

To QC or not to QC: the key to a consistent laboratory?

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Abstract. A limiting factor in every embryology laboratory is its capacity to grow ‘normal’ embryos. In human *in vitro* fertilisation (IVF), there is considerable awareness that the environment of the laboratory itself can alter the quality of the embryos produced and the industry as a whole has moved towards the implementation of auditable quality management systems. Furthermore, in some countries, such as Australia, an established quality management system is mandatory for clinical IVF practice, but such systems are less frequently found in other embryology laboratories. Although the same challenges of supporting consistent and repeatable embryo development are paramount to success in all embryology laboratories, it could be argued that they are more important in a research setting where often the measured outcomes are at an intracellular or molecular level. In the present review, we have outlined the role and importance of quality control and quality assurance systems in any embryo laboratory and have highlighted examples of how simple monitoring can provide consistency and avoid the induction of artefacts, irrespective of the laboratory’s purpose, function or species involved.

Introduction

Quality management systems encompassing quality control (QC) and quality assurance (QA) programmes are routinely used in diagnostic laboratories as cornerstones of everyday practice. In human *in vitro* fertilisation (IVF) clinics worldwide, there is an increase in the use of quality management systems to guarantee high-quality patient care, as well to ensure that the laboratory is maintaining consistency, thus maximising embryo quality and pregnancy rates (Gardner *et al.* 2005; Mortimer and Mortimer 2005). However, there is a great deal of variation in governmental regulations relating to quality standards in different countries. A key role of a QA and QC system in the laboratory is to facilitate repeatability and accuracy of results and provide an auditable system to ensure good laboratory practice (GLP). Therefore, although increasingly common in a commercial setting, such programmes are less frequently found in a research setting. However, because measurements of embryos are increasing on a smaller scale, with perturbations being examined on a molecular level, there is an increasing need for QC and QA in a research setting to ensure repeatability of embryo quality and responses. Therefore, whether the aim of the laboratory is to culture embryos for replacement to the mother (human or animal) with the ultimate goal of producing offspring or whether the outcome is the assessment of ‘normal’ levels of gene expression or functioning of the embryos, we argue that there is an essential role for a quality system to ensure high levels of consistent and accurate outcomes.

Susceptibility of the preimplantation embryo to the culture environment

The ultimate role of the embryology laboratory is to maintain the inherent viability of the gametes and embryos in an environment outside the female reproductive tract. The dynamic nature of preimplantation embryo development is unique because, unlike somatic cell culture, embryos are constantly changing, both in morphology and function, every day (Leese 1991; Bavister 1995). It is during this time that the preimplantation embryo changes rapidly, in just a matter of days, from a metabolically quiescent, undifferentiated single cell under genetic control from maternal transcripts into a dynamic, multi-celled embryo that has developed homeostatic mechanisms and its own functioning genome (Leese 1991; Lane 2001; Gardner *et al.* 2005). The embryo changes from a pyruvate-based metabolism solely dependent on mitochondrial oxidative phosphorylation for energy production and resembles a unicellular organism lacking many key regulatory functions for pH and osmotic control. After compaction at the eight- to 16-cell stage (dependent on species), there is a change in metabolic control to a highly glycolytic metabolism. Concomitantly, there is also a marked transition in the functional complexity of other cellular mechanisms as the embryo’s physiology becomes more like that of a somatic cell. It is the initially crude nature of homeostatic regulation in the early embryo and its subsequent development through later stages of preimplantation development that pose significant challenges in the laboratory when researchers

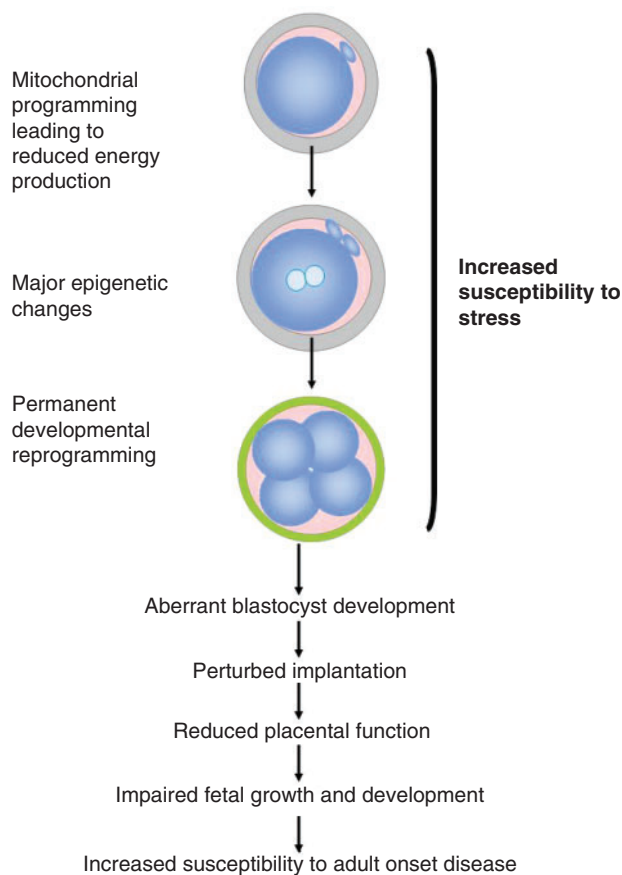


Fig. 1. Stress applied to the precompaction-stage embryo (from the pronuclear stage to the time of compaction, eight- to 16-cell stage, depending on species) appears to have lasting effects on development of the mammalian embryo. A stress applied at this early stage of development has effects on metabolic and epigenetic regulation, all of which have lasting impact on blastocyst viability and fetal growth parameters.

endeavour to optimise viability. Thus, the maintenance of a favourable *in vitro* environment that meets the changing requirements of the embryo through preimplantation development is essential for maximising viability (Bavister 1995; Gardner and Lane 1998; Gardner *et al.* 2000a, 2000b). Perturbations to the environment surrounding the embryo during development in culture, relative to 'normal' conditions encountered in the reproductive tract, result in reduced embryo viability and impaired development (Thompson *et al.* 1995). Furthermore, increasing evidence indicates that conditions or stresses encountered *in vitro* may have far-reaching effects on fetal and placental development, as well as offspring health (Fig. 1; Sinclair *et al.* 1999; Khosla *et al.* 2001; Zander *et al.* 2006; Watkins *et al.* 2007). Well-controlled conditions for the preimplantation embryo are critical for normal cellular physiology and, therefore, the maintenance of viability and future pregnancy outcomes.

The exquisite sensitivity of the early embryo, particularly before compaction, is a result of early mammalian embryos lacking many key homeostatic mechanisms that are generally found in most somatic cells (Lane 2001). Prior to compaction,

the early embryo has been shown to lack robust mechanisms for the regulation of intracellular pH and osmotic control is poor (Baltz *et al.* 1990, 1991; Phillips and Baltz 1999; Steeves and Baltz 2005). It has been demonstrated, using several different *in vitro* models, that the susceptibility of the mammalian embryo to the environment is greater at the precompaction stage (Edwards *et al.* 1998; Zander *et al.* 2006; Rooke *et al.* 2007). Therefore, perturbations in the environment, such as alterations in CO₂ levels, pH or osmotic pressure, can have major effects on the precompaction embryo, altering cellular physiology and compromising viability (Fig. 1). Using exposure to varying ammonium levels as a model, we have shown that the most susceptible stage of development is the cleavage division that coincides with activation of the embryonic genome (Zander *et al.* 2006). A stress applied at this stage results in major changes in embryo physiology, such as a decrease in blastocyst cell number and an increase in apoptosis (Zander *et al.* 2006). These cellular changes are accompanied by a significant loss in viability after transfer (Table 1). Notably, however, embryo morphology and the capacity to form a normal-appearing blastocyst are not altered by this stress (Fig. 2). The same stress applied at the post-compaction stages had no effect on embryo development, physiology or viability (Table 1). Very small alterations to the culture environment at these early precompaction stages can result in major changes in the development and functioning of the embryo.

