturer.
- Inclusion of phenol red remains variable and is generally unnecessary in media used for extended culture.

In practice the ART field is faced with the challenge of improving the composition of culture media while acknowledging that there is no suitable model in which to prove the principles first. Harper and colleagues, when providing recommendations for how to bring new culture media to market, stated: 'If we aim to change the design of culture media in significant ways, we must carefully assess the risk of those changes, as well as the potential benefits.' Yet new single-step media with significant changes in design were recently introduced with no published animal studies and little clinical oversight (*Hardarson et al., 2015*; *Sfontouris et al., 2017*). The composition of new culture media, such as a new single-step medium with low lactate, are shrouded in mystery, leaving practitioners, and thus patients, with little evidence or assurance of safety (*Sunde et al., 2016*). An area where we most lack consensus is how to continue to make improvements to culture media in a safe yet effective manner.

Culture media – sequential or 'single-step' media for human embryo culture

Two different approaches to developing embryo culture media have been proposed, more than a century after the first cell tissue culture system (simple salt solution) was described by Ringer and Ludwig. Aiming to mimic the reproductive environment (the so-called 'back to nature' principle), experimental embryologists in the 1970s tailored culture medium composition to closely match the chemistry of the female reproductive tract. An example of the back to nature principle is the sequential embryo culture system. The second group of media was designed to optimize growth *in vitro*, having embryo development as an endpoint to optimize composition, to some extent ignoring existing formulations and principles. These 'simplex optimization', or SOM, media were described by scientists supported by the National Institutes of Health in the USA during the 1990s, in a team effort that was colloquially referred to as 'The Culture Club'. To formulate SOM media the performance of each ingredient is evaluated separately using the 'simplex optimization' process – a mathematical system developed first for the mouse in the laboratory of John Biggers at Harvard University 25 years ago. The advantage of this approach over the sequential system is that there is no consideration of fallopian and uterine conditions, and that embryos can be left alone without altering conditions for 5 days or longer. The core question is whether this perceived advantage comes at a cost.

Gardner and Lane (1997) were the first to suggest that 'in order to optimize mammalian embryo development in culture, sequential media are required, each designed to meet the changing requirements of the developing embryo'. The need for sequential changes in their culture media was a logical conclusion of their experiments. This compelling principle and the 'back to nature' approach in general have been challenged in part because the approach is reliant on the analysis of oviductal and uterine components, and such analyses may have shortcomings (*Biggers, 2002*; *Cohen and Rieger, 2012*).

The suggestion that either the single-step SOM approach or sequential culture is better is not based on scientific arguments and cannot be decided after evaluation of the literature. More than a thousand papers and abstracts have been written about culture media systems and a considerable number of thorough reviews have been published comparing the two approaches (*Dieamant et al., 2017*; *Sfontouris et al., 2016*; *Youssef et al., 2015*). Analyses like this are unfortunately compromised by the quality of the original comparative studies and a multitude of confounders affecting laboratory results. Common confounders such as support media for gamete handling, protein supplementation and details of incubator QC aspects are often ignored by authors, and are hence unavailable to reviewers.

The recent systematic reviews plead for additional studies (including repeat trials), larger investigations and improved quality of evidence. There are five important aspects known to affect the reliability of RCT: (i) appropriate randomization; (ii) allocation concealment; (iii) blinding; (iv) limiting technological bias (such as rigorously applying manufacturer's instructions and reducing the effect of known confounders); and (v) independent statistical evaluation. Trials evaluating the two culture systems are often lacking in most of these areas. The debate over evidence-based practice has been long and tedious in reproductive medicine and particularly the use of the RCT as the sole argument to prove or disprove a hypothesis has been questioned (*Cohen and Alikani, 2013*; *Harper et al., 2012*).

Harper and coworkers (2012) suggested not just paying attention to RCT studies and the typical evidence base when evaluating new and existing clinical embryology technologies. This model of medicine and evidence by itself was not new, but the context was refreshing. They considered seven premises: (i) Is

the technology based on an appropriate hypothesis? (ii) Were studies in animals performed? (iii) Were spare gametes and embryos evaluated? (iv) Was the technology validated in small series studies? (v) Were RCT performed? (vi) Is the technology relevant from a routine application point of view? (vii) Is the technology economically sensible?

