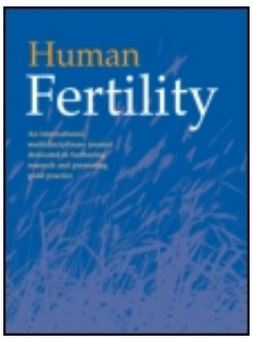

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Human Fertility

an international, multidisciplinary journal dedicated to furthering research and promoting good practice

ISSN: 1464-7273 (Print) 1742-8149 (Online) Journal homepage: https://www.tandfonline.com/loi/ihuf20

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To cite this article: Virginia N. Bolton, Rachel Cutting, Helen Clarke & Daniel R. Brison (2014) ACE consensus meeting report: Culture systems, Human Fertility, 17:4, 239-251, DOI: 10.3109/14647273.2014.944417

To link to this article: https://doi.org/10.3109/14647273.2014.944417

Human Fertility, 2014; 17(4): 239–251

ISSN 1464-7273 print/ISSN 1742-8149 online
DOI: 10.3109/14647273.2014.944417

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ORIGINAL ARTICLE

ACE consensus meeting report: Culture systems

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Abstract
The UK Association of Clinical Embryologists held a workshop on Culture Systems for assisted conception in Sheffield on 22 May 2013. The meeting was organised in the light of the availability of numerous commercial products for the culture of human preimplantation embryos *in vitro* and the absence of data comparing the performance of these products. Expert opinions were presented, along with survey data provided by participating IVF Centres. The workshop highlighted the lack of a sound evidence base to support the selection of any one commercial product over another, and raised concerns over the lack of information defining precisely the composition of media, and the potential for adverse long-term effects of such products following their use in assisted conception.

Keywords: *Human embryo, IVF, embryo culture*

Introduction

Since the use of a simple balanced salt solution for the fertilisation *in vitro* and culture of the embryo that led to the birth of Louise Brown in 1978, culture systems used in human *in vitro* fertilisation (IVF) and other assisted reproduction technologies have been modified, and now there are numerous products available commercially. With this in mind, the UK Association of Clinical Embryologists (ACE) held a workshop on Culture Systems for assisted conception in Sheffield on 22 May 2013, in order to consider various aspects of current and future practice. The focus of the meeting included the following:

- the rationale behind inclusion of different components of culture media, and their validity in light of current knowledge;
- which commercial products are used, in what manner, by how many centres and with what results?
- the potential effects and modes of use of various media, for prolonged culture;
- whether it is possible to improve current outcomes, as measured by
    - embryo quality (e.g., cell numbers in blastocyst cell lineages; embryo metabolism, identification and measurement of biochemical markers of embryo viability),
    - live birth rate per embryo transferred,
    - number of embryos cryopreserved, and
    - impact on live birth weight/long-term health of offspring
- Should commercial companies be required to provide precise information on the composition of their media? and
- the need for thorough product evaluation through rigorously designed trials for currently available, and future, culture media

Expert contributions

As Chair of the meeting, ***Daniel Brison*** *(St Mary's Hospital, Manchester)* introduced the proceedings by considering the key events of preimplantation human embryogenesis. The many pivotal events that occur during this time include fertilisation, syngamy, embryonic genome activation, extensive DNA demethylation and remethylation, maintenance of genomic imprinting, and blastocyst formation and the derivation of distinct cell lineages starting with the inner cell mass and trophecto-

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(Received 10 July 2014; accepted 10 July 2014)

derm. The question to address is how good are the culture systems used routinely in assisted conception, that is, to what extent do they sustain the normal physiology of the embryo? In order to answer this, it is important to focus on critical end points, which traditionally have included measuring rates of embryo development, implantation, and successful pregnancy. More recently, attention has been drawn towards evaluation of the long-term health of offspring, since a number of variables in ART have been reported to affect birth weight in infants conceived by assisted conception, including potentially the culture medium used (Dumoulin et al., 2010; Nelissen et al., 2012). This may have consequences for long-term child health and the early onset of adult diseases, following the Barker hypothesis (Barker & Osmond, 1986) subsequently termed the Developmental Origins of Health and Disease (DOHaD) phenomenon. Such reports have not gone unnoticed, with organisations such as the UK Human Fertilisation and Embryology Authority (HFEA), the European Society of Human Reproduction and Embryology (ESHRE) and the European Commission (via funding for the EpiHealth project) concerned about the quality of culture media and possible associations between IVF culture conditions and offspring health.