Is QC necessary? Is embryo morphology a good marker?

The complexity of embryo development at a molecular level and the critical role that molecular signalling has in normal viability is only beginning to be understood. We, among others, have shown that severe cellular perturbations that occur when embryos are exposed to a suboptimal environment cannot be determined using morphology as a marker (Thompson *et al.* 1995; Doherty *et al.* 2000; Wrenzycki *et al.* 2001; Feil *et al.* 2006; Zander *et al.* 2006). In many instances, embryos can develop to apparently morphologically normal blastocysts. However, at a cellular level, these embryos can be severely compromised and have a reduced capacity to subsequently implant and produce a successful term pregnancy (Table 1). Therefore, it is clear that the collection and culture environment that an embryo is exposed to can significantly alter its developmental potential and cellular regulation, without altering morphology, making assessment of the quality of the culture system difficult.

To counteract this, human IVF laboratories regularly use extensive processes and equipment that help maintain the *in vitro* environment of the embryo (Mortimer and Mortimer 2005). However, the vast resources required to provide such high levels of monitoring cannot usually be replicated in a research setting. It is not uncommon for embryos to be collected and manipulated in a research laboratory in the absence of specialised equipment, such as calibrated warm stages. Unfortunately, this action alone can have a major impact on the embryo and result in adaptations to normal development. We collected mouse zygotes (C57/BL6xCBA) in either the presence or absence of specialised

Table 1. Effect of temporal exposure of mouse embryos to ammonium in the culture medium on embryo development and viability

Control, zygotes were cultured to the blastocyst stage in medium G1/G2; precompaction, zygotes were exposed to ammonium (300 μM) for 19 h to the two-cell stage before being returned to control medium; post-compaction, zygotes were cultured under control conditions until compaction and then cultured in the presence of ammonium (300 μM) to the blastocyst stage; throughout development, zygotes were cultured to the blastocyst stage in the presence of ammonium (300 μM)

Stage of exposure to ammonium	Blastocyst development (%)	Apoptotic index (%)	Fetal development per embryo transferred (%)
Control (no exposure)	72.8	2.4 \pm 0.6	41.7
Precompaction	70.4	5.8 \pm 1.8 ^A	18.1 ^A
Post-compaction	70.8	2.7 \pm 0.6	31.8
Throughout development	69.2	6.5 \pm 1.9 ^A	19.7 ^A

^A $P < 0.05$ compared with control.

Data from Zander *et al.* (2006).

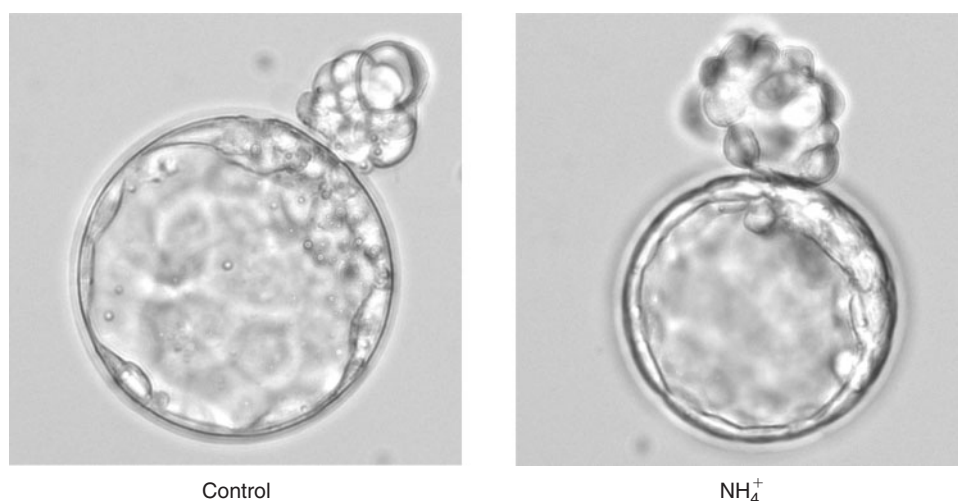


Fig. 2. Similar morphology of typical mouse blastocysts after 96 h culture, without (control) or in the presence of ammonium (NH_4^+).

warm stages calibrated to 37°C before culture under our standard conditions. Oviducts that were collected from females were allocated one to each treatment. The collection took less than 5 min and performed simultaneously by two operators who worked at the exact same pace. There was no difference in blastocyst development rates (Fig. 3a) whether embryos were collected in warm calibrated conditions *v.* those collected at room temperature, nor was there a significant difference in blastocyst cell numbers. However, there were significant alterations in gene expression by these blastocysts (Fig. 3b). For collections in well-controlled conditions, where the temperature was kept constant at 37°C, there was no difference in gene expression between cultured blastocysts and *in vivo*-produced blastocysts. In contrast, there were significant increases in the expression levels of insulin-like growth factor (IGF2) and IGF2 receptor (IGF2R) genes from blastocysts that were collected in the absence of warm stages (Fig. 3b). Therefore, significant changes in the gene expression of blastocysts could be introduced by simply collecting embryos under different temperature conditions. We conclude that care should be taken to ensure that the temperature

is constant for embryo collections and manipulations, as well as culture.

Therefore, it is critical that due consideration is given to providing consistent conditions under which embryos are collected and maintained. In our experience in a research setting, the implementation of rigid QC and QA can meet with skepticism, because embryos, particularly bovine and mouse embryos, have the ability to grow to the blastocyst stage under a wide variety of conditions. However, simply monitoring developmental parameters, such as blastocyst development, is unlikely to establish the real quality of the culture system. Therefore, in order to accurately determine that each 'run' of embryo development is consistent and of a sufficient quality, it would be necessary to examine a series of molecular markers with each experiment. Further, such measurements are usually destructive and therefore would not always be reflective of a heterogeneous group of embryos, such as bovine embryos. Alternatively, a simple solution can be the implementation of a quality management system to provide a level of assurance that no outside factor has crept into the experiment to confuse or confound the outcome.