It is suggested that the 'Harper model' be considered seriously, as there is sufficient merit to each of the 'common sense' premises. If each technology is evaluated for these seven themes, and one aspect is not considered more important that any of the others, the lack of RCT might be considered acceptable (in some cases), and technologies can be evaluated (and compared) according to their Harper model score. Using this model, a score can be derived within a few years after the introduction of the technology, rather than the evidence-based approach, which can drag out over decades, often causing considerable debate and confusion. This was illustrated by the work of David Gardner and colleagues describing the first sequential culture medium system G1 and G2, nearly 20 years ago (reviewed in *Niederberger et al., 2018*). Those studies fulfilled the Harper model criteria in short order, although later RCT comparing sequential media against single-step KSOM-derived culture in systematic reviews did not show differences (*Dieamant et al., 2017*; *Sfontouris et al., 2016*).

Use and management – cold chain and storage

Manufacturers provide specific guidelines regarding transport and storage of human IVF culture media products, with little real difference between suppliers. Guidelines for storage and handling are based on manufacturers' experience, professional organization directives, and an example set by the associated field of cell tissue culture. General recommendations are:

- Culture media products must be stored at refrigerated temperatures according to the manufacturer's instructions (2–8°C) and kept away from light, particularly sunlight.
- Storage conditions and expiry date should be clearly shown on labels and product inserts.
- Products should be used in order of batch or lot numbers.
- Products should never be used after expiration of the shelf life indicated on the product or inserts.
- Bottles of ready-to-use culture medium should not be opened once, closed and reopened again for use later.

Culture media effects for ART do not just start during egg retrieval, but are a function of transportation as culture media may go out of their specified tolerance range during transport and storage. Culture medium is produced at room temperature and may be kept for a while at ambient environments before final packaging and refrigerated storage. It is obviously safe to assume that culture medium tolerates some changes in temperature, but potential detrimental effects, if any, are a function of exposure changes, duration and frequency of temperature fluctuations. There are two extreme events that may occur in cold chain failure: freezing or becoming overheated.

Effect of freezing

When the ambient temperature drops below 0°C it may take a while before a medium freezes, due to the shipping box and its thermal mass, which reduce the impact of temperature excursions. The general composition of culture media is such that they will not freeze immediately but will first super-cool, without inducing ice crystallization. Recommended storage conditions are +2°C to +8°C, indicating that manufacturers rely on a safety margin. Once a culture medium freezes, it will have to be discarded because the freezing can cause the liquid part to become non-homogeneous and subsequently the medium might not reconstitute properly. If the culture medium is protein supplemented, there is an increased risk of denaturation, as the liquid portion might have a sub-optimal osmolality and pH. Low temperatures do not have much effect on mineral oil.

Effect of elevated temperature

The cold chain might be broken due to transportation delays or temporary storage at elevated temperatures. A slightly elevated temperature between refrigeration and room temperature for up to a week might not be detrimental. The main effect of elevated temperature exposure is oxidation and a reduction in the concentration of active components. Through deamination amino acids and proteins might slowly release ammonium ions that have been shown to have a negative impact on the embryo; an effect that is worse when the monopeptide version of glutamine is used (as opposed to the more stable alanyl-glutamine or glycyl-glutamine dipeptide forms) or in protein-supplemented culture media. The process is slow, but effects may be noticeable after several days, and occurs even when samples are held at stable refrigerator temperatures: protein-free culture media do not have any marked ammonium ion build-up *in vitro* at 37°C or during 6 weeks in the refrigerator, but undiluted protein supplements showed considerable deamination after just a few days at 37°C (*Kleijkers et al., 2016a*). The actual levels of ammonium ions and specific effects at very low concentrations are unknown and effects could be brand-dependent. The reactions are O_2- and temperature-dependent and degradation can be calculated. A similar problem can occur in the event of a laboratory refrigerator malfunctioning.

Logging thermometers are sometimes sent in shipments. From their data, the mean kinetic temperature (MKT) can be calculated, a measure routinely used in the pharmacological industry as a simplified way of expressing the overall effect of temperature fluctuations during storage or transit of perishable goods.

There are no known international recommendations for storing IVF culture media. Regular residential refrigerators and freezers, which have very poor temperature stability, are still seen in IVF laboratories. Appropriate refrigeration can only be achieved with clinical grade ('pharmacy') refrigerators, which generally reduce fluctuations and failures. These units have wire shelves and compartments for improved circulation, and the doors do not have any shelves for storing samples. Combined side-by-side refrigerator/freezer units should not be used. Storage efficiency is dependent on load, so that empty and completely full refrigerators under-perform. A built-in unit should have a fan in the front.

Monitoring refrigerators and freezers using an external/secondary method is highly advisable and typically required by accreditation bodies. Monitoring should be periodic (at least daily). The Centers for Disease Control and Prevention (CDC) recommends NIST-calibrated certified thermometers using a glycol-filled bottle in addition to

display temperature. Dataloggers may be helpful. An audible alert is useful, as are self-closing doors, open door alerts and keyed locks.