Examination of the components of the various commercially available culture media reveals a wide range of concentrations of different ingredients, not all of which can be determined from the information provided by the manufacturer. In the UK alone, a wide range of culture media products are used, and while most centres use a single system from one source, others may a combination of different products.

The aim of the meeting was to present expert opinion on the relative importance of different components of culture systems, to share experience and data among UK clinics, including a comparison of different commercial products and modes of use, and to attempt deriving a consensus for good practice.

Henry Leese *(Hull York Medical School)* provided the context for the workshop by summarising the major events in the development of human IVF (reviewed by Biggers, 1987; Table I).

Prior to 1949, attempts to culture mammalian embryos *in vitro* used the rabbit model and a variety of poorly defined biological fluids, such as coagulated blood plasma. Later, the mouse embryo became the focus of research, with the first success achieved by Hammond, who was able to culture embryos from the 8-cell stage to blastocyst stage in physiological saline supplemented with hen's egg yolk and white. Following further developments by Whitten, and by Brinster and Biggers, which included the substitution of egg white for bovine serum albumin, incorporation of an energy substrate (first glucose, later lactate) and antibiotics, adjusting the pH to 7.4 using 5%CO_2, and optimising osmolarity, a breakthrough was achieved in 1958, when McLaren and Biggers demonstrated the successful development and birth of mice following culture of embryos *in vitro* in Whitten's medium. Their farsighted paper predicted that "*the study of the cultivation and transfer of embryos is …of the greatest interest, both from the point of view of pure science, and because the techniques associated with it are potentially of immense value in the investigation of many biological problems in medicine and agriculture"* (McLaren & Biggers, 1958; Biggers, 1987).

Other breakthroughs in the 1950s included the development of techniques to induce ovulation (Fowler & Edwards, 1957) and to capacitate spermatozoa *in vitro* (Austin, 1951; Chang, 1951), as well as extensive metabolic studies with Wales and Whittingham joining the field (Biggers et al., 1967) serving to pave the way for the eventual successful fertilisation of human eggs *in vitro* (Edwards et al., 1969). Throughout the 1970s and 1980s, research concentrated on attempts to refine the composition of culture media, to mimic more closely the environment in the female reproductive tract (the so-called Back to Nature approach, reviewed by Leese in 1998). Although sampling the female tract is technically challenging and prone to artefacts, making it difficult to define its microenvironments (reviewed by Leese et al., 2008), the notion of a human embryo

Table I. The development of human IVF.

1561 First correct anatomical description of the Fallopian tube: G. Fallopius
1677 Discovery of mammalian spermatozoa: A van Leeuwenhoek
1797 Recovery of embryos from rabbit Fallopian tube: W C Cruikshank
1827 Identification of an egg in a mammalian ovarian follicle: von Baer
1878 Understanding that fertilisation requires the fusion of one sperm with one egg: Hertwik (sea urchin), Van Beneden (rabbit) & Fol (starfish)
1890 First embryo transfer (in rabbit): Heape
1912 First culture of mammalian embryos: Brachet
1930 First experiments on IVF (rabbits): Pincus
1932 Publication of *Brave New World*: A Huxley
1944 First attempt at IVF using human oocytes: Rock & Menkin
1949 Culture medium in which 8-cell mouse embryos developed to blastocysts: J Hammond Jr
1951 Capacitation in sperm: Chang; Austin
1958 Transfer of cultured mouse blastocyst to the uterus of another female followed by birth of live young: McLaren & Biggers
1959 Unequivocal demonstration of IVF in the rabbit: Chang
1969 Demonstration of human oocyte fertilisation *in vitro*: Edwards, Bavister & Steptoe
1972 Successful freezing of mammalian embryos (mouse): Wilmut, Whittingham
1976 First human pregnancy (ectopic) after IVF and embryo transfer: Steptoe & Edwards
1978 Birth of first child following IVF and embryo transfer: Steptoe & Edwards

culture medium based on human tubal fluid attracted interest as the field of human IVF and assisted conception expanded.