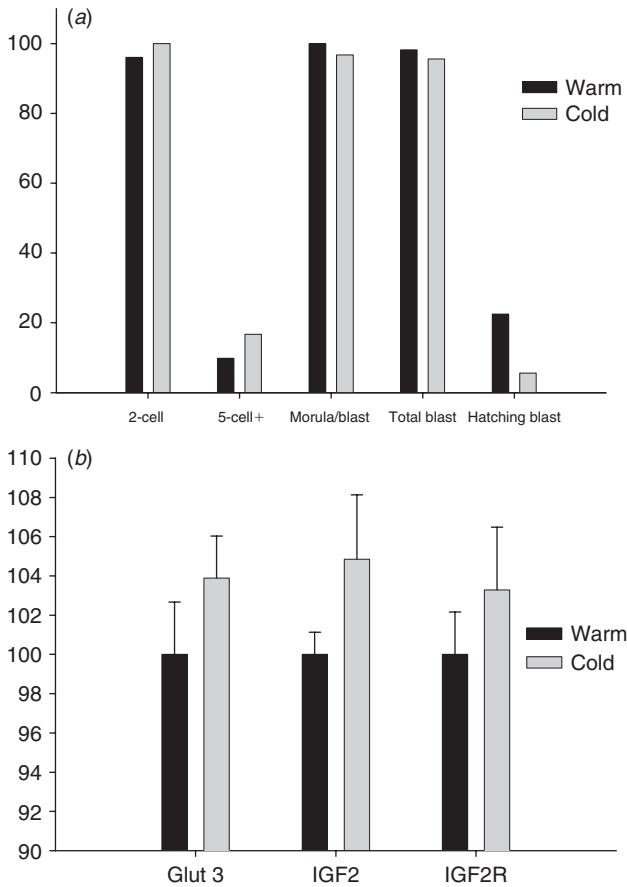


Fig. 3. Effect of culture of mouse embryos with or without a warm stage on (a) development (%) and (b) gene expression (relative levels). There was a significant increase in gene expression of IGF2 and IGF2R between treatments ($P < 0.05$).

Therefore, the establishment of a quality system that can assure the quality and consistency of the environment that the embryo is exposed to is an essential component of any laboratory that grows embryos, whether the outcomes are babies (human IVF and commercial domestic animal embryo production) or for research purposes.

Establishment of a quality management system

When establishing a quality system for the laboratory, one of the first decisions to be made is to what degree the system will be proactive *v.* reactive and what aspects should be included. The methods of how to implement such systems have been extensively detailed elsewhere (Mortimer and Mortimer 2005). The implication for a quality system with high levels of monitoring is the significant ongoing resource commitment. In our own experience of maintaining human IVF as well as research embryology laboratories, we have different standards of QC for each situation, ranging from a highly extensive, externally accredited system in the human setting to a slightly less stringent system in the research laboratory.

No matter what decision is made as to the extent of the resource commitment, it is clear that a minimal system of quality management is required in every embryo laboratory. Therefore, throughout the present review, we have attempted to identify the key aspects that need to be controlled in the laboratory and have listed appropriate monitoring options (Fig. 4).

For all the measures discussed in the present review, it is necessary to establish the tolerance levels for each individual laboratory. The tighter the tolerance levels, the more consistent and repeatable the culture environment will be. However, this also has significant resource implications, because a large personnel time is required for equipment maintenance and monitoring and perhaps also investment in additional equipment. Methods for the establishment of tolerance levels have been elegantly described

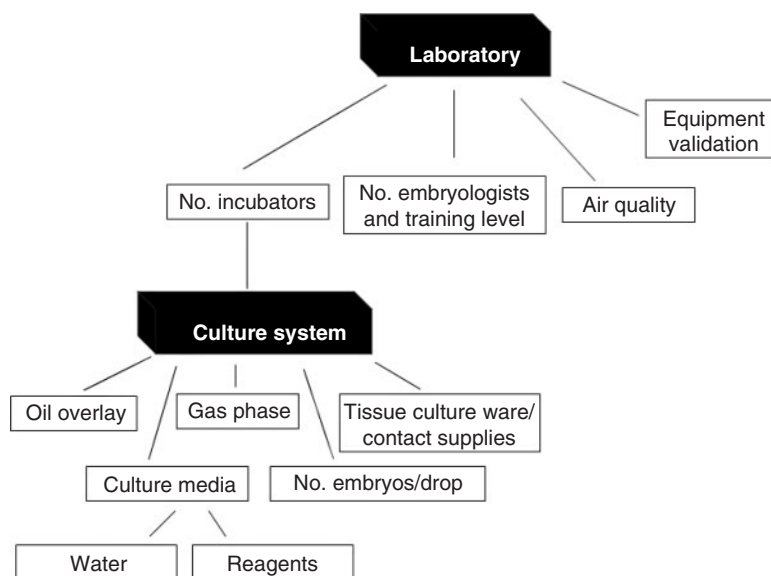


Fig. 4. Different aspects of a quality management system that should be monitored in an embryology laboratory (adapted from Gardner *et al.* 2005).

elsewhere and so are not discussed in detail here (Mortimer and Mortimer 2005).

Equipment

The maintenance of equipment is a key aspect to providing a consistent environment that ensures normal embryo development.

Incubators

The incubator environment is the single most critical aspect to be maintained because, in most cases, the embryo will spend most of its time *ex vivo* in this environment. Incubator carbon dioxide and oxygen levels (if applicable), as well as temperature, should be checked at least once a week, although daily checking is highly recommended. Levels should be checked using calibrated equipment (e.g. a Fyrite device is commonly used to check carbon dioxide levels). Although popular, Fyrite measurement has a variance of up to $\pm 0.5\%$, thus electronic meters with infrared (IR) sensors with a variance of only $\pm 0.2\%$ should preferably be used. Whichever technology is favoured, based on an acceptable tolerance level, it is essential that the first step to any equipment reading is to calibrate (or check) against a known carbon dioxide concentration (e.g. a certified gas cylinder) before taking the incubator reading. Similarly, temperature should be checked against a certified thermometer. All readings are best performed before an incubator is opened for the first time on any given day.

Opening the door of the incubator significantly alters the environment within and studies have shown that it can take up to 1 h for carbon dioxide (Avery *et al.* 2000) and 20 min for incubator temperature levels to return to set levels (Fujiwara *et al.* 2007). In addition, culture medium temperature can take up to 30 min to equilibrate (Fujiwara *et al.* 2007). The more times the incubator is opened, the less consistent the environment is for the embryos inside. Studies with mouse (Gardner and Lane 1996) and cow (Avery *et al.* 2000) embryos showed that multiple openings of the incubator door significantly reduced cell numbers of blastocysts. Furthermore, both increases and decreases in temperature can affect embryo viability (McKiernan and Bavister 1990; Wakayama *et al.* 2004; Jousan and Hansen 2007; Sugiyama *et al.* 2007). Thus, it is important to keep these things in mind when conducting experiments, because different replicates may produce different results simply as result of incubator usage (Avery *et al.* 2000).

One simple solution to reduce the number of incubator door openings is to designate incubators as either 'working' or 'longer-term culture' incubators. Working incubators can be used for incubating media and dishes, as well as for storing embryos for activities that require more frequent opening, such as embryo assessments or change overs to fresh media. The use of signage sheets on incubators ensures monitoring of the number of openings, as well as acting as a deterrent for overly frequent use. If conditions exist such that incubators are unavoidably opened frequently, modular incubation chambers can be used. These modular incubation chambers are gassed with the appropriate gas phase for embryo development and act as mini-incubators within the larger incubator. This effectively protects the gas environment surrounding the embryo when the incubator door is opened and sustains greater development.