Effect of light

Visible light is non-existent in the reproductive tract, but during laboratory exposure both gametes and embryos, as well as the products used for embryo culture, are exposed to visible light of various intensities (review by *Pomeroy and Reed, 2013*). The sources of light in ART laboratories are ceiling lights and microscopes (*Pomeroy and Reed, 2013*) and *Ottosen et al. (2007)* have shown that illuminance (lux) and irradiance are greater from microscopes than ambient light by a factor of 10–20 × . Harmful light-mediated effects have been described in invertebrates, hamsters, mice and cattle, but the direct effect of light on human embryos is basically unknown (*Ottosen et al., 2007*). Blue and ultraviolet light are found to be the most harmful and the effects can be suppressed by use of red and green filters on microscopes. Important indirect peroxidation effects of light on mineral and silicone oil have been documented (*Otsuki et al., 2007*). Indirect effects of light via hydrogen peroxide have also been found in HEPES-buffered media and culture media including essential amino acids like tryptophan and tyrosine. The intensity of the exposure (irradiance) is an important aspect to consider, as well as the duration of exposure and actual wavelength. Photo-oxidation (also known as peroxidation) of oil can occur when exposed to ultraviolet. Other compounds affected by light exposure are hydrocarbons, nitrogen and sulphur.

Test equipment – calibration and certification

Laboratory environment

VOC and air contaminant monitoring: Equipment with the proper sensitivity must be used (*Hall et al., 1998*). Meaningful VOC values are measured as ppb and not ppm. Sensitized photo-ionization detection (PID) devices are used for screening with sensitivities not lower than 100 ppb. Frequent calibration (at least semi-annually is recommended) is typically performed at the factory with a reference gas such as isobutylene. Heated metal oxide sensors (HMOS) are not recommended as their sensitivity is not acceptable. For the continuous measuring of ppb, a photoelectric absorptiometric principle is used, applying a sensor cartridge (150–1000 tests).

Particulates: Particle size to be measured should be defined. Most particle counters are certified and calibrated by the manufacturer using NIST-traceable particles; typically, in-house calibrations are not required. For ISO-certified clean rooms, particle counts should be performed according to *ISO 14644*, although a recent consensus on IVF laboratory air quality recommends a more practical approach (*Mortimer et al., 2018*).

Temperature: Temperature measurements should be tailored to the purpose and acceptable tolerance for the equipment being tested. Temperature measuring devices for working surfaces can be accepted with an accuracy of ±0.05°C. Thermometers should be calibrated at regular intervals, at least annually to conform to *ISO 15189:2012*.

Humidity: Control of room humidity is strongly recommended to minimize the risk of fungal growth and contamination, for staff comfort, and to minimize evaporation during the open handling (no oil overlay) of gametes and embryos. Inexpensive measurement devices can be used successfully, but typically last only for months. Continuous and/or wireless humidity sensors are calibrated at installation and should be recalibrated at least twice per year.

Culture environment

Temperature: When measuring the temperatures of incubators or other highly sensitive environments, a tolerance limit of no more than ±0.1°C is recommended. Thermometers should be calibrated against a NIST-traceable reference device at least twice per year.

pH: Monitoring of pH is commonly used as a means of validating culture medium performance (*Swain, 2012b*) as described above. However, with the consistency of modern culture media, the stabilizing effect of bicarbonate buffering, compromised measuring resolution and inter-measurement variation, routine single pH measurements may be of questionable value. Hand-held pH meters need to be calibrated before each use. Furthermore, one must ensure that the pH probe has a meaningful resolution and range for IVF applications, is designed for IVF micro-volumes, and that an immediate reading at 37°C can be obtained. Continuous measurement of pH *in situ* (inside the incubator) is possible using continuous sensors with certification and calibrations determined by the manufacturer. One example of continuous pH monitoring is SAFE Sens® (Blood Cell Storage Inc., Seattle, WA, USA), which employs an optical fluorescent measurement technology. Each disposable sensor contains a proprietary pH-sensitive fluorescent dye; the fluorescent signal is read continually at a set frequency and converted to a pH value. Each lot of sterile sensors lasts 7 days and is certified by the manufacturer.

Blood-gas analyzers: These instruments provide instant and accurate pH readings without having to rely on inaccurate hand-held pH meters or more expensive continuous monitoring devices (*Swain, 2013*). In addition, blood-gas analyzers provide helpful pCO_2, pO_2 and electrolyte information that can aid in the overall quality management of the culture system. The hospital-type analyzers are expensive and require daily maintenance and calibration. Newer handheld devices allow for pH, pCO_2, pO_2, and electrolyte readings, using disposable cartridges with integrated calibrators.

