human reproduction update

The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction

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BACKGROUND: Although laboratory procedures, along with culture media formulations, have improved over the past two decades, the issue remains that human IVF is performed *in vitro* (literally 'in glass').

METHODS: Using PubMed, electronic searches were performed using keywords from a list of chemical and physical factors with no limits placed on time. Examples of keywords include oxygen, ammonium, volatile organics, temperature, pH, oil overlays and incubation volume/embryo density. Available clinical and scientific evidence surrounding physical and chemical factors have been assessed and presented here.

RESULTS AND CONCLUSIONS: Development of the embryo outside the body means that it is constantly exposed to stresses that it would not experience *in vivo*. Sources of stress on the human embryo include identified factors such as pH and temperature shifts, exposure to atmospheric (20%) oxygen and the build-up of toxins in the media due to the static nature of culture. However, there are other sources of stress not typically considered, such as the act of pipetting itself, or the release of organic compounds from the very tissue culture ware upon which the embryo develops. Further, when more than one stress is present in the laboratory, there is evidence that negative synergies can result, culminating in significant trauma to the developing embryo. It is evident that embryos are sensitive to both chemical and physical signals within their micro-environment, and that these factors play a significant role in influencing development and events post transfer. From the viewpoint of assisted human reproduction, a major concern with chemical and physical factors lies in their adverse effects on the viability of embryos, and their

long-term effects on the fetus, even as a result of a relatively brief exposure. This review presents data on the adverse effects of chemical and physical factors on mammalian embryos and the importance of identifying, and thereby minimizing, them in the practice of human IVF. Hence, optimizing the *in vitro* environment involves far more than improving culture media formulations.

Key words: ammonium / embryo culture / combined stress / density / oxygen / pH / pipetting / temperature

Introduction

Assisted reproductive techniques (ART) have advanced significantly over the last three decades resulting in higher implantation and take home baby rates. Further improvements in preimplantation embryo culture systems should continue to augment success rates, while reducing the number of cycles required in achieving a pregnancy. To date such improvements in culture conditions have come largely in the form of enhanced media formulations, with relatively little attention being paid to the fact that one is trying to maintain the early events of life outside of the human body (Gardner, 2008). Fertilization and embryo development *in vitro* have the potential to introduce (often inadvertently) stresses which cannot only impair embryo development in the laboratory, but also which can have down-stream effects after transfer (Lane and Gardner, 1994). *In vivo*, the developing preimplantation embryo is exposed to gradients of nutrients, hormones, cytokines and growth factors as it progresses through the fallopian tube to the uterus (Gardner *et al.*, 1996; Hannan *et al.*, 2011; Thouas *et al.*, 2015). Within the lumen of the female tract the embryo resides in a few hundred nanolitres of a complex viscous fluid (Hoversland and Weitlauf, 1981; Salleh *et al.*, 2005), characterized by high levels of mucins, albumin and glycosaminoglycans (Lee and Ax, 1984; Leese, 1988; Laurent *et al.*, 1995; Zorn *et al.*, 1995), and by reduced levels of oxygen (typically 2–8%, Fischer and Bavister, 1993). The embryo is in constant motion, moved by gentle ciliated and muscular action of the female tract, and metabolites produced by the embryos are removed from its immediate vicinity due to its proximity to the epithelia of the female tract and hence maternal circulation. This scenario is in stark contrast to the laboratory environment, where typically gametes and embryos are exposed to relatively large volumes of culture medium (up to 100 μl per embryo, Bolton *et al.*, 2014), remain static during culture, while resting on a polystyrene substrate, and create unstirred layers where the end products of metabolism concentrate and nutrients become rate limiting (Gardner and Lane, 2012, 2014). The culture dishes themselves can release volatile organic compounds, which can adversely affect embryo developmental potential (Holyoak *et al.*, 1996). Manipulation of gametes and embryos in the laboratory is facilitated through pipetting, an act which if performed too vigorously can induce stress-activated responses (Xie *et al.*, 2007). During embryo manipulations both temperature and pH of the medium can drift, which can negatively impact gametes and embryos. In addition, if more than one stress is present in the laboratory, then there is evidence that negative synergies can result, culminating in significant trauma to the developing embryo (Awonuga *et al.*, 2013).

From the viewpoint of human ART, the major interest in chemical and physical factors lies in their adverse effects on the viability of embryos, even if exposure is for a relatively brief period of time. This review, therefore, presents data on the adverse effects of chemical and physical factors on mammalian embryos and their importance in the practice of human IVF and embryo culture. Although assisted fertilization through ICSI, embryo biopsy for genetic screening and cryopreservation can all be perceived as sources of stress on gametes and embryos, they will not be covered here.

Methods

This review presents the available evidence regarding the effect of the listed chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. A search was performed in PubMed regarding these chemical and physical factors with no limits placed on time. Keywords used included oxygen, ammonium, volatile organics, temperature, pH, oil overlays and incubation volume/embryo density. Available clinical and scientific evidence surrounding these physical and chemical factors have been assessed, interpreted and presented here.

Chemical factors

Oxygen

Analysis of oxygen within the oviduct and uterus of different mammalian species has shown that the concentration of oxygen is typically 2–8% in luminal fluids (Bishop, 1957; Mastroianni and Jones, 1965; Ross and Graves, 1974; Maas *et al.*, 1976; Fischer and Bavister, 1993). These levels are in contrast to that present in air (∼20% depending upon altitude). The first report of the beneficial effects of physiological oxygen (∼5%) for preimplantation embryo culture was published over 40 years ago by Wes Whitten. Whitten, using both inbred and hybrid mice strains, observed that whilst all 1-cell mouse embryos developed in an oxygen concentration of 5%, all arrested when the oxygen concentration was 20% (Whitten, 1969). Whitten went on to state that 'mouse embryos do have a low, but definite, oxygen requirement and that both oxygen lack and excess may damage the surface membrane'. Consequently, the gas mix of 5% O_2, 5% CO_2 and 90% N_2 was adopted as a standard in embryo culture (Whitten, 1971). Concomitantly, Edwards *et al.* (1970) reported the development of the human embryo under different oxygen conditions (5 and 20%). Comparisons were made across different media, within each oxygen concentration fertilization rates of 10.3% for 5% oxygen and 11.9% for 20% oxygen, and cleavage rates of 90.9% for the 5% oxygen group and 87.5% for the 20% oxygen group were observed (Edwards *et al.*, 1970). In a follow-up study demonstrating the growth of human blastocysts in culture, it was stated: 'we seem to have achieved this improved embryonic development through better handling of the cultures than previously . . . under reduced oxygen tension' (Steptoe *et al.*, 1971). Hence, based on the development seen in these early two studies, an environment of 5% oxygen was adopted as the standard gas mixture for subsequent cultures (Steptoe and Edwards, 1978; Edwards *et al.*, 1981; Edwards and Steptoe, 1983).

Given that relative oxygen concentration was shown to be an important factor for the development of cultured mouse embryos in these pioneering studies (Whitten, 1969, 1971), a number of investigators followed up and examined the effects of oxygen on other mammalian

species. Tervit *et al.* (1972) endeavoured to overcome the *in vitro* block in development of sheep embryos at the 8- to 16-cell stage by culturing embryos in a range of oxygen concentrations. Embryos cultured in 5% oxygen developed through the reported 8- to 16-cell block in development, with a higher proportion of late morulae and blastocysts (56%) compared with results from 10 and 20% oxygen concentrations (16 and 0%, respectively) (Tervit *et al.*, 1972). Building upon this, Quinn and Harlow (1978) examined development of inbred mouse embryos under an atmosphere containing 0, 2.5, 5, 10 or 40% oxygen and in agreement with Whitten's findings, they determined that 5% oxygen supported optimal embryo development. Of interest, the culture of mouse embryos in 20% oxygen resulted in blastocysts with fewer blastomeres than those that had developed in 5% oxygen (Quinn and Harlow, 1978; Harlow and Quinn, 1979). More recently Karagenc *et al.* (2004) demonstrated that although oxygen concentration had no effect on inbred mouse embryo implantation or resultant fetal weights, embryos cultured in the presence of 20% oxygen resulted in a dramatic decrease in fetal development per blastocyst and fetal development per implantation, compared with embryos cultured in 5% oxygen.

Over the intervening three decades several studies have gone on to demonstrate that culture in a reduced oxygen concentration, of 5–7%, improves preimplantation embryo development in a multitude of mammalian species including the sheep (Thompson *et al.*, 1990), cow (Thompson *et al.*, 1990), goat (Batt *et al.*, 1991) pig (Berthelot and Terqui, 1996) and outbred mice (Gardner and Lane, 1996). Furthermore, there is a growing body of evidence that the preimplantation embryo exhibits stage-specific sensitivity (a phenomenon highlighted throughout this review) to stress, including oxygen. Embryos exposed to atmospheric oxygen as pronucleate oocytes for as little as one hour followed by culture in 5% oxygen displayed delayed development at the morula stage (Pabon *et al.*, 1989) and blastocyst stage (Umaoka *et al.*, 1992). Subsequently, Gardner and Lane (1996) determined that even the equilibration of culture media for 6 h in atmospheric oxygen prior to culture in 5% oxygen was detrimental to subsequent embryo development (reflecting the extended time it takes to equilibrate media with 5% oxygen, which is considerably longer than that required for carbon dioxide equilibration). Consistent with these data, Karagenc *et al.* (2004) reported that exposure of pronucleate oocytes to atmospheric oxygen for 23 h resulted in a significant decrease in blastocyst cell numbers.

In order to further investigate the temporal responses of the preimplantation embryo to oxygen concentrations *in vitro*, Wale and Gardner (2010) used time-lapse microscopy to continuously assess embryo development. Mouse preimplantation embryos were cultured in 5 or 20% oxygen concentrations for the first 48 h, followed by culture in the same or reciprocal oxygen concentrations for another 48 h, resulting in four treatments: group 1 (control, 5 and 5%); group 2 (5 and 20%); group 3 (20 and 5%); and group 4 (20 and 20%). Compared with embryos cultured in 5% oxygen, embryos cultured in 20% oxygen were delayed at the first cleavage by 0.45 h, at the second cleavage by 0.84 h and at the third cleavage by 3.19 h. Of great interest, switching from 20 to 5% oxygen after 48 h could not alleviate the perturbations induced during the cleavage stages, indicating that either the pre-compaction stage is more sensitive to oxygen stress, or that the damage imparted by 20% oxygen to the cleavage stage embryos is irreversible. Partial or complete culture in 20% oxygen resulted in a significant reduction in blastocyst cell numbers compared with control.

These data reveal that oxygen can influence mouse embryo development at both the pre- and post-compaction stages (Wale and Gardner, 2010), and that the detrimental effects of 20% oxygen on the early embryo were not only irreversible but cumulative (Fig. 1a and b). Kirkegaard *et al.* (2013) subsequently replicated these study conditions with human embryos, with the early exposure to 20% oxygen reduced to 24 h. Similar results were seen, with delayed development after culture in 20% oxygen, with the pre-compaction embryo unable to recover from exposure to 20% oxygen, and culture in 20% O_2 reducing developmental rates and delaying the completion of the third cell cycle (Kirkegaard *et al.*, 2013).