pH

The maintenance of culture media pH in the incubator is also important, because small changes in the CO₂ level within the incubator can have major effects on the pH of the bicarbonate buffered culture medium, based on the Henderson–Hasselbalch equation. An example of this is shown in Table 2, where we examined the effects of growing mouse embryos in a bicarbonate (25 mM)-buffered medium system in an incubator calibrated to have a CO₂ concentration of either 6% or 5%, giving an extracellular pH level based on the Henderson–Hasselbalch equation of 7.30 and 7.40, respectively (M. Lane and D. L. Zander-Fox, unpubl. obs.). Measurements of embryos from all species measured to date indicate that intracellular pH levels are in the range 7.2–7.3 (Baltz *et al.* 1991; Dale *et al.* 1998; Lane *et al.* 1998; Lane and Bavister 1999). Therefore, a CO₂ level of 5% indicates a small pH elevation of between 0.1 and 0.2 pH units above optimal. Because embryos have a limited capacity to regulate intracellular pH, this would result in a stress on the embryo to maintain intracellular pH at optimal levels. When embryos were cultured in an atmosphere of 5% CO₂, there was a significant reduction in blastocyst cell numbers compared with culture in 6% CO₂ (72.1 ± 2.1 v. 84.5 ± 2.4 , respectively; $P < 0.05$, Student's *t*-test). Furthermore, and perhaps more significantly, there was a significant reduction in the implantation rates and fetal development rates after embryo transfer when embryos were cultured in 5% CO₂. It is clear that there is a significant effect of the CO₂ concentration within the incubator on pH and it is essential that the incubator is set consistently to maintain pH and ensure reliable experimental outcomes. We use CO₂ level monitoring by an IR sensor whenever possible and maintain our incubators between 6% and 6.5% CO₂ in order to preserve medium containing 25 mM bicarbonate in a pH range of 7.2–7.3, as per the Henderson–Hasselbalch equation.

The removal of bicarbonate-buffered culture medium from a controlled CO₂ environment results in an immediate degassing and loss of dissolved CO₂ from the medium. Thus, there is an immediate increase in media pH, which can reach levels that are detrimental to embryo development in 1–2 min. One simple method to avoid such rapid changes is the use of an oil overlay, which can significantly slow the loss of CO₂ from the medium and allow pH to be maintained while embryos are manipulated or assessed outside the incubator. However, an oil

Table 2. Effect of incubator CO₂ levels on the development and viability of mouse zygotes cultured to the blastocyst stage

There was no difference in blastocyst development; however, resultant blastocysts had significantly lower cell numbers and significantly reduced fetal development rates after transfer. All other conditions of the culture were maintained consistently and therefore the effect seen is a result of the different set-point of the CO₂ within the incubator. $n > 100$ embryos cultured per treatment

Incubator CO ₂ level	Implantation rates on Day 18 (%)	Fetal development per embryo on Day 18 (%)
5%	68.5 ^A	38 ^A
6%	82.3	62.5

^ASignificantly different from 6% CO₂ as determined by Fisher's exact test.

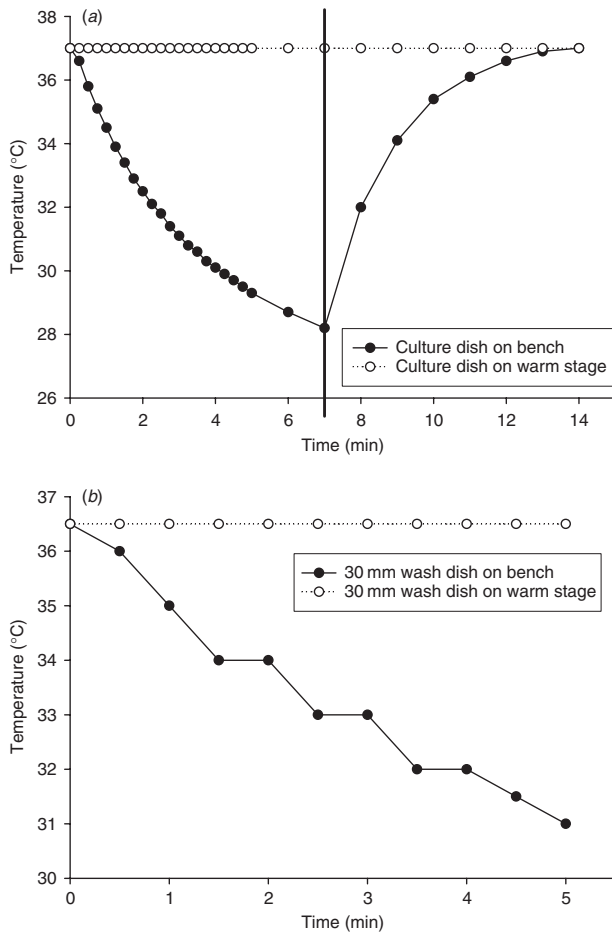


Fig. 5. Measurement of heat loss of media in culture dishes after removal from the incubator and placement on either on a calibrated warm stage or at room temperature for (a) 50- μ L drops of medium under oil. After 7 min, dishes that had been removed from the warm were returned to the warm stage and observation was continued. (b) 3 mL medium without an oil overlay. After just 2 min at room temperature, there is a highly significant loss in temperature of the medium resulting in significant cooling and stress on the cultured embryos.

overlay does not prevent the loss of CO₂; rather, it reduces the rate of CO₂ diffusion and, therefore, the time that dishes are removed from incubator should be kept to a minimum. For embryo manipulation and collection, a medium buffered with *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) or 3-(*N*-morpholino) propanesulfonic acid (MOPS) should be used. Such media maintain pH in an air environment, are commonly available in formulations similar to culture medium (i.e. containing amino acids) and are an effective way of maintaining pH during prolonged (>5 min) manipulations.

Microscope warm stages and heating blocks

As mentioned above, the use of microscope warming stages for embryo culture can have a major impact on the cellular physiology of developing embryos. Replicates may differ if heated stages are not used or if they differ in temperature. Microscope warm stages and heating blocks should be calibrated in a manner

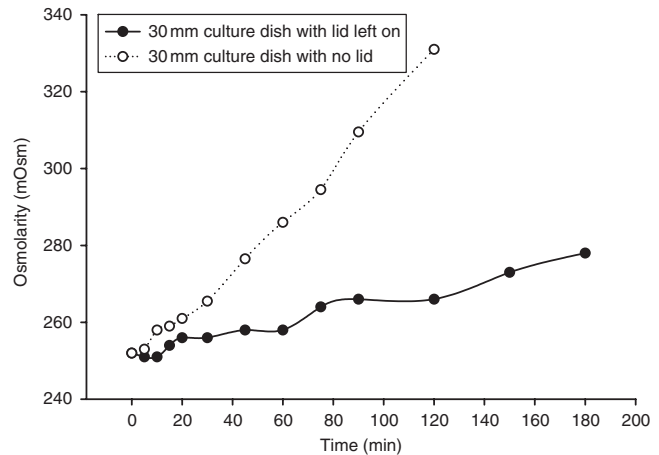


Fig. 6. Determination of the increase in osmolality that occurs when medium is incubated in a non-humidified environment. When 3 mL medium is placed in a dish on a warm stage at 37°C without a lid, there is a 5% increase in osmolality after just 30 min, which increased to a 14% rise after 1 h. This increase would be greater with smaller volumes or increased temperature. The increase in osmolality was less pronounced in a dish covered with a lid.

that takes into account the loss of heat across the culture dish (i.e. the heated stage should be set at a slightly higher temperature than is required for culture; Fig. 5). A study conducted by Cooke *et al.* (2002) showed that media at 37°C in a four-well dish can decrease up to 2°C in 30 s, depending on the type of heated stage. However, all heating stages tested lost at least 1°C in the first 5 min and some lost >3°C during this time. Even a short time at an increased or decreased temperature can cause irreversible stress to embryos (Pickering *et al.* 1990; Kimmel *et al.* 2002); therefore, it is essential that heated stages are set correctly and maintained. A further consideration is the culture dish itself, because dishes without a flat bottom do not come into direct contact with the heating stage, facilitating heat loss. Consequently, flat-bottomed dishes should be used to provide even heating where possible.