Oxygen: O_2 can be measured reliably with mechanical devices, electronic measuring devices, or the pO_2 can be determined with a blood gas analyser. Electronic hand-held devices should be calibrated regularly (daily, monthly, periodically) as specified by the manufacturer. A calibration gas mixture is frequently required for proper calibration. In a growing number of countries, the classic Fyrite® fluid for measuring O_2 or CO_2 requires hazardous goods shipment or is not even available.

Amperometric (galvanized or voltage-sensitive) and optical sensors are used in these applications, with an optical sensor requiring the least maintenance. Over time, both types of O_2 sensors can suffer from ageing effects leading to measurement drifts. A reference electrode (amphoteric) or reference LED (optical) or backup control unit is always advisable.

Laboratory equipment and real-time monitoring

Real-time monitoring (RTM) has long been considered impractical because of

the lack of accurate CO_2 sensors, the difficulty of connecting a large number of sensors, and the cost of cabling and adding sensors and data transmitters. Today, with the universal availability of low-cost wireless technology, internet, smart phones and tablets this is no longer the case, and there are now affordable solutions that provide vital information in real time. RTM systems can reduce 'loss' by equipment failure and thus provide the Embryology Laboratory Manager with increased safety and reliability. Also, regulators see the benefits of monitoring and this requirement is now integrated in professional guidelines (*Magli et al., 2008*), regulatory requirements (*Commission Directive 2006/86/EC*) and accreditation standards (*ISO 15189:2012*).

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

Air pressure, relative humidity, air flow sensors and particle counters can be connected to an RTM system but these parameters are usually integrated into a building management or monitoring system (BMS). Laboratory ambient air monitoring should be part of the laboratory's RTM system because deviations in ambient temperature have consequences on the temperature regulation of microscope heated stages.

Digital signals that can be monitored in real time are door status, equipment alarm signals. It is even possible to read digital RS232 or RS485 interfaces.

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

Blood gas analysers provide instant and accurate pH readings without having to rely on inaccurate hand-held pH meters or more expensive continuous monitoring devices (*Swain, 2013*). In addition, blood gas analysers provide helpful pCO_2, pO_2 and electrolyte information that can aid in the overall quality management of the culture system. The hospital-type analysers are expensive and require daily maintenance and calibration. Newer hand-held devices allow for pH, pCO_2, pO_2 and electrolyte readings, using disposable cartridges with integrated calibrators.

Validation: In *ISO 15189:2012*, the international standard for accreditation of medical laboratories, validation is defined as 'confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled'. IVF is a process, and 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 (consistent high pregnancy rates). Certainly, in IVF the quality of the 'end product' cannot itself be measured, so each contributing factor (infrastructure, equipment, utilities) and all the steps of the process need to be known and controlled.

Validation should be performed for new premises, laboratory equipment, utilities and processes and procedures and should result in written reports. During the validation, in-process controls should be defined to monitor the process.

Equipment needs to be validated to provide a high degree of assurance that it will consistently meet its predetermined specifications with minimal variation. Equipment validation is broken down into three phases: 'Installation Qualification' (IQ), 'Operational Qualification' (OQ), and 'Performance Qualification' (PQ). As the first step, IQ ensures that the equipment is correctly installed according to the manufacturer's specifications, e.g. a new incubator needs be installed on a solid, vibration-free surface, the room temperature should be within a defined range, the instrument should be connected to CO_2 and mains power. During the next step, OQ, the equipment is calibrated and tests are performed to document a baseline of the critical parameters of the equipment. For the incubator, this is defining set-points for CO_2, temperature, O_2 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 verification should be traceable to international standards. Calibrated equipment should be labelled, coded or otherwise 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.

Consensus guidelines

During the consensus meeting, the attendees formed statements based on the best available evidence and, if lacking, their expert opinion. In our deliberations it was not possible to conclude that there were certain aspects which were more important than others. Rather we agreed on an important fact first formulated by Don Rieger, that everything is important in the IVF laboratory. In TABLE 2 we summarize some of the points we focused on. General recommendations were aimed at creating a scenario whereby the laboratory or culture of eggs and embryos is more a case of limiting the cumulation of stresses. It is accepted that the whole process may not be optimal, however, the fewer the stresses the higher the chance of normal offspring. In many discussions, it was noted that high-level evidence was lacking. It was also a general observation that properly designed, conducted and reported RCT were lacking. This is unfortunate, especially because randomizing what we do in the IVF laboratory should not be considered a 'scientific experiment', but a mere evaluation of what we do