Such data raise the question; *how* does atmospheric oxygen impair embryo development? Extensive analyses have revealed that oxygen regulates events during the preimplantation period, and affects all aspects of cell function. Analysis of mouse blastocyst gene expression (Gardner and Lane, 2005; Rinaudo *et al.*, 2006) demonstrated that atmospheric oxygen has a causal role. When Gardner and Lane (2005) cultured zygotes from inbred mice to the blastocyst stage in complex medium

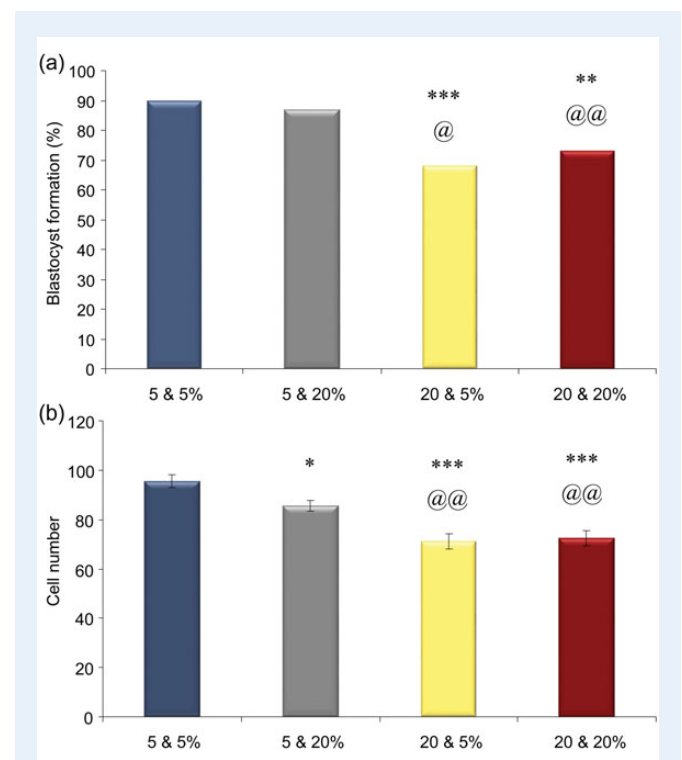**Figure 1** (**a**) Effect of oxygen on mouse blastocyst development rates. Embryos were cultured in either 5 or 20% oxygen for 48 h and then either transferred to the same or different oxygen tension, creating four groups of embryos. Significantly different from embryos incubated in control (5 and 5%): **$P < 0.01$, ***$P < 0.001$. Significantly different from embryos incubated in 5 and 20% treatment: @$P < 0.05$, @@$P < 0.01$. Data from Wale and Gardner (2010). (**b**) Effect of oxygen on mouse blastocyst total cell numbers. Embryos were cultured in either 5 or 20% oxygen for 48 h and then either transferred to the same or different oxygen tension, creating four groups of embryos. Significantly different from embryos incubated in control treatment: *$P < 0.05$, ***$P < 0.001$. Significantly different from embryos incubated in 5 and 20% treatment: @@$P < 0.01$. Data from Wale and Gardner (2010).

under 20% oxygen, 19 genes were up-regulated and 12 were down-regulated compared with the 5% oxygen group. Rinaudo et al. (2006) cultured outbred mouse zygotes to the blastocyst stage in simple and complex medium, under both 5 and 20% oxygen. Embryos cultured in 5% oxygen displayed fewer perturbations in their global pattern of gene expression, and embryos cultured in complex medium showed gene expression patterns more closely resembling those of the in vivo controls. Li et al. (2014a) subsequently examined the effects of atmospheric oxygen on global methylation patterns in bovine embryos. Compared with embryos cultured at 5% oxygen, embryos cultured at 20% exhibited a significant increase in global DNA methylation at the 4-cell and blastocyst stages, indicating that oxidative stress can induce changes in the embryonic epigenome. Additionally, chronic exposure to 20% oxygen induces irreversible X chromosome inactivation (XCI) in human embryonic stem cells (hESC). In contrast, hESC isolated under 5% oxygen do not undergo premature XCI and are more able to retain pluripotency (Lenger et al., 2010). Of note, XCI is considered a model epigenetic event (Whitelaw, 2006; Lee, 2011). More recently it has been determined that culture in an atmosphere of 5% oxygen is required for epigenetic stability in hESC, and that culture in the presence of 20% oxygen leads to aberrant methylation of the DLK1-DIO3 (Delta-like homologue 1/type 3 iodothyronine deiodinase) gene cluster, an imprinted region (Xie et al., 2014). Defects in this imprinted region are associated with developmental delay, mental retardation and even post-natal death in humans (Mo et al., 2015).

Of note, changes in the transcriptome do not always directly reflect changes in cellular function, nor do they account for post-translational modifications or protein−protein interactions. Consequently Katz-Jaffe et al. (2005) investigated the effects of oxygen on the embryonic proteome. Consistent with transcriptomic data, protein profiles from embryos cultured at 5% oxygen concentration more closely resembled profiles from in vivo developed embryos, whereas those blastocysts developed in atmospheric oxygen exhibited a divergent proteome. Looking at specific proteins, Rodina et al. (2009) determined that culture of bovine embryos in 5% oxygen, but not 20%, maintained blastocyst viability and facilitated constitutive and inducible interferon-tau production, which promotes uterine implantation. It is important to consider that although both transcriptomic and proteomic data indicate that embryos that developed in 5% oxygen are similar to those developed in vivo, the in vivo-derived blastocysts have to be flushed from the uterus. Consequently they will experience some stress during the collection through to their extraction/analysis. How much this alters the true in vivo phenotype is unknown and impossible to determine with current technologies.

More recently Meuter et al. (2014) demonstrated that the oxygen concentration employed during preimplantation embryo culture induces markers of permanent cell cycle arrest (cellular senescence). Mouse blastocysts were derived both in vitro under 5 or 20% oxygen, and in vivo. Blastocysts were assessed for primary markers of senescence: the phosphorylated histone family member H2A.X (γ-H2A.X), a marker of DNA oxidative damage and senescence-associated β-galactosidase (SA-β-gal), as well as senescence-associated genes p21, p16, and interleukin 6. Compared with in vivo-derived blastocysts, in vitro embryos (from both oxygen concentrations) had higher levels of SA-β-gal, nuclear γ-H2A.X, and p21 mRNA expression, indicating that a senescence-like phenotype is induced by in vitro culture. However, blastocysts cultured in 5% oxygen had low levels of both

SA-β-gal and γ-H2A.X compared with blastocysts cultured in 20% oxygen. Meuter et al. (2014) concluded that energy dependent cell responses to stress, particularly to DNA damage, may lead to cell cycle arrest and ultimately may affect viability or long-term development. This is relevant to human ART as these data suggest that the senescence-like phenotype is largely due to culture in atmospheric oxygen (Meuter et al., 2014).

Consistent with transcriptomic and proteomic analyses, atmospheric oxygen also induces metabolic perturbations during the preimplantation period. The first evidence came from Khurana and Wales (1989) who demonstrated that culture of mouse embryos in 5% oxygen was associated with higher levels of glucose oxidation. Wale and Gardner (2012) investigated the effects of oxygen on carbohydrate and amino acid utilization, both of which are linked to the ability of the human and mouse embryo to develop in culture (Gardner et al., 2001; Houghton et al., 2002) and to subsequent viability of embryos after transfer (Lane and Gardner, 1996; Brison et al., 2004; Gardner et al., 2011). The pre- and post-compaction periods of embryo development were again of interest, as the cleavage stage embryo displayed a greater sensitivity to oxygen, and distinct differences were recorded in embryonic metabolism before and after compaction. Consistent with the biphasic results during embryo culture (Wale and Gardner, 2010) the metabolic response of the embryo to the oxygen concentration employed was different between pre- and post-compaction stages (Wale and Gardner, 2012). When cultured in 20% oxygen the cleavage stage embryo exhibited higher rates of pyruvate uptake and amino acid turnover compared with embryos cultured in 5% oxygen. Interestingly, the increase in amino acid turnover by cleavage stage embryos as a result of culture in 20% oxygen could be largely accredited to higher amino acid consumption.

In contrast, post-compaction embryos cultured in 20% oxygen exhibited lower rates of glucose uptake and a lower uptake of most amino acids (Wale and Gardner, 2012). The rate and fate of glucose consumed was also different: blastocysts cultured in 20% oxygen displayed higher rates of lactate production from glucose, i.e. a high rate of glycolysis. Of note, low rates of glycolysis are consistent with blastocyst viability (Gardner and Wale, 2013; Lee et al., 2015). That 5% oxygen is associated with lower rates of glycolysis in the blastocyst is consistent with the data of Khurana and Wales (1989), i.e. 5% oxygen is linked with higher levels of glucose oxidation. The carbohydrate and amino acid data from Wale and Gardner (2012) support the hypothesis that viability depends not only on the amount (or rate) of nutrient(s) consumed (Renard et al., 1980; Gardner and Leese, 1987; Brison et al., 2004), but also on which metabolic pathway (or fate) the embryo uses to metabolize nutrients (Lane and Gardner, 1996; Gardner and Wale, 2013; Lee et al., 2015). Furthermore, these findings again highlight differences in the physiology of the embryo before and after compaction, and the embryo's increased susceptibility to stress during the cleavage stages. Given that metabolism is a biomarker of viability (Gardner et al., 2011; Gardner and Wale, 2013), it is of significance that atmospheric oxygen has such a profound effect on metabolic function. Consistent with its effects on blastomere function, data indicate that embryo culture in a reduced oxygen environment has benefits post-partum (Iwata et al., 2000; Fischer-Brown et al., 2005).

Significantly, changes in cell function as a result of exposure to atmospheric oxygen are not limited to embryonic cells. Oxygen also regulates the energy metabolism of hESC, and is intrinsic to the self-renewal of hESC. The concentration of oxygen significantly affects hESC physiology

with coordinated changes in gene expression, in the absence of detectable alterations in undifferentiated marker expression (Forristal et al., 2013). Similarly, Harvey et al. (2014) reported that under atmospheric oxygen conditions glucose consumption was reduced in hESC.