Osmolality

Balances and osmometers that are used to make and test media should be calibrated against known standards to ensure correct readings. Both increases and decreases in osmolality have been shown to negatively affect embryo development (Li and Foote 1996; Hadi *et al.* 2005; Fong *et al.* 2007). The calibration of pipettes is also important to ensure accurate measurement when preparing media, because the use of uncalibrated pipettes can alter concentrations of solutes, as well as media osmolality. A further consideration for the maintenance of osmolality is temperature, because the osmolality of the medium can change when media are left on a warm stage in a non-humidified environment. Placement of medium in a dish on a warm stage over time can result in a significant and unacceptable elevation in osmolality (Fig. 6). Similar to pH, the use of an oil overlay is an effective way to reduce the increase in osmolality.

Air quality

Air quality is difficult to completely control in the laboratory, but should always be considered for the provision of a quality embryology research environment. Poor air quality has a range of effects, from fertilisation failure and delayed embryonic development to a reduction in the viability of embryos, which may or may not be evident morphologically (Cohen *et al.* 1997).

Air quality can fluctuate depending on events both within and external to the laboratory. To best protect the quality of the air in the laboratory and ensure that as much incoming air as possible undergoes HEPA filtration, the ceilings and walls of the laboratory should have a minimum number of penetrations (e.g. a solid ceiling, sealed lighting and airtight utility connections; Cohen *et al.* 1998; Hall *et al.* 1998; Boone *et al.* 1999). Seals and sweeps should be fitted to doors, which should be lockable or have restricted access. The spacing of air inlets and outlets is important to avoid drafts that can result in localised temperature fluctuations, which emphasises the importance of the physical location of workstations in the laboratory. Laminar flow hoods and manipulation workstations should not be located too close to air supply vents, thus minimising disturbances to the sterile field and preventing cooling of microscope and handling warm stages. In order to further protect the internal laboratory space, storage areas should be independent of the laboratory, particularly for newly sterilised items, which release multiple deleterious compounds for prolonged periods of time.

Ideally, air within the laboratory should undergo HEPA filtration. However, at a minimum it is important to be aware of, and ideally limit, the external activities in the vicinity of the embryo laboratory that may affect air quality (Cohen *et al.* 1998; Hall *et al.* 1998; Boone *et al.* 1999). Activity related to any type of construction, especially paints, adhesives and other solvents, industrial hazards, general pollution and wind direction can be important in protecting the internal laboratory environment. In situations where air quality may be affected by external activities, measures to prevent a decrease in air quality can be taken, such as increasing the frequency of the routine HEPA filter changes or by protecting cultures with the use of modular incubation chambers gassed with certified gas to protect the quality of the air for culture.

A large proportion of the air in incubators is ambient air from the laboratory, dependent on the gaseous environment being used. Therefore, maintaining good-quality ambient air in the laboratory also affects the quality of the air contained in incubators. In addition to this, the source and quality of gases used to maintain appropriate incubation conditions should be considered as part of the quality of the environment for incubating gametes and embryos. Often producers of these gases may not be aware of the high standards required for such specialised laboratory techniques. Gas lines should consist of as little plastic or rubber tubing as possible to minimise alterations in concentration due to leakage and contain carbon filters to absorb some of the volatile agents dissolved in the atmosphere. There have been some reports that the quality of the air within the incubator can be improved by the use of specialised filters within the incubator that purify the gas entering the chamber (Merton *et al.* 2007).

Plastic-ware and consumables in the research laboratory: what should we test?

The culture of gametes and embryos is an integral part of any reproductive research laboratory, as is the use of plastic-ware and other consumables, such as gloves, media, chemicals and oil. For some time now, it has been understood in the human IVF setting that the QC of all consumable and plastic-ware is essential for maintaining an optimal environment for embryo culture, thus ensuring normal embryo physiology and subsequent pregnancy rates. The QC system in an IVF laboratory not only ensures the consistency of outcomes, but also allows the tracing of events during a laboratory procedure (Gardner *et al.* 2005). Recently, scientific societies and regulating bodies have strongly recommended that continual evaluation of the culture environment in the IVF laboratory becomes routine, if not mandatory, and that laboratory protocols should include in-house QC procedures using appropriate bioassays, such as the sperm motility bioassay or the mouse embryo assay (MEA; Tucker and Jansen 2002).

Despite the importance of QC in the human IVF setting, little or no attention has been paid to consumable and plastic-ware QC in the research setting. Multiple studies have demonstrated the effect of various toxic sources on embryo quality and growth. For example, exposure of embryo culture media to powdered latex gloves can completely inhibit blastocyst development (Reddy *et al.* 1999) and exposure to ethylene oxide, a commonly used sterilising agent, can inhibit embryo cleavage, despite a 12-day aeration period (Holyoak *et al.* 1996).

The purchasing of embryo toxicity tested plastic-ware is essential, including all plastics that are used for media storage, and these should be discarded after use and never re-used. However, because there is no standard testing regimen, the type of embryo toxicity testing should be evaluated against internal standards and tolerance levels determined for the research laboratory, because different conditions can affect the sensitivity of the assay (see below).

Culture media

Media preparation is a time-consuming and often complex task and requires appropriate training and technical competence. In clinical settings, it is now highly unusual for individual laboratories to prepare their culture medium 'in-house' because of the enormous burden of QC that accompanies each batch. This helps ensure that no artefact is introduced into the system that may disturb embryo physiology and alter pregnancy potential. Therefore, IVF clinics routinely purchase aseptically produced media formulated under good manufacturing procedures (GMP) under the control of various regulatory, QC and inspection systems.

However, concerns surrounding the introduction of artefacts into the culture system exist in all laboratories in the practice of growing embryos, irrespective of whether the laboratory is a clinical IVF laboratory or one growing embryos for commercial production or research purposes. Therefore, every batch of medium that is used for the development of embryos should undergo a basic set of QC and QA to determine consistency. This aids in reducing batch-to-batch variation and provides consistent and repeatable results. The storage of media, with regards

to temperature, moisture and humidity for example, is also an important consideration that should not be overlooked.

The quality of the chemicals, water and equipment is paramount to producing high-quality media. Ideally, all new chemicals should be tested for embryo growth compatibility, using the MEA for example, to ensure they are not embryo toxic. Wherever possible, chemicals should be of pharmacopeia grade, stored correctly (i.e. at the correct temperature, humidity and light conditions) and are not cross-contaminated. The equipment required to make the media, such as glassware, must also be clean and sterile to prevent the transfer of impurities to the media (Gardner and Lane 1999). The water used to make the media should be of the highest quality and be free of bacterial contamination and endotoxins, which themselves can affect embryo development (Rinehart *et al.* 1988). Each new batch of medium should be tested. We routinely test our 'in-house' media preparations for osmolarity, pH, bacterial load and endotoxin levels; in addition, we use MEA.