TABLE 2 ALTHOUGH EVERYTHING IS IMPORTANT IN THE IVF CULTURE SYSTEM, A LIST OF 20 SUGGESTIONS IS PRESENTED HERE; THESE ARE NOT PRESENTED IN ORDER OF IMPORTANCE, NOR ARE THESE THE ONLY POINTS COVERED IN THE CONSENSUS DOCUMENT

IVF culture aspect	Consensus suggestion
1	The chance of every patient having a healthy baby is the most important outcome. Cumulative live birth rate per woman or started cycle and other parameters should be monitored. High-level evidence is still lacking.
2	Gamete and embryo assessment practice should be based on expert or professional body, peer-reviewed, published and evidence-based guidelines.
3	Physicochemical conditions must be maintained during assessment, but evidence for optimal timing and frequency is lacking.
4	Evaluation of embryo culture must include embryo viability (in-vivo development) after transfer. Appropriate measures are implantation rate, live babies born per embryo transferred, embryo and fetal loss rate.
5	Temperature validation is critical for each step of IVF. Evidence supports maintenance of 37°C during all aspects of culture, but humidified and non-humidified incubator environments may both be supportive depending on conditions.
6	Measurement of pH and of CO_2 concentration can provide effective QC. Evidence supports culture of embryos in a low oxygen (normally 5% O_2) iso-atmospheric condition.
7	Micromanipulation validation should include temperature mapping of the stage (dish) to determine the maximum period the oocyte can be maintained at ~37°C and the lowest light setting should be chosen.
8	It is critical that mechanical (shear) stresses, as well as temperature changes and pH shifts, be minimized during all assessment and pipetting procedures.
9	Validated QM (SOP) programmes for routine monitoring of incubator performance should include daily assessment of temperature, CO_2, O_2 and humidity. Pre-established targets with tolerances (warning and control limits) for each variable are required as benchmarks.
10	Incubator supply gases should be filtered to remove particulates and contaminants. For pre-mixed incubator supply gases, the individual gas levels in the mixture should be verified and liquefied CO_2 level must be monitored.
11	Select buffers (i.e. HEPES and MOPS) appear to be safe for stabilizing pHe outside the culture incubator, but consequences of prolonged exposure are unknown.
12	Commercially manufactured culture medium intended for human ART should be used with adherence to protein supplementation guidelines from manufacturers.
13	There is currently insufficient evidence to support the addition of bioactive compounds, such as growth factors, to gamete and embryo culture medium. Further safety and efficacy studies are required before their routine inclusion.
14	SOP to verify acceptability for receipt and use of all contact materials, in accordance with best practice and local regulations. This includes keeping permanent records of Certificates of Analysis, lot/batch numbers. The laboratory should monitor ongoing performance of these materials.
15	Single-step media and sequential culture are both supportive of development and high outcomes. Current evidence is limited, and insufficient to demonstrate one culture system to be superior over the other.
16	Culture media must be kept and monitored under manufacturer's recommended storage conditions. Appropriately maintained and manufactured storage refrigerators must be monitored diligently.
17	Independent continuous monitoring of critical parameters of laboratory infrastructure and equipment is desirable and may be mandatory in certain countries.
18	Culture media batches can be validated by using accepted KPIs such as the Vienna consensus.[a]
19	Spermatozoa for ART must be efficiently separated from the seminal plasma environment as soon as possible after ejaculation.
20	Spermatozoa preparation for ICSI should be held at ambient temperature to minimize ROS generation.

ART = assisted reproductive technology; ICSI = intracytoplasmic sperm injection; KPI = key performance indicator; QC = quality control; QM = quality management; ROS = reactive oxygen species; SOP = standard operating procedure.

[a] *ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*.

every day. When discussing what is best to do in the IVF laboratory, the group considered cumulative live birth rate to be the outcome of interest (*Maheshwari et al., 2015*; *Wilkinson et al., 2016*). Ultimately not only the cumulative live birth rate, but also data on safety (for both the woman and the forthcoming child), time to pregnancy, and costs need to be taken into account. For many of the topics discussed, high-level evidence on outcomes was lacking. Also, the group primarily relied on data reported per woman or per started cycle, rather than per embryo or per transfer, as the latter could be misleading when evaluating any part of the IVF treatment (*Griesinger, 2016*). Examples mentioned during the meeting were reports on increased fertilization rates, and increased implantation rates, while in these same studies a decrease of live birth rate was reported (*Kleijkers et al., 2016b*; *Mastenbroek et al., 2007*). The group acknowledged the shortcomings of these outcome measures in evaluation of laboratory treatment efficacy, while at the same time recognizing their value for validation and monitoring of laboratory performance (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*). Validation and monitoring are used to ensure laboratory performance within predefined limits, but the decision on what to do comes down to treatment efficacy.