In spite of the available data, clinical debate continues around whether exposure to atmospheric oxygen throughout the preimplantation period has a cumulative effect on the preimplantation embryo, or whether specific embryonic stages are more vulnerable to non-physiological oxygen. Nanassy et al. (2010) concluded that extended culture of the preimplantation embryo increases stress on the embryo and, as such, reduced oxygen (5%) should be employed for culture of the post-compaction embryo but not before. However, several studies have reported no difference in blastocyst development rates when the preimplantation embryo was only exposed to atmospheric oxygen concentration at post-compaction stages (Karagenc et al., 2004; Kind et al., 2005; Feil et al., 2006). When development of human embryos to the blastocyst stage under reduced or atmospheric oxygen was compared, both pregnancy and implantation rate increased as a result of the reduced oxygen environment (Catt and Henman, 2000). Whilst it is clear that embryos are able to form blastocysts when cultured in atmospheric oxygen, these data highlight the importance of measuring the developmental competency and viability of the resultant embryos to further evaluate the complete pathological response of the embryo to culture conditions. This is especially true as the clinical practice of selecting the highest quality embryos for transfer may contribute to the inconclusiveness of the human studies, as a comparison of fresh pregnancy rates may not reflect the overall viability of a cohort. In support of this, two prospective RCTs have demonstrated that the culture of human embryos in low oxygen is associated with an overall increase in live births; Meintjes et al. (2009a) reported an increase from 30.7 to 42.9% for implantation rate and 42.6 to 57.4% for live birth rate in 20 and 5% oxygen, respectively. Similarly, Waldenström et al. (2009) reported a 10% increase in live birth rate when embryos were cultured in 5% oxygen. A recent Cochrane evaluation of whether culturing preimplantation human embryos in a physiological oxygen compared to atmospheric improves the treatment outcome concluded; 'The results of this systematic review and meta-analysis suggest that culturing embryos under low oxygen concentrations improves the success rates of IVF/ICSI, resulting in an increase in the live birth rate' (Bontekoe et al., 2012). This conclusion is consistent with the data from other species (mouse, cow, sheep, goat and pig) where employment of physiological oxygen concentration throughout development results in superior embryo transfer outcomes.

Of additional interest are the observations that oxygen appears to have a greater detrimental effect on female embryos than male. It is well established that male and female embryos differ with respect to gene expression, their proteome and metabolism (Review by Gardner et al., 2010, 2011). Consequently, it is not surprising that gender differences exist in response to cellular trauma induced by 20% oxygen. Meintjes et al. (2009b) observed a significant skewing in the sex ratio of babies following embryo culture in atmospheric oxygen and blastocyst transfer, with significantly more males (58.5%) being born. However, when human embryos were cultured in the presence of 5% oxygen, the sex ratio at birth was restored to 51.9% males (Meintjes et al., 2009b). Subsequently, Gardner and Kelley (2013) used a mouse model, with males carrying an X chromosome with a green fluorescent protein tag to facilitate sex determination by fluorescence, combined with time-lapse microscopy, to determine if the effects of oxygen on embryos were influenced by their sex. It was determined that atmospheric oxygen induced significant delays in female embryo development as early as the 7-cell stage, and that this persisted through blastocyst formation (Gardner and Kelley, 2013). Recent data on the developmental kinetics of human embryos cultured in atmospheric oxygen also show that male embryos develop faster than females in non-physiological conditions (Bronet et al., 2015).

In light of the overwhelming experimental data that mammalian preimplantation embryo development, physiology and viability are significantly compromised by atmospheric oxygen, why was the practice of using a gas phase of carbon dioxide in air adopted so widely for human IVF, and why has the employment of atmospheric oxygen persisted for so long? Sadly, there is not a clear-cut answer to this question, and perhaps one must consider the technologies available in the early years of human IVF in order to deduce how atmospheric oxygen became adopted as standard practice for so long. In the pioneering studies and early days of human IVF and embryo culture, both atmospheric oxygen (Veeck et al., 1983; Wortham et al., 1983a,b) and 5% oxygen (De Kretzer et al., 1973; Trounson et al., 1980; Lopata et al., 1982; Lopata, 1983) were employed. To create the stable atmosphere of 5% O_2, 5% CO_2 and 90% N_2, premixed gas-cylinders and desiccators, or equivalent sealable chambers, were used. Such chambers were cumbersome and consumed a lot of space in each incubator. The rapid growth and widespread application of ART led to a practical move from desiccators/sealed chambers to incubators which could handle larger numbers of culture dishes for an increasing number of patients' embryos. In the late 1980s this was achieved using tissue culture incubators, designed to create an atmosphere of 5% CO_2 in air. Given the relative paucity of data on the adverse effects of atmospheric oxygen on embryonic and fetal development in the early years of human IVF, clinical IVF laboratories all over the world adopted the use of atmospheric oxygen (20%). Fortuitously, the last 10–15 years have seen the development of excellent incubators capable of maintaining both carbon dioxide and oxygen concentrations. Such chambers include tissue culture-styled chambers, as well as new bench-top designs, including several that are also able to perform time-lapse microscopy.

Finally, given the documented negative impact that oxygen has on cell physiology, it would appear prudent to consider the inclusion of antioxidants in IVF/culture media, to ensure levels of protection are conferred during the first days of life. Serendipitously, pyruvate, which is present in all culture media, is a powerful antioxidant itself (Andrae et al., 1985; Kouridakis and Gardner, 1995; O'Fallon and Wright, 1995). Several antioxidants have now been shown to have protective/stimulatory effects during embryo culture or cryopreservation including ascorbate (Umaoka et al., 1992; Lane et al., 2002), glutathione (Legge and Sellens, 1991; Hansen and Harris, 2014), carnitine (Phongnimitr et al., 2013; Takahashi et al., 2013), cysteine (Li et al., 2014b), cysteamine (Elamaran et al., 2012) and lipoate (Linck et al., 2007). Their roles in human ART wait to be fully elucidated.

Given that there are no longer any technical restrictions in using physiological levels of oxygen (i.e. below 10%), together with the substantial data from animal models covering all aspects of embryo physiology, it is time to confine the use of atmospheric oxygen to the annals of human IVF. Of note, the vast majority of studies on lower oxygen levels for embryo culture have focused on 5%. However, the optimum oxygen concentration for human embryo development has yet to be

elucidated, and further, we do not know whether stage-specific differences exist.

Ammonium

Amino acids are abundant in the fluids of the female reproductive tract (Fahning et al., 1967; Perkins and Goode, 1967; Menezo and Laviolette, 1972; Casslén, 1987; Miller and Schultz, 1987; Gardner and Leese, 1990; Moses et al., 1997; Harris et al., 2005), and hence are key components of mammalian embryo culture media (Gardner, 2008). Amino acids fill key physiological niches as development and differentiation proceed, including acting as osmolytes (Dumoulin et al., 1992; Lawitts and Biggers, 1992; reviewed by Baltz, 2001), as buffers of internal pH (Edwards et al., 1998a) and the regulation of carbohydrate metabolism (Gardner and Sakkas, 1993; Lane and Gardner, 2005). Prior to the 2-cell stage, the inability of the pre-compaction embryo to utilize lactate as the sole energy source can be attributed to low activity of the malate-aspartate shuttle. It has been determined that aspartate is the rate-limiting factor affecting the activity of the malate-aspartate shuttle and that the addition of exogenous aspartate (10 mM) enables the mouse zygote to utilize lactate in the absence of pyruvate and continue development (Lane and Gardner, 2005). Inclusion of amino acids in modern culture media has, therefore, in no small way contributed to the steady increase in success rates for human IVF over the past two decades (Bavister, 1995; Gardner and Lane, 1997; Devreker et al., 2001; Gardner, 2008). Unfortunately, there is a downside to the inclusion of amino acids due to their lability.

When present in any culture medium and incubated at 37°C, amino acids spontaneously break down in a time dependent manner to release ammonium, which subsequently affects cell physiology. This phenomenon is well-characterized in the tissue culture literature, and ammonium generated from amino acids represents a significant problem for the maintenance of somatic cell function and the maintenance of lines (Newland et al., 1990; Schneider et al., 1996). Hence this is not a phenomenon unique or peculiar to mammalian embryo culture. For example, hybridoma cells utilize glutamine as their primary nitrogen source and release ammonium as a metabolic waste product. This ammonium quickly accumulates to toxic levels in hybridoma culture media, thereby severely reducing monoclonal antibody production (Ozturk and Palsson, 1991). Gardner and Lane (1993) were first to identify the presence of increasing quantities of ammonium over time in embryo culture media and to reveal that the developing embryo also produced measurable levels of ammonium from the transamination of amino acids. It was subsequently determined that the generation of ammonium within the culture medium had adverse effects on mammalian embryo development, physiology and viability (Gardner and Lane, 1993; Lane and Gardner, 1994, 1997, 2003; McEvoy et al., 1999; Sinawat, 2001; Sinawat et al., 2003; Zander et al., 2006; Rooke et al., 2007).

In embryo culture media ammonium accumulates to levels from 50 μM (Gilbert et al., 2012) to 300 μM (Gardner and Lane, 1993) depending on the concentration of amino acids present and duration of embryo culture. In the human, ammonium has been shown to induce not only a negative effect on blastocyst formation (Virant-Klun et al., 2006), but also to alter metabolic activity and subsequent gene expression of the human embryo (Gardner et al., 2013), with the former being affected by as little as 75 μM. Hence, there is a trade-off between the highly beneficial effects of amino acids and the detrimental

effects of ammonium. Glutamine is the greatest contributor to the ammonium pool in vitro, as the most labile amino acid, and in early formulations of human embryo culture media was typically present at 1–2 mM, thereby representing a source of significant levels of ammonium in vitro. The substitution of this amino acid with the more stable dipeptide forms alanyl-glutamine or glycyl-l-glutamine has greatly reduced the accumulation of ammonium in modern embryo culture media (Gardner and Lane, 2003; Biggers et al., 2004). Interestingly, it is also possible to transaminate enzymatically the ammonium in situ to the non-toxic glutamate (Lane and Gardner, 1995). Ammonium build-up can be further reduced by renewal of the culture medium after 48 h of culture (Gardner and Lane, 1993; Lane and Gardner, 1995). In reality, production of ammonium during embryo culture cannot be prevented. However, considerable efforts have been made to ensure that the concentration of ammonium stays below 75 μM (the lowest concentration reported to have an adverse effect on the cleavage stage human embryo) by titrating down the levels of amino acids present in culture medium and substituting glutamine with heat stable di-peptide forms. It is not known at what concentration ammonium has no detectable impact on blastomere function, nor has a systematic study been performed to evaluate the importance of the frequency of medium renewal, i.e. 24 versus 48 h. Interestingly, this study may not be forthcoming with the move to time-lapse microscopy and uninterrupted culture. At least the move to use a reduced oxygen concentration during embryo culture can help alleviate the impact of ammonium (as outlined below).