Storage of media and the invisible demon, ammonium

It has been clearly established across all mammalian species that amino acids are important regulators of development and, as such, all media designed to culture mammalian embryos should now contain amino acids. One of the consequences of including amino acids, in particular glutamine, is that it breaks down at 37°C to produce ammonium in the culture medium (Gardner and Lane 1993; Lane and Gardner 2003). The high levels of ammonium that are produced by medium containing glutamine (1 mM) incubated at 37°C in just 24 h are sufficient to inhibit embryo development (Gardner and Lane 1993; Lane and Gardner 2003; Orsi and Leese 2004; Virant-Klun *et al.* 2006). As mentioned earlier, although the build up of ammonium in the medium does not alter blastocyst development and morphology, the cellular health of embryos is significantly affected, including altered levels of gene expression, epigenetic regulation and viability (Gardner and Lane 1993; Zander *et al.* 2006).

Although glutamine is quite labile in culture media, it can be substituted with a stable dipeptide of glutamine, such as alanyl-glutamine, which provides the benefits of glutamine without the problem of the toxic build up of ammonium. Therefore, care should be taken to ensure that media containing glutamine are never stored at high temperatures.

Mouse embryo assay: the QC 'gold standard'?

Although the preimplantation MEA is one of the most widely used bioassays for screening laboratory consumables, media and equipment in the human IVF setting, it is not used routinely in the research environment. The ability of the MEA to detect toxins has been the subject of some contention and this is primarily due to differences in various MEA bioassay protocols, which vastly influence the effectiveness of the assay. Embryo stage (i.e. one- or two-cell embryos) can influence assay sensitivity, as can media composition and the presence of serum albumin (Fig. 7).

Many companies and IVF laboratories use the two-cell mouse bioassay to detect toxins in consumables, but it is now widely accepted that the one-cell stage bioassay is more sensitive in responding to toxins in the culture environment (Davidson *et al.*

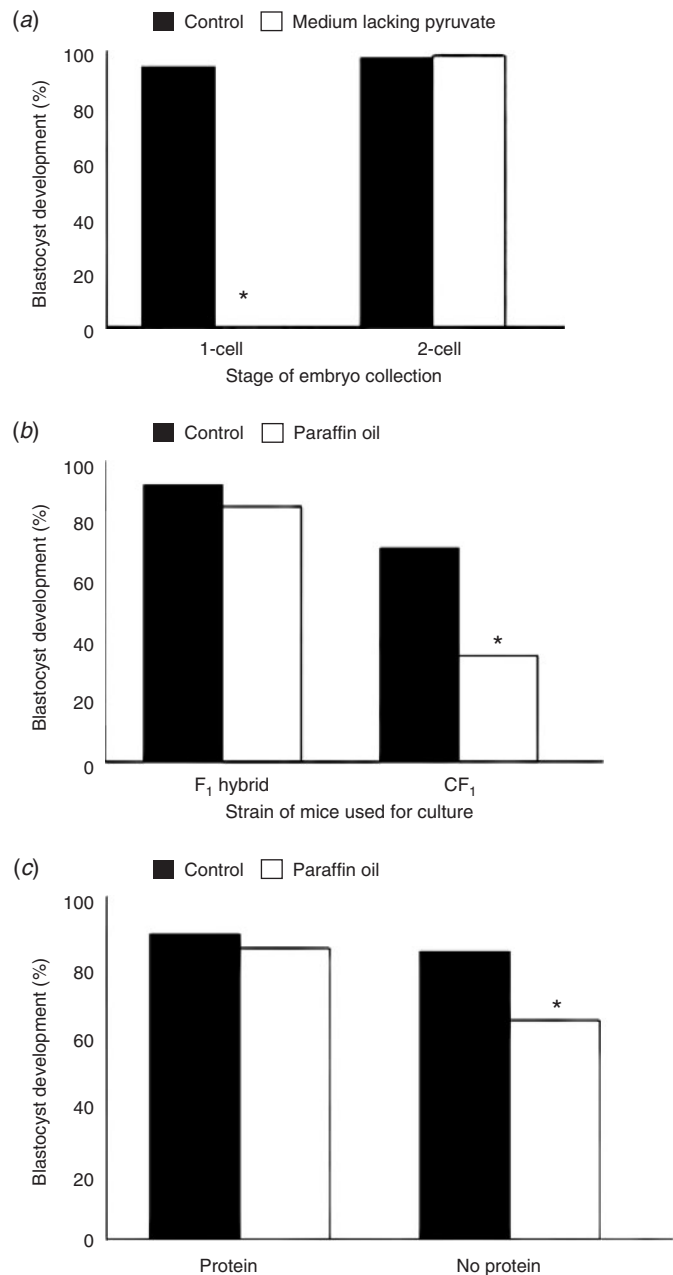


Fig. 7. Parameters of culture conditions on the sensitivity of a mouse embryo assay (MEA). For each MEA test, the outcome (i.e. whether blastocyst development rates of >80% are achieved) is altered by the conditions of the assay. (a) Stage of embryo collection: one- or two-cell stage. (b) Strain of mouse: F₁ hybrid females or CF₁ outbred females. (c) Presence or absence of serum albumin. *, significantly different from control ($P < 0.05$).

1988; Tucker and Jansen 2002). This is because the first cell division in the mouse preimplantation embryo appears to be the most sensitive stage to *in vitro* stress (Zander *et al.* 2006). Mouse strain can also influence assay sensitivity, with inbred strains and F₁ hybrids being less sensitive to their environment than outbred strains, whose embryos can block at the two-cell stage if certain media are used.

The presence of amino acids and albumin can also affect the sensitivity of the MEA because both have the ability to chelate toxins and perhaps mask their presence (George *et al.* 1989). Therefore, it has been postulated that the ideal MEA assay uses an inbred hybrid mouse strain from the zygote stage of development, cultured in a simple medium (lacking in amino acids) in the presence of albumin from the zygote to the two-cell stage and then without albumin through to the blastocyst stage to prevent the masking of any toxins (Gardner *et al.* 2005).

Because blastocyst development alone is not an accurate marker of embryo viability (Lane and Gardner 1997; Zander *et al.* 2006), multiple time-points of on-time embryo development should be used. It is suggested that embryo morphology should be determined at 24, 78 and 96 h of culture to indicate that blastocyst development may be both morphologically different and delayed. Cell number, cell allocation (to the inner cell mass and trophectoderm lineages) and birth rate after transfer can also be used as other more robust end-points because these assess not only embryo development, but also blastocyst viability.

The importance of the MEA in human IVF suggests that it has a place in the reproductive research setting. Although many plastics and media manufacturers test their products for embryo toxins, some consumables used in the research setting are not tested or are tested under less-sensitive MEA bioassay protocols. Thus, there are a number of toxins commonly in the research setting that may negatively impact results.

Although it may be a little extreme to suggest that research laboratories that routinely culture gametes and embryos should practice QC to the same extent as human IVF laboratories, it is strongly advised that all plastic-ware and consumables that come into contact with gametes or culture media should be embryo toxin tested, if not by the manufacturer, then by an in-house MEA bioassay system. This will lead to an increase in the consistency of experiments without the influence of external embryo toxins, which may otherwise confound results and lead to false research outcomes.

Conclusions

Maintaining a consistent environment in the laboratory is an essential goal for preserving normal embryo physiology and, therefore, development and viability after transfer. Small perturbations in the laboratory environment can result in stress and the resultant adaptation to conditions during preimplantation stages can affect pregnancy loss and fetal growth. It is also clear that such stressors can also cause subtle alterations in the cellular physiology and genetic control of the cells of the embryo that are not evident by assessing morphology. Therefore, the establishment of robust QC mechanisms within the laboratory is essential to maintain consistency, whether the goal of the laboratory is to grow embryos for transfer to a recipient or in a pure research context, where study of the cellular or molecular make-up of the embryo is the primary objective.