In 1991 Lawitts and Biggers (1991a, 1991b) achieved another landmark in defining the requirements of the embryo *in vitro*, by carrying out a series of elegant experiments in mice using engineering-based strategies to optimise the constituents of the culture medium. However, their experiments required up to 1,000 mouse embryos to optimise a single component of the medium, rendering this approach unrealistic in the investigation of human embryos. Nonetheless, these early studies were key to supply a sound evidence base from which to derive culture media for mammalian embryos, in establishing that their nutrient requirements are relatively simple, that the early embryo can survive in a variety of media, and is highly adaptable (reviewed by Summers and Biggers, 2003).

In spite of this early work demonstrating the resilience and adaptability of the early embryo, more recent attempts to improve culture systems have focussed on the concept of the changing substrate requirements of the embryo throughout development, as suggested by mouse work using CZB medium (Chatot et al., 1989), in which, for example, the omission of glucose during culture of early mouse embryos, and its addition only after the 8-cell stage, was shown, alongside other modifications (notably, high lactate/pyruvate ratio; presence of glutamine and EDTA) to overcome developmental arrest. This demonstration of a change in the mouse embryo's requirement for an energy substrate together with other work formed the basis of the use of sequential media for the facilitation of development of firstly mouse and then human blastocysts *in vitro* (reviewed by Gardner & Lane, 1997). This was further encouraged by the drive to improve the identification of embryos with the potential to implant, transfer and for cryopreservation, thus leading to improved success rates of assisted conception.

However, a fundamental problem in the derivation of sequential media for human embryo culture is that the majority of development and optimisation work has been carried out not in human, but in mouse embryos. If sequential media are indeed necessary for human embryo culture, it might be postulated that there should be five following distinct media in the sequence (reviewed by Leese, 1995; Biggers & Summers, 2008):

(i) *For oocyte aspiration*: a medium mimicking human follicular fluid at the appropriate hormonal stage
(ii) *For ovum incubation*: a medium mimicking the fluid in the ampulla of the human Fallopian tube post-ovulation
(iii) For fertilisation: a medium corresponding to that at the ampullary-isthmic junction post-mating
(iv) *For preimplantation embyo culture*: a medium corresponding to that in the oviduct post-fertilisation
(v) *For embryo transfer*: a medium similar to that in the uterine lumen on day 5 post-insemination

Interestingly, most sequential media systems that are available commercially use only 2 of the possible 5 listed. Might it be possible that the widespread adoption of the use of sequential media has taken place without sufficient rigorous scrutiny of the arguments in its favour? The evidence base for culture media that are currently used widely is weak, and use of a single medium throughout preimplantation embryogenesis has returned to favour.

In the development of the "new generation" of culture media, amino acids have emerged as being critical components, a notion supported by work in the mouse and sheep (Gardner et al., 1994), and human (Devreker et al., 2001), with the following caveats: the distinction between essential and non-essential amino acids, which has become an accepted dogma, does not match the requirements of human embryos, which make no distinction between them; the concern over the toxicity of ammonia levels accumulating in media through breakdown of amino acids depends critically on the concentrations of amino acids and in particular one or two which are highly labile, for example, glutamine; that the supplementation of media with certain growth factors is a concern since physiologically, the embryo will be exposed to numerous such factors; and the importance of autocrine/paracrine factors in "cross talk" between early embryos, already shown for mouse (Paria & Dey, 1990), porcine (Stokes et al., 2005), bovine (Gopichandran & Leese, 2006) and human (Ebner et al., 2010) embryos, will need to be addressed.

Analysis of the constituents of some of the media marketed for the use of human IVF illustrates how novel constituents have been introduced and accepted without sound supporting evidence. One such medium, for example, contains 4 vitamins, but the selection of these 4 would appear to be based on mouse work with no human evidence; another medium has phosphate omitted, again based on mouse studies with little supporting data on human embryos. While the derivation of media for human use from animal models has been necessary, owing to the scarcity of human embryos for definitive studies, and while such models have undoubtedly been pivotal in the development of human IVF, it is important to be wary of over-extrapolation from animal species to human systems. For example, in terms of nutrition, mouse embryos are not as resilient as those of larger species, surviving for a relatively short period when cultured in the absence of exogenous nutrients compared with the rabbit or cow embryo (reviewed by Leese & Ferguson, 1999).