Similar to the stress associated with atmospheric oxygen, it is the pre-compaction embryo which appears most sensitive to ammonium. Even a short exposure during the cleavage stages can result in significant disruptions to development and to gene expression at later stages (Zander et al., 2006). There is evidence that the preimplantation embryo may have an increasing ability to adapt or become tolerant to ammonium as development progresses beyond compaction (Zander et al., 2006). Edwards et al. (1998b) identified that the preimplantation embryo's ability to regulate pH stress increases significantly post-compaction. Pre-compaction embryos exposed to a weak acid showed a decrease in intracellular pH, while post-compaction embryos were able to maintain pH at the physiological level (Edwards et al., 1998b). Ammonium is a weak acid and would therefore significantly inhibit the ability of a pre-compaction embryo to regulate intracellular pH. Consequently, this inability to maintain normal levels of intracellular pH could be associated with reduced developmental competence and viability (Lane and Gardner, 2003; Zander et al., 2006).

Although ammonium has been shown to be embryo toxic, few studies have investigated the mechanism(s) by which the early embryo can regulate ammonium. Alanine and glutamine have been proposed as having a role in preventing the build-up of ammonium ions in culture medium. Pyruvate, after transamination to alanine, has been suggested as an ammonium sink (Donnay et al., 1999; Orsi and Leese, 2004). However, alanine production did not increase when bovine embryos were exposed to increasing concentrations of ammonium (Orsi and Leese, 2004). Glutamate metabolism, catalysed by glutamine synthetase, could remove free ammonium ions through conversion to glutamine (Orsi and Leese, 2004). Glutamine production at the blastocyst stage has been reported in cattle (Orsi and Leese, 2004; Sturmey et al., 2009), mice (Lamb and Leese, 1994) and pigs (Humpherson et al., 2005). Interestingly, there have also been reports on the varying effect of ammonium on preimplantation embryo development when different

oxygen concentrations were used by different investigators (Lane and Gardner, 1994; Biggers et al., 2004).

Wale and Gardner (2013) therefore considered the effect of ammonium on the physiology of the embryo under different oxygen concentrations (as a second source of stress in a culture system). Glutamine and alanine synthesis were investigated as possible pathways used by the preimplantation embryo in ammonium sequestration as well as the effect of oxygen on the regulation of these pathways. Amino acid utilization by blastocysts was determined after culture from the post-compaction stage with 0, 150 and 300 µM ammonium (in either 5 or 20% oxygen), and with or without 500 µM L-methionine sulfoximine (MSO), an inhibitor of glutamine synthetase. In the presence of MSO, ammonium production was significantly increased, glutamate was no longer consumed and glutamine formation decreased (Fig. 2a). Ammonium and oxygen

Figure 2 (**a**)Effect of L-methionine sulfoximine (an inhibitor of glutamine synthetase) or ammonium (150 and 300 µM) on glutamine turnover of mouse blastocysts, with all cultures performed under 5% oxygen. Notches represent the confidence interval of the median, the depth of the box represents the interquartile range (50% of the data), and whiskers represent the 5 and 95% quartiles. The line across the box is the median uptake or release. Significantly different from embryos incubated in control medium: **$P < 0.01$. Data from Wale and Gardner (2013). (**b**) Effect of oxygen with and without 150 µM ammonium on glutamine turnover of mouse blastocysts. Notches represent the confidence interval of the median, the depth of the box represents the interquartile range (50% of the data), and whiskers represent the 5 and 95% quartiles. The line across the box is the median uptake or release. Positive values reflect glutamine production, negative values reflect consumption. Significantly different from embryos incubated in control medium: **$P < 0.01$. Data from Wale and Gardner (2013).

independently altered overall amino acid turnover. Together, 5% oxygen and ammonium promoted glutamine production, whereas in the presence of atmospheric oxygen and ammonium, glutamine was consumed (Fig. 2b). These data reveal that both ammonium and oxygen affect the amino acid utilization by the developing embryo and that atmospheric oxygen appears to have a greater detrimental effect. Consequently, mouse blastocysts can alleviate ammonium stress by its transamination to both glutamine and alanine, but only under physiological oxygen conditions (Wale and Gardner, 2013). Hence when stresses collide the outlook for the developing embryo can be bleak.

Importantly, these newly identified interactions between ammonium and oxygen resolve an apparent paradox in the literature with regards to differing results on the effect of ammonium exposure (either continuously in medium containing Eagle's concentration of amino acids (Eagle, 1959) or in medium supplemented with ammonium chloride) on mouse embryo and fetal development. Lane and Gardner (1994) reported a 20% incidence of exencephaly following culture of mouse embryos for 96 h from Day 0.5 in atmospheric oxygen and in a non-renewed medium containing amino acids and 1 mM glutamine. When embryos were cultured with exogenous ammonium in atmospheric oxygen but for less time (either 72 h from Day 0.5 with 300 µM or 48 h from Day 1.5 with 600 µM), Sinawat et al. (2003) reported exencephaly at a rate of 4.5%. The interaction of ammonium and atmospheric oxygen could therefore be associated with the reported high frequency of exencephaly cases (Lane and Gardner, 1994). In support of this, Biggers et al. (2004) employed 5% oxygen and reported a 1% incidence of exencephaly when embryos were cultured in physiological oxygen in non-renewed medium containing amino acids at half Eagle's concentration and using glycyl-glutamine. These results highlight the importance of understanding how specific culture conditions (e.g. media composition, length of exposure, developmental stage to which treatment is applied and the concentrations of oxygen employed) interact and impact on the physiology of the preimplantation embryo, resulting in different experimental outcomes in terms of embryo and fetal normality. Notably, experimental conditions need to be precisely reported and replicated if data are to be correctly interpreted.

Of clinical significance, ammonium-induced changes have also been observed in human IVF. For example, when Dumoulin et al. (2010) investigated the effects of culturing human embryos in two different culture media under physiological oxygen, one medium contained free glutamine and the second contained alanyl-glutamine (a heat-stable form of glutamine). Embryo transfer primarily occurred on Day 2 during which free glutamine would break down to produce up to 150 µM ammonium in the culture medium, while the alanyl-glutamine would produce <10 µM ammonium (Lane and Gardner, 2003). Therefore, data from the Dumoulin et al. (2010) study may plausibly be interpreted as an investigation into the effect of exposure to different ammonium concentrations during in vitro culture of human embryos. Indeed, Gardner et al. (2013) determined that 150 µM of ammonium has a negative impact on the metabolism of cleavage stage human embryos. Despite the limited numbers of children born, Dumoulin et al. (2010) reported both lower pregnancy outcomes and reduced birthweight following culture of human embryos in a medium with free glutamine, indicating that ammonium not only delays development in vitro, but could affect the birthweight of live born singletons (Zandstra et al., 2015). It is important to note that the Dumoulin study was a comparison of non-equivalent culture media and there were also differences
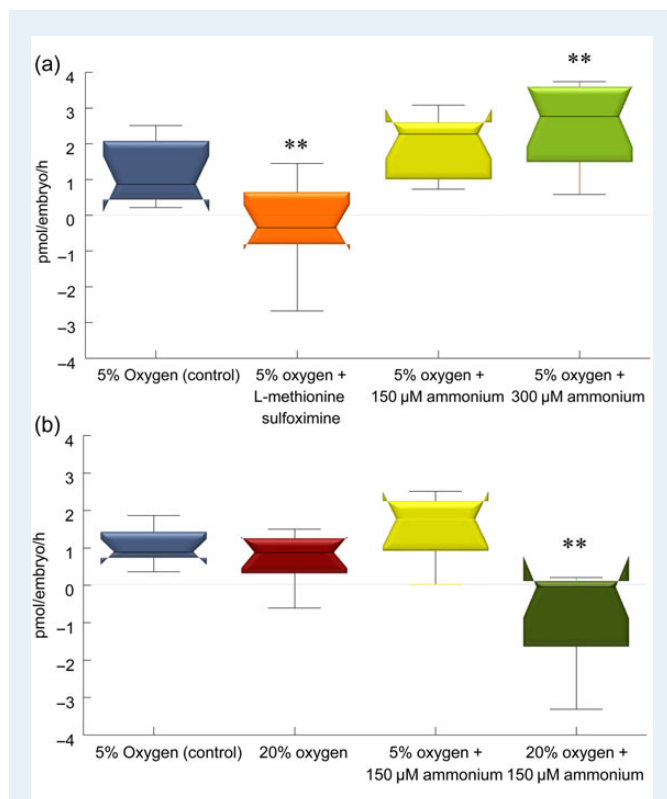
in parental phenotype of the study groups which could therefore influence the outcome in this study, i.e. the parents in the alanyl-glutamine group were heavier, and heavier parents have heavier children. De Vos et al. (2015) compared singleton live births resulting from singleton pregnancies sourced from two different sequential culture media, which both utilize a stable dipeptide source of glutamine. In this large (total of 2098 births) retrospective study no significance differences in mean singleton birthweight were observed (De Vos et al., 2015) giving credence to the proposition that the difference in outcomes described by Dumoulin's group may therefore be attributed to a chronic exposure of embryos to ammonium and/or differences in parental weights.

Of physiological significance, the observation of ammonium-induced changes to the preimplantation embryo is not limited to studies on in vitro culture. The supply of high levels of protein or urea (2.5%) during peri-conception increases levels of ammonium in plasma (150 μM) and the reproductive tract (McEvoy et al., 1997; Gardner et al., 2004), thus creating an environment that is disruptive to embryo development in vivo. In mouse and livestock models a high maternal protein diet during the peri-conception period affects fertilization (Blanchard et al., 1990), embryo development (Sinclair et al., 2000; Powell et al., 2006) and quality (Gardner et al., 2004). Additionally, altered preimplantation development and subsequent expression of type II insulin-like growth factor receptor (IGF2R), an imprinted gene, can be affected by high maternal dietary nitrogen levels (Powell et al., 2006). Given the strength of data from controlled in vitro studies, combined with data from dietary interventions, it is plausible to suggest that elevated ammonium may have negative influences in vivo, however the extent to which the periconceptual environment is buffered against this is unknown.

In closing this section, it is important to acknowledge that there are reports that ammonium does not affect embryo development or physiology (Menezo and Guerin, 2007). However, in light of data from both in vivo and in vitro studies, and data from somatic cell cultures, it is hard to support the argument that ammonium is something that one can simply disregard with regards to the development of the mammalian preimplantation embryo.

Volatile organics

Poor laboratory air quality is a recognized hazard to the culture of human gametes and embryos (Legro et al., 2010; Perin et al., 2010). However, the cumulative data on volatile organic compounds (VOCs) and chemical airborne contaminants (CACs) are rather anecdotal, with the ability to investigate potential physiological effects limited by design. As such, the concentrations of the contaminants, such as acrolein or nitrogen dioxide, which result in gamete or embryo toxicity, remain poorly defined, but their effects on other tissues have been documented: exposure to the VOC 1-octen-3-ol and its enantiomers caused a dose-dependent decline in cell viability and cytotoxicity of human stem cell line H1, as determined by a MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay to establish cell toxicity and a 'live and dead' stain (Inamdar et al., 2012). Further, exposure of murine bone marrow stromal cells to oct-1-en-3-ol and a 2nd VOC (E)-2-octenal caused a shift to unsaturated fatty acids and lower cholesterol levels in the membrane, which is an indication of increased membrane fluidity, and changes to the cell membrane are known to contribute to the breakdown of normal cell function and possibly lead to death (Hokeness et al., 2014).