Assessment practices

It is critical to select and follow evidence-based guidelines for good laboratory and assessment practice. Gamete and

embryo assessment practice should be based on expert or professional body, peer-reviewed, published, evidence-based guidelines that are the most appropriate for each setting.

All possible measures should be taken to maintain physcochemical conditions during assessment (considering assessment timing and frequency, dish set-up/oil overlay, buffering, temperature control, consumables, equipment and laboratory environment).

It was agreed that at present there is no evidence for the optimal timing and frequency of assessments. Until RCT data are available, these practices should be established for each individual laboratory considering risks and benefits. In doing so, each laboratory should define, monitor and validate that their assessment practice is effective and fit for purpose within their operating environment, using published KPI.

Ultimately, the evaluation of embryo culture must include embryo viability (in-vivo development) after transfer. Appropriate measures of in-vivo development include implantation rate, live babies born per embryo transferred, embryo loss rate and fetal loss rate. Conversely, pregnancy rate, clinical pregnancy rate, live birth rate, miscarriage rate and any cumulative rate do not account for the number of embryos transferred and/or the number of transfers, and thus are not appropriate measures of embryo viability.

Temperature

Available evidence supports the maintenance of human oocytes and embryos at 37°C during culture. It could be that there is a range of acceptable temperatures, but this range has not yet been determined. If the available equipment cannot provide a tight temperature control, then a slightly lower temperature is probably better than a slightly higher one.

Humidity

Humidified and non-humidified incubators can both yield acceptable clinical results, but currently available comparisons in humans seem to demonstrate a benefit of humidified culture (*Fawzy et al., 2017a*); specific culture conditions, such as oil volume/depth, medium surface area to oil interface, ratio of oil to medium, room humidity, pre-humidification of oil, medium droplet size, as well as medium changes, can affect individual results (*Mortimer et al., 2018*). As part of the validation of their systems, individual laboratories are encouraged to determine whether humidity is required in their culture system, while respecting local legislation and regulations.

Further research is encouraged to identify the critical parameters in defining these systems.

CO_2 and medium pH

Technically, it is the partial pressure of CO_2, rather than its proportion of the gas mixture, that maintains the pH of a bicarbonate-buffered solution. Therefore, if reporting % CO_2 it should be clarified whether this measurement is at sea level, as the relative proportion is affected by altitude.

Medium pH is a critical factor in embryo culture. Because pH is dependent upon the CO_2 concentration in a bicarbonate-buffered solution, then the measurement of pH and of CO_2 concentration can provide effective QC.

The decision about which of these parameters to measure and the frequency of measurement should be based on the laboratory's policies and SOP (with reference to established targets and tolerances). Measurements should be conducted using calibrated and validated sensors that are independent of the incubator (i.e. in addition to the incubator's displays).

Oxygen

It was the general consensus that there is robust evidence to support culture of embryos in a 5% O_2 atmosphere (although one expert noted that they felt the evidence is equivocal for Day 3 culture). There is evidence that there is greater efficiency in embryo culture with 5% O_2, but no studies have compared the health trajectories of children born from embryos cultured in 5% versus 20% O_2, and it was the consensus that these studies should be performed using retrospective data.

Micromanipulation

There are very few studies that address the optimization of sperm preparation methods for ICSI, although there is evidence that direct pelleting of spermatozoa from whole semen should be avoided if possible.

During the ICSI procedure, the aim is to maintain the temperature of each oocyte in the dish as close to 37°C as possible throughout the duration of the procedure, regardless of microscope stage temperature. This is best achieved with the use of a heated stage or warming plate. Validation of the micromanipulation rig should include temperature mapping of the stage and the dish to determine the maximum period that an oocyte can be maintained at 37°C in the injection area. Achieving this is only possible if the ambient environment is constant. If the external environmental conditions are changed, the system must be revalidated. For this reason, micromanipulation rigs should not be located in areas of active air flow.

Oocytes are sensitive to the blue-green end of the visible light spectrum, so these wavelengths should be avoided where possible. The ICSI procedure should be performed at the lowest intensity of light possible while still being able to visualize the gametes precisely.

It is critical that mechanical (shear) stresses be minimized. Preparation of oocytes for ICSI requires removal of cumulus oophorus and corona radiata cells. To minimize exposure to mechanical (shear) stress, complete removal of these cells is unnecessary beyond that required to perform the task effectively. In addition, the diameter of the stripping tip should be appropriate for the diameter of the particular oocytes.