A systematic review of studies comparing different culture media used in human IVF (Mantikou et al., 2013) excluded many published studies on the grounds that they were under-powered or poorly designed, and concluded that there is no evidence to suggest that one medium is better than another, in terms of ongoing clinical pregnancy or live birth rate. Clearly, rigorously designed, randomised, controlled

trials are needed for evaluation of both currently available and newly introduced culture media. Such studies must examine a variety of endpoints, including molecular, cellular and physiological, as well as the short- and long-term health of offspring. The ideal studies would begin with rodent models, move to larger animals, and finally to spare human embryos (Harper et al., 2012), with different media products only being introduced commercially once proven through such research, followed by clinical trials. Unfortunately, the realistic prospects for such a "back to basics" route are relatively low.

Leese concluded that while early animal studies have provided a good evidence base from which to derive culture media for use in human IVF, the mouse embryo is in many ways a poor model for the early human embryo. However, it is clear that the nutritional requirements of preimplantation mammalian embryos are relatively simple, and that the early embryo is highly adaptable, and can survive in a variety of culture media. The various commercial products that are available for use in human IVF are very similar to one another, while the introduction, and putative benefits of any '*novel*' constituents are based on insufficient evidence (Harper et al., 2012).

Roger Sturmey *(Hull York Medical School)* addressed the question of embryo metabolism, defining the metabolic needs of the human embryo, namely, a means of generating ATP and a source of biosynthetic precursors, and examining how these needs are met. In examining these, it is necessary to identify key developmental milestones, in terms of which, one can ask the following questions: (i) are any substrates including glucose, lactate, pyruvate, oxygen and amino acids required at each stage? (ii) do these requirements vary between individual embryos? and (iii) are they defined by variation in the quality of the egg itself?

Key milestones in development of the embryo from the zygote to the blastocyst include the following: early rapid, mitotic cleavage divisions and the absence of embryo growth, with cell (blastomere) size reducing at each division; embryonic gene activation occurring between the 2- and 8-cell stages depending on the species; compaction, with concomitant formation of gap and tight junctions, at around the 8-cell stage, after which the blastomeres, until now having behaved independently, act in conjunction with each other; and blastocyst formation entailing (i) the differentiation of inner cell mass (ICM) and trophectoderm (TE) lineages, (ii) a major increase in the level and variety of protein synthesis, (iii) a significant increase in activity of the enzyme Na+,K+, ATPase, and (iv) the commencement of true growth of the embryo. How does the embryo regulate and provide the metabolic requirements for all these processes?

In terms of energy substrates, glucose, pyruvate and lactate are included in all commercial culture media, and present in the female reproductive tract. The accepted dogma is that as the embryo forms a blastocyst it becomes glycolytic (at least *in vitro*: Leese, 2012), converting glucose to lactate in the generation of ATP; this follows the demonstration that glucose consumption and lactate production spike around the time of blastocyst formation in animal embryos (e.g. Houghton et al., 1996). However, in terms of energy production, glycolysis is relatively inefficient, generating only 2 ATPs through the conversion of glucose to lactate compared with ~32ATPs generated by the complete oxidation of glucose. This raises the question why, at a time when ATP requirements are high and increase throughout development, and when oxygen consumption has been shown to increase, would the embryo utilise glycolysis when oxidation of glucose is much more efficient?

This increase in glycolysis and the production of lactate, despite high oxygen consumption, are also seen in cancer cells, which can switch from oxidative metabolism to glycolysis even in the presence of adequate oxygen (the Warburg effect: reviewed by Smith & Sturmey, 2013). Pig embryos have been found to express two of the same gene variants that are known to be involved in regulation of metabolism in rapidly dividing cancer cells (HK2 and the M2 variant of PKM2; Redel et al., 2012). Expression of both HK2 and PKM2 M2 slows down glycolysis, so that glucose is metabolised to lactic acid through the anabolic pentose phosphate pathway, and it seems likely that these gene products are involved in the same processes in the developing embryo as in cancer cells. This leaves the question: how does the embryo meet its requirements for ATP? Amino acids may provide the answer.