Avoiding the negative effects of poor air quality requires an understanding of how toxicants can infiltrate the laboratory, the incubator, and ultimately the culture media. With this in mind, careful consideration should be given to the site and location of human IVF laboratories as there is potential for VOCs and CACs to significantly affect the developing embryo (Cohen et al., 1997; Hall et al., 1998; Khoudja et al., 2013) and appropriate design of the laboratory is important (Boone et al., 2010). The use of low odour specialized paints and the avoidance of sealants and toxic glues is also recommended (Cutting et al., 2004; Cohen et al., 2012). Positive pressure airflow in the laboratory coupled with the use of air purification systems (Khoudja et al., 2013) and appropriate in-line filters for incubators (Merton et al., 2007) will help to minimize the level of airborne contaminants and improved outcomes. Pragmatically, the use of appropriate in-line air filtration together with certified gas cylinders, and suitable bench-top incubators, means it is feasible to effectively isolate gametes and embryos from the gaseous environment of the laboratory.

VOCs can be emitted by various sources within the laboratory, such as consumables, and a VOC meter should be used to establish off-gassing time for consumables (including packaging) within the laboratory. Many laboratories ensure the off-gassing of their culture wares in a separate laboratory to the clinical IVF laboratory, where dishes and tubes can be placed in a laminar flow hood overnight to facilitate the safe emission of VOCs. Packaging, therefore, has a major impact on the immediate use-ability of such plastics, with gas-permeable packaging allowing for use 'straight out of the box'. Further, laboratory personnel can also introduce VOCs in the form of perfumes and deodorants, hence discretion is required. Finally, given the concept of when stresses collide in the laboratory, the use of a reduced oxygen environment should help to reduce possible toxic effects of VOCs.

Albumin; blood versus recombinant

The early years of human IVF were characterized by the inclusion of patient or fetal cord serum in the culture media used. This was done irrespective of whether the medium was a simple salt solution, such as human tubal fluid medium (Quinn et al., 1985), or a complex tissue culture medium, such as Ham's F-10 (De Kretzer et al., 1973; Leung et al., 1984; Zamboni et al., 1986). Although the use of sera facilitated early embryo development during a time of sub-optimal laboratory conditions, it introduced many unknown variables into the culture system (due to different diets of the patients and their physiological status at the time of collection), making standardization of culture impossible (Gardner and Lane, 2007). Furthermore, over the years there have been growing concerns about the use of whole sera for embryo culture given its documented adverse effects on the embryos of laboratory and domestic animals. In mice, the addition of serum is associated with altered expression of growth-related imprinted genes, which culminates in aberrant fetal development (Khosla et al., 2001). In sheep and cattle the inclusion of serum (human or autologous) induces premature cavitation of morulae (Walker et al., 1992) and damages the intracellular integrity of embryos, disrupting mitochondrial membrane organization, and thereby compromising metabolism (Gardner, 1994; Thompson et al., 1995). Of greater concern, the presence of serum induces the development of abnormally large offspring, whereby the lambs or calves can be up to 50% greater than normal birthweight (Behboodi et al., 1995; Thompson et al., 1995; Sinclair et al., 1999; Rooke et al., 2007).

Consequently, the move from whole serum to serum albumin reduced a significant amount of the variability, and was shown to be equally, or more, effective as a protein source (Laverge et al., 1997). However, with serum albumin significant lot to lot differences were evident with regard to their ability to support embryo development (Kane, 1983; Batt et al., 1991). Further, the use of serum albumin carries with it the finite risks of disease transmission associated with the use of blood-derived products. From a chemical perspective it is evident that serum albumin brings to the culture medium far more than bound fatty acids and other metabolites, such as citrate (Kane, 1983) and steroids.

A variation on the use of human serum albumin (HSA) in human embryo culture was considered by Pool and Martin (1994), who used a plasma protein fraction, Plasmatein, characterized by a high globulin content. This concept was developed further by Weathersbee et al. (1995) who compared the efficacy of HSA to a synthetic serum substitute (SSS), characterized by high levels (16%) of both α and β globulins. Using this approach accelerated growth of human embryos at 38 h post insemination was reported. Subsequently, several forms of enhanced albumin have become commercially available. In a randomized trial Meintjes et al. (2009c) observed an 11% increase in pregnancy rate when SSS was used in a blastocyst culture system as opposed to HSA alone (Meintjes et al., 2009c). Anecdotal evidence indicates that certain batches of such products work well, whereas others are perhaps less effective than serum albumin alone, indicating that there may be factors in such albumin preparations other than globulins that have embryo trophic effects.

Of note, two recent and independent analyses have determined that commercially used serum albumin preparations used in human IVF contain an abundance of non-declared proteins as well as transition metals (Dyrlund et al., 2014; Morbeck et al., 2014). Further, the processing of albumin can lead to the introduction of known chemicals, such as octanoic acid, which has been shown to be detrimental to embryo development (Leonard et al., 2013). From our own analyses we have also determined high levels of albumin-bound compounds in culture medium, such as ethanol and caprylate, which are used in the extraction and stabilization of albumin, respectively (Sheedy and Gardner, unpublished observations). Additionally, there are now concerns that serum albumin, added as the protein supplement, is the source of detectable levels of Di(2-ethylhexyl)phthalate and mono(2-ethylhexyl)phthalate, as well as polybrominated diphenyl ethers in human embryo culture media (Takatori et al., 2012; Akutsu et al., 2013). Such data infer that the use, and/or preparation, of serum albumin in human IVF warrants renewed consideration. To this end, recombinant human albumin has been shown to be an effective replacement for serum-derived albumin in IVF and embryo culture (Bavister et al., 2003; Lane et al., 2003) and its clinical efficacy validated (Bungum et al., 2002). Given the growing concerns surrounding the safety of human IVF, it would appear that a move towards recombinant albumin to minimize risk and to increase consistency of function, much like the move from urinary to recombinant gonadotrophins, is timely. When the use of recombinant albumin was first considered, the cost of the recombinant protein was almost one hundred times that of serum-derived albumin. However, over the past two decades the costs for such recombinant materials has decreased considerably, making the inclusion of recombinant albumin in human IVF and embryo culture media a feasible proposition. Hence it is time to re-evaluate its use clinically.

Physical factors

Temperature and pH

Although at first glance ensuring constant temperature and pH would appear to be straightforward, it remains a technically demanding challenge to minimize variations of both parameters around their set points. The importance of temperature regulation is of greatest significance for the oocyte, followed by the cleavage stage embryo, with increased thermotolerance increasing after compaction. If the temperature goes over 37°C by a couple of degrees, even for just a transient time of 20 min, then in the metaphase II oocyte the spindle starts to disassemble and does not always completely reconstitute when the temperature is brought back to 37°C (Sun et al., 2004). The embryo responds to heat by expression of stress response genes, associated with loss of developmental competence (Hansen, 2007), whilst an earlier study suggests that a fall in temperature during oocyte handling is inconsequential (Bernard et al., 1992) when fertilization and culture for a further 24 h are the measured end-points. More recent studies have reported a decrease in temperature, whether drastic (Zenzes et al., 2001) or mild (Wang et al., 2002), has the potential to affect the stability of the meiotic spindle of the oocyte. The reported consequence of this alteration to the oocyte's meiotic spindle is reduced fertilization rates (Wang et al., 2002), delayed embryo development (Wang et al., 2001) and decreased clinical pregnancy rates (Wang et al., 2002).

It would therefore seem prudent to take rigorous steps to ensure that all warming stages undergo daily monitoring using appropriately accurate and calibrated equipment. Furthermore, stages on microscopes should not necessarily be set to 37°C. Rather the temperature to which the gametes and embryos are exposed to in the dish depends on the type/design of the dish, and whether there is a lip on the base, which will create an air pocket, and affects the thermal conductivity of the dish itself. Consequently, the temperature of warming stages needs to be calibrated within the culture medium inside the dish itself. This frequently translates to a temperature of >38°C on the warming stage. As will be discussed with regards to pH, a possible solution to variations in temperature is to work within an isolette/humidified chamber, which can maintain air temperature (and consequently any equipment housed within the heated chamber) close to 37°C, therefore preventing equipment within from acting as heat sinks.

More recently it was proposed that working at a temperature of 36°C may mimic more closely the temperature of the female reproductive tract (Bahat et al., 2005), and that consequently a lower temperature better supports a 'quieter' embryo development (Leese et al., 2008). However, in a RCT, there was no improvement in human embryo development or clinical outcomes when 36°C was used compared with the standard of 37°C (Hong et al., 2014). To date, therefore, the data indicate that maintaining gametes and embryos at 37°C is advisable and effectual.

In order to regulate the pH of fluids, when working outside of the body it is typical to employ a bicarbonate/carbon dioxide buffering system. By manipulating the concentration of carbon dioxide and the concentration of bicarbonate, typically present as the sodium salt, it is readily feasible to create the required pH. This approach has the advantage that it is easy to manipulate the set pH of the culture medium by changing either the bicarbonate or carbon dioxide concentration. However, in reality it takes careful attention to detail with regards to calibration of the

incubator and pH measuring device, in order to ensure that the pH of the culture medium inside the incubator is what one expects it to be. Calibrated digital carbon dioxide analysis units are preferred over a liquid-based system such as Fyrite. Significantly, as the accuracy of electronic-based gas analysis unit falls during its lifetime, one must regularly check the accuracy of these monitors against a known standard. The analysis of medium pH is best performed with a blood-gas analyser, or an optical device inside the incubator. Standard pH meters can be used for such measurements, but it is technically challenging given that the pH of the media will rapidly increase outside of the incubator. Consequently, when measuring pH it is important to snap the tube of test media closed whilst inside the incubator prior to injection into a blood gas analyser. If a pH probe is used then it needs to be at 37°C to obtain a meaningful reading, or alternatively use a probe with temperature adjustment.

An increase in the pH of the culture media can have a highly detrimental effect on oocyte and embryo physiology and development (reviewed by Swain, 2012). Of note, even a transient exposure to acidified media can have ensuring effects on both fetal weight and length (Zander-Fox et al., 2010). Therefore, although the human embryo possesses several intracellular mechanisms to regulate its internal pH (Phillips et al., 2000) care must be taken to minimize fluctuations of pH. An excellent, and pragmatic, means of doing this is to use isolettes capable of maintaining a more stable medium pH through the provision of carbon dioxide gas within the chamber. This approach has proven to be effective. If isolettes are not available and one needs to keep embryos out of an incubator, then the inclusion of either HEPES and/or MOPS can serve to maintain a constant pH for embryo manipulations (Gardner and Lane, 2007; Swain and Pool, 2009). Whilst numerous studies indicate HEPES is able to support oocyte maturation, fertilization and embryo development at room atmosphere, only short exposure to HEPES and/or MOPS is recommended as long-term exposure has been reported to result in oocyte degeneration, lower fertilization rates, and/or compromised blastocyst formation (reviewed by Will et al., 2011). Similar to data on the effects of temperature, the effects of pH drifts appear greater on the oocyte than the cleavage stage embryo (Lane and Gardner, 2000), while at compaction (and the formation of a transporting epithelium) the embryo develops greater ability to maintain intracellular pH (Edwards et al., 1998b). Furthermore, temperature is a key affecter of intracellular pH (Lane, 2001). Consequently, one needs to address both parameters with equal gravitas.