References

Avery, B., Melsted, J. K., and Greve, T. (2000). A novel approach for *in vitro* production of bovine embryos: use of the Oxoid atmosphere generating system. *Theriogenology* **54**, 1259–1268. doi:10.1016/S0093-691X(00)00432-5

- Baltz, J. M., Biggers, J. D., and Lechene, C. (1990). Apparent absence of Na^+/H^+ antiport activity in the two-cell mouse embryo. *Dev. Biol.* **138**, 421–429. doi:10.1016/0012-1606(90)90208-Z
- Baltz, J. M., Biggers, J. D., and Lechene, C. (1991). Two-cell stage mouse embryos appear to lack mechanisms for alleviating intracellular acid loads. *J. Biol. Chem.* **266**, 6052–6057.
- Bavister, B. D. (1995). Culture of preimplantation embryos: facts and artifacts. *Hum. Reprod. Update* **1**, 91–148. doi:10.1093/HUMUPD/1.2.91
- Boone, W. R., Johnson, J. E., Locke, A. J., Crane, M. M., and Price, T. M. (1999). Control of air quality in an assisted reproductive technology laboratory. *Fertil. Steril.* **71**, 150–154. doi:10.1016/S0015-0282(98)00395-1
- Cohen, J., Gilligan, A., Esposito, W., Schimmel, T., and Dale, B. (1997). Ambient air and its potential effects on conception *in vitro*. *Hum. Reprod.* **12**, 1742–1749. doi:10.1093/HUMREP/12.8.1742
- Cohen, J., Gilligan, A., and Willadsen, S. (1998). Culture and quality control of embryos. *Hum. Reprod.* **13**(Suppl. 3), 137–144.
- Cooke, S., Tyler, J. P., and Driscoll, G. (2002). Objective assessments of temperature maintenance using *in vitro* culture techniques. *J. Assist. Reprod. Genet.* **19**, 368–375. doi:10.1023/A:1016394304339
- Dale, B., Menezes, Y., Cohen, J., DiMatteo, L., and Wilding, M. (1998). Intracellular pH regulation in the human oocyte. *Hum. Reprod.* **13**, 964–970. doi:10.1093/HUMREP/13.4.964
- Davidson, A., Vermesh, M., Lobo, R. A., and Paulson, R. J. (1988). Mouse embryo culture as quality control for human *in vitro* fertilization: the one-cell versus the two-cell model. *Fertil. Steril.* **49**, 516–521.
- Doherty, A. S., Mann, M. R., Tremblay, K. D., Bartolomei, M. S., and Schultz, R. M. (2000). Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol. Reprod.* **62**, 1526–1535. doi:10.1095/BIOLREPROD62.6.1526
- Edwards, L. J., Williams, D. A., and Gardner, D. K. (1998). Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum. Reprod.* **13**, 3441–3448. doi:10.1093/HUMREP/13.12.3441
- Feil, D., Lane, M., Roberts, C. T., Kelley, R. L., Edwards, L. J., Thompson, J. G., and Kind, K. L. (2006). Effect of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development. *J. Physiol.* **572**, 87–96.
- Fong, B., Watson, P. H., and Watson, A. J. (2007). Mouse preimplantation embryo responses to culture medium osmolarity include increased expression of CCM2 and p38 MAPK activation. *BMC Dev. Biol.* **7**, 2. doi:10.1186/1471-213X-7-2
- Fujiwara, M., Takahashi, K., Izuno, M., Duan, Y. R., Kazono, M., Kimura, F., and Noda, Y. (2007). Effect of micro-environment maintenance on embryo culture after *in-vitro* fertilization: comparison of top-load mini incubator and conventional front-load incubator. *J. Assist. Reprod. Genet.* **24**, 5–9. doi:10.1007/S10815-006-9088-3
- Gardner, D. K., and Lane, M. (1993). Amino acids and ammonium regulate mouse embryo development in culture. *Biol. Reprod.* **48**, 377–385. doi:10.1095/BIOLREPROD48.2.377
- Gardner, D. K., and Lane, M. (1996). Alleviation of the ‘2-cell block’ and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. *Hum. Reprod.* **11**, 2703–2712.
- Gardner, D. K., and Lane, M. (1998). Culture of viable human blastocysts in defined sequential serum-free media. *Hum. Reprod.* **13**(Suppl. 3), 148–159.
- Gardner, D. K., and Lane, M. (1999). Embryo culture systems. In ‘Handbook of *In Vitro* Fertilization’. (Eds A. O. Trounson and D. K. Gardner.) pp. 81–92. (CRC Press: Boca Raton.)
- Gardner, D. K., Lane, M., and Schoolcraft, W. B. (2000a). Culture and transfer of viable blastocysts: a feasible proposition for human IVF. *Hum. Reprod.* **15**(Suppl. 6), 9–23. doi:10.1093/HUMREP/15.3.694
- Gardner, D. K., Pool, T. B., and Lane, M. (2000b). Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation,