Measurement of amino acid metabolism has been explored extensively as a means of evaluating preimplantation development, including embryo viability (Houghton et al., 2002; Brison et al., 2004; Stokes et al., 2007), sex (Sturmey et al., 2010), aneuploidy (Picton et al., 2010), and DNA damage (Sturmey et al., 2009). The importance of amino acids is attributed to roles in protein and nucleotide synthesis, in acting as osmolytes and as precursors for signalling molecules. However, amino acids can also be used as substrates for energy metabolism.

Of the many different amino acids that are utilised at different levels during embryogenesis by different animal species, glutamine has attracted considerable attention: the requirement for glutamine changes as development proceeds (pig; Humpherson et al., 2005; human: Houghton et al., 2002; Brison et al., 2004). It is added to most commercial culture media, often as the more stable synthetic dipeptide glutamax, owing to concerns over ammonia accumulation through degradation (see above). In bovine embryos, glutamine added as a single amino acid may be used as an oxidative substrate (Rieger & Guay, 1988), and it is highly likely that rather than glucose, embryos utilise amino acids as oxidisable energy substrates.

Finally, as the largest diameter cell in the mammalian body, the oocyte contains its own endogenous energy stores, including droplets of energy-efficient lipid. Thus in some species, the addition of exogenous substrates to culture media may not merely be unnecessary, but det-

rimental. Certainly, work using the bovine model suggests that excess exogenous lipids in culture media may have negative effects on oocyte maturation and embryo development (Van Hoeck et al., 2011).

Thus, whilst recognising that the needs of the developing human embryo, in terms of the production of ATP and of biosynthetic precursors, must be met by whichever culture medium is used, and whilst knowledge of the precise nature of those needs is increasing, uncertainty persists concerning the extent to which they are met by endogenous substrates inherited by the oocyte and/or require a source of exogenous substrates. While the embryo is able to adapt and utilise selective substrates that have been added to culture media, the extent of this adaptability, and the levels and nature at which different additives may be detrimental, remains uncertain.

Michael Summers *(Bridge Fertility Centre, London)* considered the use of single-step and sequential media for culture of embryos to the blastocyst stage. The use of sequential media derives largely from observations in the mouse, namely that glucose utilisation by the embryo increases between early and late cleavage stages, and the associated suggestion that to relatively high levels of glucose during early cleavage is detrimental (Gardner & Lane, 1997, 2003). Yet there is a wealth of evidence to suggest that varying glucose levels have no effect on development in the mouse (Summers et al., 1995). Despite this, there has been widespread adoption of the dogma that sequential media should be used if the optimum conditions for development of human embryos to the blastocyst stage are to be achieved. Thus, currently available commercial products for the culture of human embryos promote alternatively: uninterrupted culture using a single medium throughout the 5–6 days of preimplantation embryo development (*nonrenewal single medium protocol*); interrupted culture using a single medium, but replacing ("refreshing") the same medium after 72 hours (*renewal single medium protocol*); interrupted culture using two media of different composition in a sequential fashion (*sequential media protocol*). In spite of widespread use of sequential media, and acceptance of the premise behind it, the rationale and necessity for sequential media has been questioned (Biggers & Racowsky, 2002). Indeed, critical analysis of the results of studies used to justify the use of specific sequential media (G1/G2 type; Gardner & Lane, 2003) led to the conclusion that while two-step culture systems may be sufficient to support development, they may not be necessary (Summers & Biggers, 2003). Using mouse embryos, no significant differences were observed in the rates of blastocyst formation and hatching, or in the numbers of cells in the inner cell mass (ICM) and trophectoderm (TE), whether embryos were cultured in single-step or sequential media (Biggers et al., 2005). Moreover, a review of the literature (Biggers & Summers, 2008) revealed that in the 7 studies evaluated, the rate of human blastocyst development tended to be greater using a single-step culture system compared to a sequential system; in four of the studies, the comparison was statistically significant. No statistical significance was shown between single-step and sequential media in terms of pregnancy rate, suggesting strongly that use of a single-step culture system is at least as effective as that of sequential systems. More recent publications in the human support this conclusion (Sepulveda et al., 2009; Reed et al., 2009; Summers, 2014).