The significant impact that the percentage of carbon dioxide has on maintaining medium pH is sometimes overlooked, and too many patients' embryos are all too commonly cultured in the same incubator, resulting in multiple door openings, thereby compromising the level of carbon dioxide and consequently medium pH. Therefore, it is recommended to ensure sufficient chambers are made available to safely accommodate the number of cycles performed. Hence working quickly outside the incubator, together with careful attention to safe and gentle pipetting of embryos, is essential, and needs to be instilled and reinforced as a fundamental of successful embryo culture. If standard tissue culture type incubators are selected, then it is best to ensure that they have an infra-red CO_2 sensor, rather than a thermocouple-based sensor which is humidity dependent and therefore much slower to react to changes in gas composition and has a slower recovery time (Swain, 2014). Factors such as altitude will affect the partial pressure of carbon dioxide in the medium, and hence it is paramount to measure pH rather than simply relying on the carbon dioxide measurement.

Similarly, the addition of amino acids and protein to a medium will also affect medium pH.

Oil overlay

Due to the small volumes of medium typically used for IVF and embryo culture (between 20 and 250 μl) an oil overlay is used to prevent evaporation and hence stop the medium becoming hyperosmotic. Although mammalian embryos can tolerate a wide range of osmolality (200–350 mOsmol) (Gardner and Lane, 2007; Baltz, 2012), without an oil overlay the osmolality would rapidly become too high for the embryo to survive. The inclusion of such an overlay also provides stability with regards to medium pH, reducing the magnitude of medium pH oscillations. Consequently, oil has a very important place in the IVF laboratory. The oil typically used in embryo culture is mineral (also known as paraffin) oil. Mineral oils are derived through the production of petroleum, and can be classified according to viscosity. Given its industrial origin it is essential that the quality of oil used is ensured, which can be achieved through an appropriate bioassay (Gardner et al., 2005). It is our experience that embryo development is enhanced at higher oil viscosity (Gardner and Wale, unpublished observations). Oils are not chemically inert; rather oil readily removes hydrophobic compounds, such as steroids. Further, it has the capacity to oxidize over time, so it should be stored in a cool dark place. Alternatives to mineral oils include silicon, but there has been limited clinical validation of such reagents (Erbach et al., 1995; Van Soom et al., 2001). Oils also can come with many impurities, some of which have been shown to be embryo toxic (reviewed by Morbeck and Leonard, 2012). Consequently, protocols have been developed to wash oils, either with medium or with a solution of chelators such as EDTA, in an attempt to improve the quality of the oil used in the IVF lab. Whilst Morbeck et al. (2010) provide clear evidence (including a thorough protocol) that washing reduces the toxicity of mineral oil, the washing of oils, although it appears an attractive concept, warrants a considerable amount of time and resources. Alternatively, if oil can pass a suitable bioassay, such as a 1-cell mouse embryo (Hughes et al., 2010), in a reduced volume (2–5 μl) in order to increase surface area to volume ratio in order to increase sensitivity, then washing should not alter its efficacy.

Incubation volume/embryo density

Human embryos are cultured in volumes ranging from a few microliters up to ~1 ml (typically when using test tubes or 4-well plates, Bolton et al., 2014). Further, with the advent of both preimplantation genetic screening (PGS) and time-lapse microscopy, there has been a move to single embryo culture. Interestingly, animal studies have revealed that culturing embryos in smaller volumes and/or groups significantly increases blastocyst cell number and elevates embryo viability (Wiley et al., 1986; Paria and Dey, 1990; Lane and Gardner, 1992; Doherty et al., 1997; Donnay et al., 1997). However, Spyropoulou et al. (1999) could not replicate the positive effects of group culture with human embryos, although it should be noted that embryos were only cultured to Day 2. In regards to blastocyst development, Rijnders and Jansen (1999) cultured human embryos from day 3 to the blastocyst stage individually, or grouped in large and small volumes, and found no significant different in blastulation rates between the four groups. They concluded that whilst all of the four treatments yielded similar results individual culture in a small volume allowed for direct and individual assessment

Table 1: Summary table of key human embryo culture studies of individual and/or group culture.

Paper	Summary	Start	End	Culture medium	Protein source	Individual conditions	Group conditions	Oxygen concentration	Outcome
Moessner and Dodson (1995)	Individual culture versus group culture, same volume	Day 1	Day 2	Modified Ham's F10	15% human serum	1 ml	2–5 embryos per 1 ml	20%	Significantly greater MCN from group culture, positive correlation between size of the co-culture groups and cell number
Almagor et al. (1996)	Individual culture versus group culture, same volume	Day 1	Day 2	Ham's F10	10% serum – cord blood	700 μl	3–6 embryos per 700 μl	20%	Increased pregnancy and implantation rates from group culture
Spyropoulou et al. (1999)	Individual culture versus group culture, same volume	Day 1	Day 2	Earle's balanced salt solutions containing 0.11 mg/ml pyruvate	Synthetic serum replacement (0.001%) + 1% (v/v) human serum albumin	20 μl	3–5 embryos per 20 μl	20%	No statistical significance difference
Rijnders and Jansen (1999)	From Day 3, four conditions: single culture in small volume or group culture in large volume and vice versa	Day 3	Day 5	Mixture of Earle's and Ham's F10, without hypoxanthine and thymidine	Pasteurized plasma solution (8.7%)	(a) Small volume (5 μl) (b) Large volume (160 μl)	(c) 8–12 embryos (5 μl per embryo) (d) 160 μl total volume	20%	No statistical significance difference
Rebollar–Lazaro and Matson (2010)	Individual culture versus group culture through cleavage stage, all embryos cultured individually from day 3 to blastocyst stage	Day 1	Day 3 (culture continued to Day 5)	Cook SIVF medium	Not specified	15 μl	3–5 embryos per 15 μl	Not specified	Similar pregnancy and implantation rates Group culture (up to Day 3) increased blastocysts utilized
Ebner et al. (2010)	Individual culture (with and without contact–using specially designed culture dishes that allow for individual identification of embryos) and group culture	Day 1	Day 5	EmbryoAssist – Medicult	Not specified	30 μl (OWI) 4 embryos per 30 μl (CW)	3–5 embryos per 30 μl (OWG)	Not specified	Group culture was superior for compaction and blastulation as well as overall blastocyst quality Trend to higher live birth rate with group culture
Tao et al. (2013)	Group culture from Day 3 embryos in which good or poor quality embryos were separately grouped	Day 3	Day 5	Vitrolife G1 and G2, v5)	Not specified	Not applicable	2–5 embryos per droplet 50 μl	5%	Group culture of good embryos from day 3 significantly promoted blastocyst development
Restelli et al. (2014)	Group culture with embryos either randomly grouped or grouped based on pronuclear pattern	Day 1	Day 5	Sage Quinn's sequential media	Not specified	Not applicable	'Random Group' 3-4 embryos per 20 μl 'Definite Group' upto 4 embryos per 20 μl	Not specified	Similar results in terms of blastulation rate and the random grouping of zygotes improved pregnancy and implantation rates in IVF-cycles

| Minasi et al. (2015) | Sibling 2PNs were cultured in either 35 μL or 15 μL, or in 35 μL or 7 μL drops. Two end points were analysed; day 3 (before medium change) and day 5 | Day 1 | Day 5 | Sage Quinn's Advantage protein plus medium | Incorporated in culture medium | 35 μl vs 15 μl or 35 μl vs 7 μl | Not applicable | Not specified | Significantly higher blastocyst formation rate observed from embryos cultured in 7 μl drops, with differences observed during day 3 to day 5 phase |

MNC, mean cell number; OWI, outer well individual; CW, centre well; OWG, outer well group; 2PN, pronucleate oocyte.

of embryo morphology and therefore was preferable. Subsequently, Ebner et al. (2010) performed a much larger prospective study comparing single and grouped culture using specially designed culture dishes that allowed for the individual identification of embryos. Group culture was superior for both compaction and blastulation as well as overall blastocyst quality, with Ebner et al. (2010) concluding that culture volume should be reduced and embryo density increased. Table I lists studies on human embryo group versus individual culture, including key information such as duration of culture, embryo density and the outcome observed.

This supportive effect of group culture is plausibly the result of the preimplantation mammalian embryo producing a factor(s) which can stimulate development. Several factors have been identified which have bioactivity in vitro (reviewed by Thouas et al., 2015). Secreted factors, such as platelet activation factor (PAF) and ubiquitin, have been identified and in some cases quantified in culture medium conditioned by human embryos; PAF has been identified in culture medium conditioned by human, murine, hamster, rabbit and sheep embryos with roles in cell cycle progression, embryo metabolism and viability (reviewed by O'Neill, 2005). Of interest, in relation to the human embryo a positive correlation between clinical pregnancy and the level of PAF in culture medium was reported by Roudebush et al. (2002). Ubiquitin is secreted by human embryos and is detectable in culture medium, and has been correlated with blastocyst development (Katz-Jaffe et al., 2006). Other possible factors include insulin-like growth factor 1 (IGF1) and IGF2, platelet-derived growth factor alpha, basic fibroblast growth factor, transforming growth factor beta and interferon, as the appropriate mRNA for these growth factors are expressed by the bovine preimplantation embryo (Watson et al., 1992). However, the physical presence of several of these growth factors in embryo culture media has yet to be demonstrated.

Perhaps not surprisingly paracrine factors appear to be common amongst mammalian species. Spindler et al. (2006) demonstrated that companion mouse and cattle embryos conferred a benefit to singleton cat embryos, whilst the number (and quality) of companions embryos necessary to grant an advantage may be species dependent (Spindler et al., 2006). Other groups have also demonstrated that paracrine factors have a limited effective range. Stokes et al. (2005) used a novel method to investigate the development of porcine embryos, which allowed distance between adjacent embryos to be varied by securing the embryos to the base of a petri dish coated with Cell-Tak. The development of individual porcine embryos to the blastocyst stage was optimal when they were cultured 81–160 μm apart and as the distance between the embryos was increased, blastocyst rates declined significantly, reaching zero beyond 640 μm (Stokes et al., 2005). Using the same novel method, Gopichandran and Leese (2006) investigated the development of bovine embryos. Interestingly, similar to the porcine embryos, the optimal bovine blastocyst formation rate occurred when embryos were cultured 165 μm apart, which lends further support to the notion that paracrine factors are common amongst mammalian species.