Workstations

Workstations should be designed and operated to maintain oocytes and embryos as close to 37°C as possible while also minimizing pH shifts. When working with dishes without oil, every possible precaution must be taken to minimize evaporative cooling.

Travel distances between workstations and incubators should be as short as possible. This is an important matter of ergonomics in laboratory design.

Incubators

A validated QM programme for regular, routine monitoring of incubator performance should use independently calibrated devices for daily assessment of temperature and CO_2, with O_2 and humidity monitored as appropriate according to SOP.

Pre-established targets with tolerances (warning and control limits) for each variable are required as benchmarks for assessing performance. Data should be captured with dataloggers or hand-entered into control charts. Readings that cross warning or control limits should be investigated promptly.

The use of incubators that have thermal conductivity sensors for CO_2 measurement is discouraged. If incubators with thermal conductivity CO_2 sensors *are* used, humidity measurement and management are mandatory.

Incubator supply gases should be filtered before they enter the incubator, to remove particulates and gaseous contaminants. For pre-mixed incubator supply gases, the individual gas levels in the mixture should be verified. For incubators with internal gas mixers, there must be verification of the gas mixture achieved within the incubator.

CO_2 supply pressure must be monitored to ensure that there is always liquefied gas in the supply cylinder. This is critical to reduce the risk of release of VOC that are dissolved in the liquid CO_2 (*Mortimer et al., 2018*).

Handling practices

Every attempt should be made to maintain the temperature of the oocytes and embryos as close as possible to 37°C in every step of the IVF and in vitro culture process. This could be achieved through the use of a controlled environment chamber, but there are no RCT data available at this time to establish this benefit.

Care should be taken to manage heat loss in pipettes and oocyte collection needle sets. For example, holding multiple COC in the pipette while searching for other oocytes during the retrieval procedure should be avoided.

If a carrier is to be used when moving dishes between an incubator and a workstation, the choice of carrier type should be based on results from simulated procedures in which temperature of the dish contents was assessed.

The frequency of oocyte and embryo assessment has already been addressed in other consensus publications (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*; *ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*).

Culture media – buffering and pH

Maintaining a narrow and stable pHe is important for gamete and embryo quality. Ideal pHe is difficult to define and may vary based on media and ingredients (single versus sequential media; 7.20–7.35), as well as temperature. The CO_2 level appropriate for each laboratory's culture conditions must be determined and maintained closely. The appropriate CO_2 level will vary based on elevation and media composition.

Select buffers (i.e. HEPES and MOPS) appear to be safe for stabilizing pHe outside the culture incubator.

Culture media – general composition and protein supplementation

IVF laboratories should use products in general conformance to the manufacturer's instructions, and to local regulations.

Culture and micromanipulation media should contain protein, carbohydrates, a complex mixture of amino acids with glutamine in the form of a dipeptide, and usually antibiotic(s). The use of phenol red as a pH indicator is optional.

For reasons of quality, consistency and reproducibility, laboratories should use commercially-manufactured culture medium intended for human ART use. In certain jurisdictions this is a regulatory requirement. It should be noted that supplementation of media with exogenous protein beyond the manufacturer's recommendations could change the composition and performance of media.

A majority of the practising embryologists in the group expressed a belief that manufacturers should provide the formulations of culture media based on the need for a consensus regarding what constituent should be added to a culture medium and at what concentration; there were concerns that undeclared constituents of a culture medium can lead to unpredictable harm to future generations. Several members of the consensus, including Dr Don Rieger, abstained from this discussion due to potential conflicts and for other reasons.

It was the panel's opinion that there is currently insufficient evidence to support the addition of bioactive compounds, such as growth factors or complex proteins, to gamete and embryo culture medium, and that safety and efficacy studies are required before their routine inclusion.

IVF laboratories must have policies and SOP to verify acceptability for receipt and use of all contact materials, in accordance with best practice and local regulations. This includes keeping permanent records of certificates of analysis, and lot/batch numbers. The laboratory should monitor ongoing performance of these materials.

Culture media – sequential or 'single-step' media for embryo culture

A recent Cochrane Review (*Youssef et al., 2015*) concludes that there are no differences in outcomes between the two culture systems, and this conclusion is supported by other systematic reviews (*Dieamant et al., 2017*; *Sfontouris et al., 2016*). However, *Sfontouris et al. (2016)* concluded that: 'Although using a single medium for extended culture has some practical advantages and blastocyst formation rates appear to be higher, there is insufficient evidence to recommend either sequential or single-step media as being superior for the culture of embryos to days 5/6. Future studies comparing these two media systems in well-designed trials should be performed.'