- and viability. *Semin. Reprod. Med.* **18**, 205–218. doi:10.1055/S-2000-12559
- Gardner, D. K., Reed, L., Linck, D., Sheehan, C., and Lane, M. (2005). Quality control in human *in vitro* fertilization. *Semin. Reprod. Med.* **23**, 319–324. doi:10.1055/S-2005-923389
- George, M. A., Braude, P. R., Johnson, M. H., and Sweetnam, D. G. (1989). Quality control in the IVF laboratory: *in-vitro* and *in-vivo* development of mouse embryos is unaffected by the quality of water used in culture media. *Hum. Reprod.* **4**, 826–831.
- Hadi, T., Hammer, M. A., Algire, C., Richards, T., and Baltz, J. M. (2005). Similar effects of osmolarity, glucose, and phosphate on cleavage past the 2-cell stage in mouse embryos from outbred and F₁ hybrid females. *Biol. Reprod.* **72**, 179–187. doi:10.1095/BIOLREPROD.104.033324
- Hall, J., Gilligan, A., Schimmel, T., Cecchi, M., and Cohen, J. (1998). The origin, effects and control of air pollution in laboratories used for human embryo culture. *Hum. Reprod.* **13**(Suppl. 4), 146–155.
- Holyoak, G. R., Wang, S., Liu, Y., and Bunch, T. D. (1996). Toxic effects of ethylene oxide residues on bovine embryos *in vitro*. *Toxicology* **108**, 33–38. doi:10.1016/S0300-483X(95)03268-K
- Jousan, F. D., and Hansen, P. J. (2007). Insulin-like growth factor-I promotes resistance of bovine preimplantation embryos to heat shock through actions independent of its anti-apoptotic actions requiring PI3K signaling. *Mol. Reprod. Dev.* **74**, 189–196. doi:10.1002/MRD.20527
- Khosla, S., Dean, W., Reik, W., and Feil, R. (2001). Culture of preimplantation embryos and its long-term effects on gene expression and phenotype. *Hum. Reprod. Update* **7**, 419–427. doi:10.1093/HUMUPD/7.4.419
- Kimmel, G. L., Williams, P. L., Claggett, T. W., and Kimmel, C. A. (2002). Response–surface analysis of exposure–duration relationships: the effects of hyperthermia on embryonic development of the rat *in vitro*. *Toxicol. Sci.* **69**, 391–399. doi:10.1093/TOXSCI/69.2.391
- Lane, M. (2001). Mechanisms for managing cellular and homeostatic stress *in vitro*. *Theriogenology* **55**, 225–236. doi:10.1016/S0093-691X(00)00456-8
- Lane, M., and Bavister, B. D. (1999). Regulation of intracellular pH in bovine oocytes and cleavage stage embryos. *Mol. Reprod. Dev.* **54**, 396–401. doi:10.1002/(SICI)1098-2795(199912)54:4<396::AID-MRD10>3.0.CO;2-6
- Lane, M., and Gardner, D. K. (1997). Differential regulation of mouse embryo development and viability by amino acids. *J. Reprod. Fertil.* **109**, 153–164.
- Lane, M., and Gardner, D. K. (2003). Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biol. Reprod.* **69**, 1109–1117. doi:10.1095/BIOLREPROD.103.018093
- Lane, M., Baltz, J. M., and Bavister, B. D. (1998). Regulation of intracellular pH in hamster preimplantation embryos by the sodium hydrogen (Na⁺/H⁺) antiporter. *Biol. Reprod.* **59**, 1483–1490. doi:10.1095/BIOLREPROD.59.6.1483
- Leese, H. J. (1991). Metabolism of the preimplantation mammalian embryo. *Oxford Rev. Reprod. Biol.* **13**, 35–72.
- Li, J., and Foote, R. H. (1996). Differential sensitivity of one-cell and two-cell rabbit embryos to sodium chloride and total osmolarity during culture into blastocysts. *J. Reprod. Fertil.* **108**, 307–312.
- McKiernan, S. H., and Bavister, B. D. (1990). Environmental variables influencing *in vitro* development of hamster 2-cell embryos to the blastocyst stage. *Biol. Reprod.* **43**, 404–413. doi:10.1095/BIOLREPROD.43.3.404
- Merton, J. S., Vermeulen, Z. L., Otter, T., Mullaart, E., de Ruigh, L., and Hasler, J. F. (2007). Carbon-activated gas filtration during *in vitro* culture increased pregnancy rate following transfer of *in vitro*-produced bovine embryos. *Theriogenology* **67**, 1233–1238. doi:10.1016/J.THERIOGENOLOGY.2006.12.012
- Mortimer, D., and Mortimer, S. (2005). ‘Quality and Risk Management in the IVF Laboratory.’ (Cambridge University Press: Cambridge.)
- Orsi, N. M., and Leese, H. J. (2004). Ammonium exposure and pyruvate affect the amino acid metabolism of bovine blastocysts *in vitro*. *Reproduction* **127**, 131–140. doi:10.1530/REP.1.00031
- Phillips, K. P., and Baltz, J. M. (1999). Intracellular pH regulation by HCO₃⁻/Cl⁻ exchange is activated during early mouse zygote development. *Dev. Biol.* **208**, 392–405. doi:10.1006/DBIO.1999.9199
- Pickering, S. J., Braude, P. R., Johnson, M. H., Cant, A., and Currie, J. (1990). Transient cooling to room temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. *Fertil. Steril.* **54**, 102–108.
- Reddy, V. R., Thomas, T. S., Wright, H. R., Fisher, M. D., and Edlich, R. F. (1999). The scientific basis of surgical glove selection in an *in vitro* fertilization laboratory. *J. Biomed. Mater. Res.* **48**, 569–571. doi:10.1002/(SICI)1097-4636(1999)48:4<569::AID-JBM24>3.0.CO;2-G
- Rinehart, J. S., Bavister, B. D., and Gerrity, M. (1988). Quality control in the *in vitro* fertilization laboratory: comparison of bioassay systems for water quality. *J. In Vitro Fertil. Embryo Transf.* **5**, 335–342. doi:10.1007/BF01129569
- Rooke, J. A., McEvoy, T. G., Ashworth, C. J., Robinson, J. J., Wilmot, I., Young, L. E., and Sinclair, K. D. (2007). Ovine fetal development is more sensitive to perturbation by the presence of serum in embryo culture before rather than after compaction. *Theriogenology* **67**, 639–647. doi:10.1016/J.THERIOGENOLOGY.2006.09.040
- Sinclair, K. D., McEvoy, T. G., Maxfield, E. K., Maltin, C. A., Young, L. E., Wilmot, I., Broadbent, P. J., and Robinson, J. J. (1999). Aberrant fetal growth and development after *in vitro* culture of sheep zygotes. *J. Reprod. Fertil.* **116**, 177–186.
- Steeves, C. L., and Baltz, J. M. (2005). Regulation of intracellular glycine as an organic osmolyte in early preimplantation mouse embryos. *J. Cell. Physiol.* **204**, 273–279. doi:10.1002/JCP.20284
- Sugiyama, S., McGowan, M., Phillips, N., Kafi, M., and Young, M. (2007). Effects of increased ambient temperature during IVM and/or IVF on the *in vitro* development of bovine zygotes. *Reprod. Domest. Anim.* **42**, 271–274. doi:10.1111/J.1439-0531.2006.00776.X
- Thompson, J. G., Gardner, D. K., Pugh, P. A., McMillan, W. H., and Tervit, H. R. (1995). Lamb birth weight is affected by culture system utilized during *in vitro* pre-elongation development of ovine embryos. *Biol. Reprod.* **53**, 1385–1391. doi:10.1095/BIOLREPROD.53.6.1385
- Tucker, K. E., and Jansen, C. (2002). The mouse embryo bioassay: is it the ‘gold standard’ for quality control testing in the IVF laboratory? In ‘The Art and Science of Assisted Reproductive Techniques’. (Eds G. N. Allahbadia and R. Basuray Das.) pp. 249–253. (Martin Dunitz: London.)
- Virant-Klun, I., Tomazevic, T., Vrtacnik-Bokal, E., Vogler, A., Krsnik, M., and Meden-Vrtovec, H. (2006). Increased ammonium in culture medium reduces the development of human embryos to the blastocyst stage. *Fertil. Steril.* **85**, 526–528. doi:10.1016/J.FERTNSTERT.2005.10.018
- Wakayama, S., Thuan, N. V., Kishigami, S., Ohta, H., Mizutani, E., Hikichi, T., Miyake, M., and Wakayama, T. (2004). Production of offspring from one-day-old oocytes stored at room temperature. *J. Reprod. Dev.* **50**, 627–637. doi:10.1262/JRD.50.627
- Watkins, A. J., Platt, D., Papenbrock, T., Wilkins, A., Eckert, J. J., Kwong, W. Y., Osmond, C., Hanson, M., and Fleming, T. P. (2007). Mouse embryo culture induces changes in postnatal phenotype including raised systolic blood pressure. *Proc. Natl Acad. Sci. USA* **104**, 5449–5454. doi:10.1073/PNAS.0610317104
- Wrenzycki, C., Herrmann, D., Keskinetepe, L., Martins, A., Jr, Sirisathien, S., Brackett, B., and Niemann, H. (2001). Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Hum. Reprod.* **16**, 893–901. doi:10.1093/HUMREP/16.5.893
- Zander, D. L., Thompson, J. G., and Lane, M. (2006). Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol. Reprod.* **74**, 288–294. doi:10.1095/BIOLREPROD.105.046235