Whilst the identity of these factors remains largely unknown, their significance is being considered through the morphokinetic analysis of embryo development. Wydooghe et al. (2014) used time-lapse analysis to study the influence of 'neighbours' on a bovine embryo's potential to reach the blastocyst stage. They demonstrated that it is not necessarily the number of neighbouring embryos that impede an embryo's potential

to reach the blastocyst stage but rather the developmental stage of the neighbours. An embryo's development was evaluated at 45 h post insemination (hpi) with embryos which had proceeded through the third cleavage division (5–8 cells) classified as 'fast' and embryos which were between 2 and 4 cells (second cleavage division) categorized as 'slow'. Time-lapse analysis revealed that a bovine embryo's development after 45 hpi can positively affect the outcome of its neighbours, with a markedly higher percentage of 'slow' embryos developing into blastocysts by 192 hpi if they had been surrounded by many embryos that had also developed into blastocysts by 168 or 192 hpi when compared with 'slow' embryos cultured in isolation (Wydooghe et al., 2014). These results suggest that production of factors may be associated with embryo quality, and warrant evaluation clinically.

Of interest, the work by Tao et al. (2013) suggests that embryo-to-embryo communication may not always be positive, with poor-quality embryos possibly exerting a negative influence on development of good-quality embryos in group culture. Caution also needs to be exercised when considering what volume and configuration should be used for culture of embryos. When Dumoulin et al. (2010) evaluated two different media, individual embryos were cultured in only 5 μl drops of medium under oil for 2–3 days after egg retrieval (embryo transfer occurred on Day 2 or 3). The choice to employ such low volumes could have further contributed to the ammonium build in both groups, coupled with the additional ammonium from the medium containing free glutamine.

The decision to culture embryos individually or in groups is typically a decision based on historical laboratory driven protocols, convenience and/or the need to collect data on individual embryos, as in the case of PGS. Habitually, embryo density is not considered a physical factor that may affect mammalian embryo culture, and its importance in the practice of assisted human reproduction is often overlooked. Embryo density can be controlled and, as such, it can be utilized as a simple, yet effective tool to improve in vitro development of human embryos (Reed, 2012).

Pipetting induced shear stress

In vivo, embryos do not experience acute shear stress; such stress is induced by high-sustained velocity through too vigorous handing. Up-regulation of phosphorylated mitogen-activated protein kinase (MAPK)8/9 is a marker of MAPK8/9 activation in response to stress (Xie et al., 2006). Pipetting embryos can result in the up-regulation of phosphorylated MAPK8/9 in a dose-dependent manner (Xie et al., 2007). In comparison, when measured in in vivo-derived embryos, from embryonic day (E)1.5 to E4.5, phosphorylated MAPK8/9 was expressed at low levels (Xie et al., 2007). After 24 h embryos that had previously been pipetted sufficiently to induce phosphorylated MAPK8/9 displayed the same number of cells as untreated, which suggests that rapid phosphorylation of MAPK8/9 as a result of transient shear stress does not mediate long-term negative effects (Xie et al., 2007). However, it is not known if multiple handling events could impact biological outcomes (Xie et al., 2007). These data suggest that
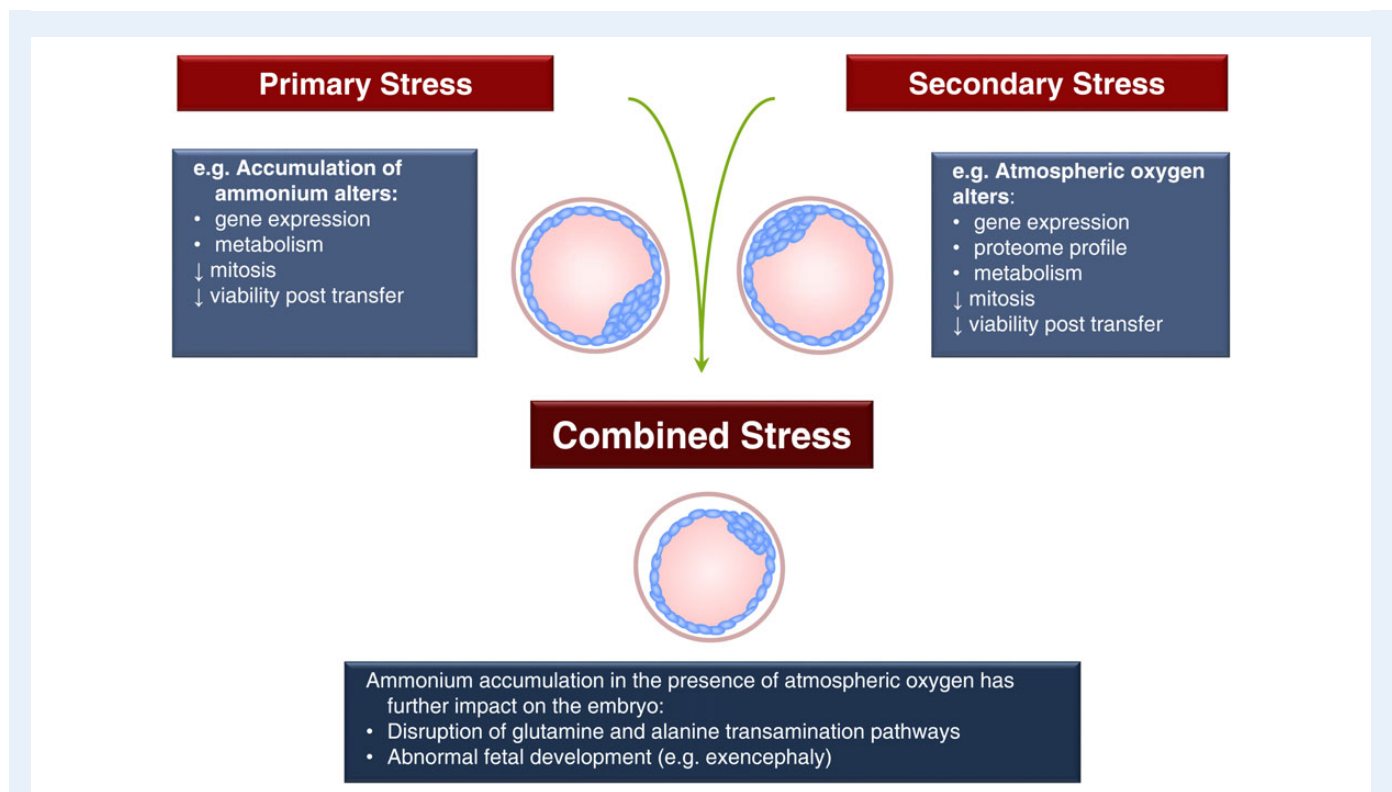
Figure 3 Synergistic effects of two stresses in an embryo culture system. In the presence of a single stress, embryo physiology can be compromised. When two stresses combine in the system then further, and potentially synergistic, negative effects become apparent. The example depicted is ammonium accumulation in the presence of atmospheric oxygen (Wale and Gardner, 2013). A further example is the exposure of embryos to light while at room temperature (Fischer et al., 1988).
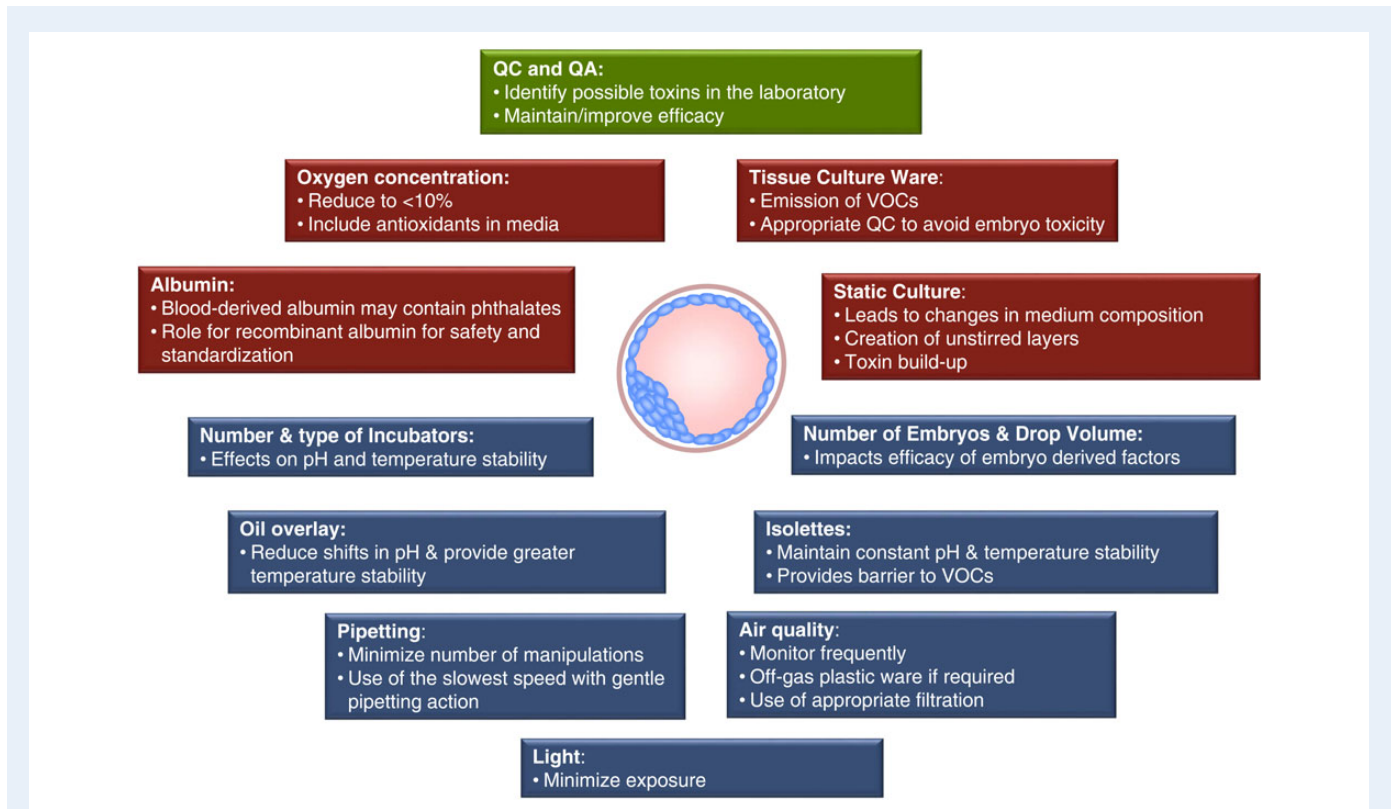
Figure 4 An overview of key chemical and physical factors that affect mammalian embryo development *in vitro*; red represents chemical factors and blue represents physical factors. QC, quality control; QA, quality assurance; VOC, volatile organic compounds.

embryo handling, a necessary aspect of human ART, should be performed with care and kept to a minimum. Coincidently, the emergent time-lapse technology offers the ability to reduce the number of handling events required whereas the introduction of routine embryo biopsy for genetic testing increases the required handling events.