The studies suggesting effects on mosaicism and follow-up cognition were judged to be of low quality. Further, the panel found no evidence of a difference between cleavage- versus blastocyst-stage transfer for cumulative pregnancy rates derived from fresh and frozen–thawed cycles following a single oocyte retrieval, but cautioned that the evidence for this outcome was very low quality.

Culture media – use and management/cold chain/storage

Culture media must be:

- stored refrigerated at 2–8°C (https://www.cdc.gov/vaccines/hcp/admin/storage/toolkit/index.html);
- kept away from light (and particularly direct sunlight);
- discarded when shelf life (i.e. expiry date) has been exceeded;
- labelled with expiry date and storage conditions; this information should also be on all product inserts;
- used in order of their lot/batch numbers

Light: To protect gametes and embryos from exposure to potentially damaging wavelengths of light, use filters in microscopes to block ultraviolet. Relative to microscope illuminations, the effect of ambient light stress is low, and this could be taken into account when planning a new laboratory.

Temperature and transportation:

- Any culture medium that has been frozen must be discarded.
- Culture oil will not freeze but oil can show cloudiness, which usually reflects reduced solubility of water or other molecules at low temperatures; this should be reversible at ambient temperature. If so, the oil does not need to be discarded.

Elevated temperature can lead to:

- Degradation through oxidation (relative loss of active ingredients).
- Amino acids and proteins may undergo deamination (ammonium ions; main sources of ammonia are unstable monopeptides of glutamine and protein sources).

Refrigerated storage of culture media (https://www.cdc.gov/vaccines/hcp/admin/storage/toolkit/index.html):

It was the consensus that IVF laboratories should use 'clinical grade' refrigerators with wire shelves for circulation, no closed bins, and no door shelves. The use of combined freezer/refrigerator units with no compartment control is discouraged. The efficiency of a refrigerator, in terms of maintenance of a stable temperature, is dependent on load, which should be in the range of 30–80% of its storage capacity.

Independent monitoring and logging of refrigerator temperature is vital, using a certified, calibrated thermometer, with remote monitoring and automatic alarm.

Equipment monitoring and validation

Independent continuous monitoring of critical parameters of laboratory infrastructure and equipment is desirable at the very least, and is mandatory for some accrediting/licensing authorities.

Validation that the equipment performs as per its specifications is normally performed by the manufacturer or their agent, at the time of installation ('Installation Qualification'). Verification of user requirement specifications and equipment performance is undertaken by the user after installation and before first use ('Operational Qualification') and again at regular intervals, including after servicing or repair ('Performance Qualification'). In these cases, the purpose is to establish that the equipment's performance is within accepted tolerance ranges of its specifications (*ISO 15189:2012*; *Mortimer and Mortimer, 2015*).

Culture medium validation and monitoring

There are different types of KPI that monitor different aspects of laboratory performance, as per the Vienna consensus (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*).

Test equipment – calibration and certification

Test equipment for the laboratory and/or culture environment should be certified and calibrated according to the manufacturer's recommendations. In the absence of recommendations, calibrations should be conducted at least twice per year, or more often if required to ensure ongoing fitness for purpose.

Accurate pH measurements may be difficult to achieve due to calibration with buffers not designed for use at 37°C and therefore should be interpreted with caution. Point-of-care blood gas analysers with autocalibration function could provide more accurate measurements. It should be noted that blood gas analysers do not measure the pH of zwitterion-buffered media reliably.

Sperm preparation for IVF

Spermatozoa for ART must be separated from the seminal plasma environment not only as soon as possible after ejaculation (allowing for liquefaction), but also as efficiently as possible (*Björndahl et al., 2010*; *Mortimer, 2000*). Depending on intended use, the spermatozoa are then suspended in culture media either capable of supporting in-vitro capacitation (e.g. for IVF) or not (ICSI).

For IVF sperm preparations a balance is required between achieving in-vitro capacitation and avoiding premature acrosome loss and sperm senescence that can occur in men who are 'fast capacitators' when their spermatozoa are incubated for prolonged periods under capacitating conditions. So, if there will be a long delay before IVF insemination (e.g. >2.5 h) then the prepared spermatozoa could be resuspended in modified medium (e.g. HEPES-buffered) and held at ambient temperature until about 2.5 h before insemination and then washed and resuspended in fertilization medium and incubated in a CO_2 incubator for the final 120 min before inseminating the oocytes.

Spermatozoa being prepared for ICSI can be processed and resuspended using a sperm 'buffer' because capacitation is irrelevant for fertilization using ICSI. The preparation should be held at ambient temperature to minimize ROS generation (e.g. in a Styrofoam box to protect it from light and cold drafts) until the ICSI dish is prepared.

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