Static nature of culture

Given that undisturbed culture will reduce the cellular trauma associated with shifts in temperature and pH as embryos are removed from the incubator, and with subsequent pipetting, it may appear at first glance that leaving embryos in the same drop of medium for extended culture is a logical means to culture human embryos. However, this approach creates a very artificial condition; a static environment. The reality is that the human embryo *in vivo* is exposed to a highly dynamic environment. Not only is the embryo moved constantly, but it is exposed to gradients of nutrients (Gardner *et al.*, 1996) and to hormones, cytokines and growth factors at stage-specific times (Thouas *et al.*, 2015). The embryo itself is metabolically highly active, and hence is constantly changing its own environment in culture through its consumption of nutrients and release of metabolites (Gardner, 2008). Consequently the formulation of a 25 μl drop of culture medium at the commencement of culture is far removed from that after 4 days of embryo culture. Furthermore, the later stage embryo will create a phenomenon known as an unstirred layer (Trimarchi *et al.*, 2000), in which the embryo creates a gradient of nutrients (particularly true in a column of fluid), which typically results in nutrient insufficiency at low substrate concentrations. Hence, continual

culture without medium replacement is associated with the accumulation of the end products of metabolism and the breakdown products of labile components, as well as any toxins coming off the culture dish. Evidently, we are faced with a 'Catch 22' with regards to medium renewal; to renew the medium allows for toxin removal and nutrient replenishment in a stage-specific fashion, while to leave an embryo undisturbed minimizes trauma from handling and shifts in pH and temperature. Both approaches have been shown to work clinically. With the advent of time-lapse incubation systems, there is a growing trend to leave embryos in the same well for 4–5 days. Further, such undisturbed culture will also avoid possible dilution of embryo-derived autocrine factors. A potential scenario to accommodate the best of both of the above approaches to culture would be to remove medium (say 75%) after 48 h from the time-lapse dish and add to the remaining medium a second phase medium designed to create a new and yet relatively undisturbed environment. This approach could also assist with the retention of embryo-derived factors in the culture media. Ultimately, embryo culture may be performed in a continual flow system as advocated over 20 years ago by Gardner (1994), facilitated by the development of microfluidic devices (Swain *et al.*, 2013), able to provide a dynamic environment and facilitate the removal of toxins whilst facilitating image collection and biomarker detection (Gardner *et al.*, 2015).

Light

Within the female reproductive tract fertilization and embryo development will occur in the complete absence of light. In contrast, under

Table II **Embryo development in the laboratory is a balance of different stresses.**

Dynamic environment	Static environment
Alleviates ammonium accumulation and diminishes the impact of unstirred layers	Ammonium and other embryo-derived metabolites accumulate, and the embryo creates extended unstirred layers
Potential loss of paracrine factors derived from the cleavage stages	Potential cumulative benefit of paracrine factors derived from the cleavage stages
More pipetting; potential for pH and temperature shifts, and shear-stress during medium change over (although this could be overcome by replacing the majority of the medium drop itself and not moving embryos)	Less pipetting; no pH or temperature drift during medium change over on Day 3, and reduced potential for shear-stress activated factors
Changing nutrient pool to mirror the gradients of nutrients to which the embryo is exposed as it progresses through the oviduct to the uterus *in vivo*	Constant level of nutrients with plausible metabolic consequences

Human embryos are currently cultured in either a dynamic environment such as that facilitated by sequential media, or in a static environment, through the use of a single medium for all stages. The use of either system encounters some form of compromise, which are considered throughout the text. The influence of one or more factors in either culture system therefore has the capacity to affect the efficacy of the type of media/environment used. Consequently, it is conceivable that optimal embryo development has yet to be achieved, and will plausibly require a device (potentially one based on microfluidics) to create an environment where all stresses discussed can be alleviated.
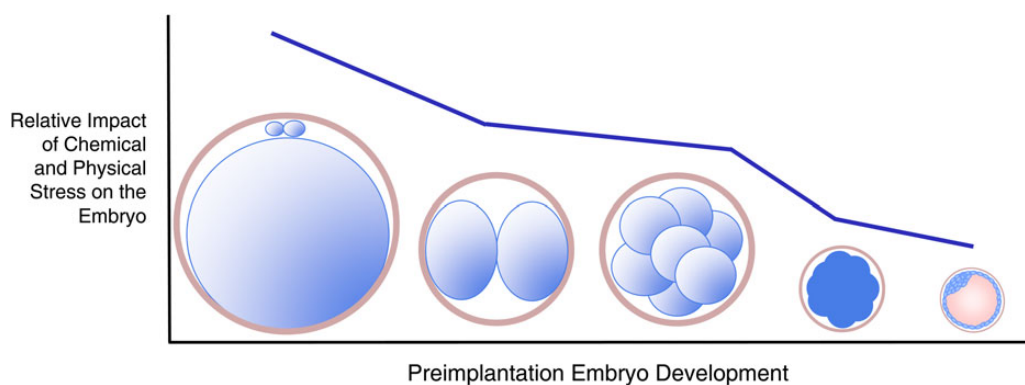

Figure 5 Relative impact of chemical and physical stress on preimplantation embryo (from the fertilized oocyte to the blastocyst stage), representing the stage-specific differences in the embryo's response to stress. The fertilized oocyte is more sensitive than the cleavage stage embryo, which in turn is more susceptible to stress than an embryo post-compaction. Of all stages, the blastocyst is least perturbed by such factors.

conventional laboratory conditions, gametes and embryos are exposed to light of various wavelengths, intensities and sources (both ambient and during assessment under a microscope). Using hamster and porcine models, it has been shown that light can directly alter embryonic development (Oh *et al.*, 2007; Li *et al.*, 2014c), or indirectly via photo-oxidation of components in medium (Li *et al.*, 2014c). Similar to previous stressors, such as oxygen and ammonium, Schumacher and Fischer (1988) demonstrated that the pre-compaction stage embryo is more sensitive to direct light than the post-compaction stage. Furthermore exposing Day 1 rabbit embryos to light for as little as 1 h resulted in decreased cell proliferation as measured by the incorporation of thymidine (Schumacher and Fischer, 1988). Light in the green spectrum (~510 nm) has higher energy and should be avoided (Boone *et al.*, 2010). The advent of time-lapse systems which typically employ light of long wavelength (625–635 nm using a red LED) and hence lower energy, combined with very short exposure times (even when successive images are added up over several days), should assist in reducing this stress on the embryo (Chen *et al.*, 2013).

With regards to the oil used to overlay culture media droplets, studies have shown that as a result of exposure to light, peroxides can form in a cascade reaction, the result of which is transfer of water soluble contaminants into the culture drops (Otsuki *et al.*, 2007, 2009). So it is important to consider that whilst commercial oil products may arrive from the manufacturer with a valid certificate of analysis, exposure to sunlight and/or viable light during transportation or improper storage may result in the defilement of the oil.

Conclusions

The impact of culture media formulations on mammalian embryo development has been extensively documented over the preceding five decades. Improvements in the success of human ART can be attributed, in no small way, to the continued efforts of laboratories around the world to optimize media formulations. However, what is evident is that media are but one aspect of the embryo culture system, which comprises other factors, both chemical and physical (Gardner and Lane, 2003). These

factors in isolation have been shown to have dramatic effects on embryo physiology and viability. Furthermore, there can be cumulative effects of such stressors which act synergistically, as documented for the negative interactions between 20% oxygen and the ability of the preimplantation mouse embryo to detoxify ammonium (Fig. 3). Similarly, when Fischer et al. (1988) investigated the adverse effects of exposure to visible light and room temperature in rabbit cleavage stage embryos and morulae, it was determined that embryonic damage was detectable in both stages of development after 1 h exposure, but that the combined exposure to light and room temperature amplified the detrimental effects (Fischer et al., 1988). Through the use of bench-top incubators and emergent technologies (time-lapse) several of the described chemical and physical factors can be addressed quite effectively, for example the use of 5% oxygen. An overview of several chemical and physical factors is represented in Fig. 4, and their presence and effects in different types of culture environment is considered in Table II.

It is also evident that there are clear stage-specific differences in the embryo's response to stress, and that the oocyte and cleavage stage embryo are far more vulnerable to their environment than the embryo post-compaction (Fig. 5). As such, the success associated with blastocyst transfer could reflect the stress associated with asynchronous transfer of the cleavage stage embryo to the uterus, a setting which provides a different environment to that which the cleavage stage requires (Barnes, 2000; Walker et al., 2015). Nevertheless, some parties continue to argue against the use of extended culture in human IVF, advocating the transfer of the cleavage stage human embryo to the uterus (Brison et al., 2014). In the publication of Scherrer et al. (2012), apparently healthy children conceived through IVF exhibited generalized vascular dysfunction associated with ART, fuelling the discussion around laboratory effects. However, these children were derived from embryos transferred at either the pronucleate oocyte or 2- to 4-cell stage following culture in medium lacking amino acids and grown in 1 ml of culture medium. Which aspect of the ART cycle induced vascular dysfunction, including the stimulation regimen, warrants consideration. It is the premise of this review that it will most likely be a combination of factors/stresses which result in the long-term consequences such as those reported by Scherrer and colleagues, with the cleavage stages most susceptible to the majority of these stresses.

Given the documented sensitivities of human gametes and embryos to several chemical and physical factors within the human IVF laboratory, ensuring that each human IVF laboratory has an adequate quality control and assurance programme in place is a prerequisite for optimizing performance and the successful maintenance of pregnancy outcome. Unless one can guarantee the quality of each component of the culture system (Gardner and Lane, 2003), and can track each lot number of media and consumables, then it is extremely difficult to maintain laboratory performance (Mortimer and Mortimer, 2015).

This review has focused on environments and factors within the laboratory. However, there are other sources of stress which affect the gametes themselves even before the patient attends for oocyte retrieval, including the administration of exogenous gonadotrophins (Huffman et al., 2015). Several research groups have also focused on the impact of parental diet on gamete and embryo quality, revealing that both maternal and paternal dietary status have a significant role in determining the outcome of assisted conception procedures (Grindler and Moley, 2013; Lane et al., 2014), and indeed that when both parents are affected, then there is an even greater effect on the developing embryo (Finger

et al., 2015). Consequently, the impact of the factors described in this review on gametes and embryos may vary according to the status of the patients, including their age and weight. Discussions arising from publications on the effects of human embryo culture media on resultant birthweights need to be tempered in light of the fact that so many variables can impact transfer outcome. Further studies on gamete quality and the interactions of all factors associated with the embryo culture system will continue to provide a greater insight into the regulation of preimplantation human embryo development, and the mechanisms associated with the programming of a healthy pregnancy and child.

Conflict of interest

P.L.W. has no conflicts of interest. D.K.G. receives research funding from Vitrolife (AB).

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