The reasons behind the adoption of sequential culture systems deserve re-examination. First, while it is known that there is a switch from the preferential consumption of pyruvate to that of glucose during mouse preimplantation development (Martin & Leese, 1995), the extrapolation of this into a requirement for differential concentrations of exogenous glucose during early cleavage may not be valid (Biggers & McGinnis, 2001); second, it has been suggested that EDTA concentration should be high in order to regulate glycolysis, as shown in the mouse embryo (Lane & Gardner 2001), but other studies using lower concentrations of EDTA in culture media do not support this (Abramczuk et al., 1977; Lawitts & Biggers, 1991a); third, concerns that the instability of glutamine results in ammonium production and consequent toxic effects during protracted culture can be resolved by substitution of glutamine with the synthetic dipeptide glutamax (Summers et al., 2005); fourth, the concept that there is a distinction between, and selective requirement for, essential and non-essential amino acids (Eagle, 1959; Lane & Gardner, 1998) requires further evaluation.

Daniel Brison examined the question of whether or not to supplement culture media with growth factors by examining the rationale behind such an approach and considering potential candidates whose effects might be of benefit, before discussing possible implications for future offspring. The embryo is inevitably subjected to stress during manipulation *in vitro*, and despite its adaptability, the consequences of such stress-induced perturbations, through modifications to gene expression and/or metabolism, may not be manifested until later in development (reviewed by Leese, 2012). Such manifestations include congenital abnormalities (Davies et al., 2012), imprinting disorders or disrupted DNA methylation (Katari et al., 2009; Rancourt et al., 2012), early-onset adult diseases such as hypertension (Ceelen et al., 2008, 2009) and pre-term birth or low birthweight where effects have been associated with embryo cryopreservation (Wennerholm et al., 2009; Nelissen et al., 2012), culture medium (Dumoulin et al., 2010; Vergouw et al., 2012; Nelissen et al., 2012) and extended culture to the blastocyst stage (Dar et al., 2013; Maheshwari et al., 2013). It is now known that poor foetal growth and low birth weight are associated with an increased risk of coronary heart disease, stroke, hypertension, Type 2 diabetes and osteoporosis (Wadhwa et al., 2009).

Evidence for earlier manifestations of the embryo's response to stress through manipulation *in vitro* is provided by the observation that mouse embryos cleave

more slowly (Bowman & McLaren, 1970), develop less frequently to the blastocyst stage, show elevated levels of apoptosis, and form blastocysts with lower cell numbers *in vitro* than *in vivo* (Jurisicova et al., 1996, 1998; Brison & Schultz, 1997). The fact that these effects may be partially reversed by group or low volume culture (Wiley et al., 1986; Paria & Dey, 1990; Lane & Gardner, 1992; Brison & Schultz, 1997; Ebner et al., 2010) suggests a role for embryo-derived growth factors and/or cytokines.

The source of the complex mixture of growth factors to which embryos are exposed *in vivo*, and that are likely to influence embryo development may be autocrine, originating from the embryo, or paracrine, produced within the reproductive tract (reviewed by Hardy & Spanos, 2002). The natural corollary of this is to suggest that the identification of relevant paracrine factors and their addition to culture media might be of substantial clinical benefit (Richter, 2008).

Studies on human embryos cultured from day 2 to day 5 in physiological concentrations of the growth factors leukaemia inhibitory factor (LIF; Dunglison et al., 1996), insulin-like growth factor-1 (IGF1; Lighten et al., 1998), and heparin-binding epidermal growth factor (HBEGF; Sargent et al., 1998) have shown that each of these growth factors promote blastocyst formation. GM-CSF also promotes human blastocyst formation, has been shown to reduce apoptosis in human blastocysts (Sjöblom et al., 2002), and in mouse, may even alleviate adverse effects of embryo culture on subsequent foetal development (Sjöblom et al., 2005). However, reduced apoptosis is not necessarily beneficial, since rescuing cells from cell death may interfere with normal processes designed to eliminate abnormal or damaged cells from development. The effects of exposure to growth factors during preimplantation development may not always be beneficial that has been demonstrated using IGF1s, where effects include increased foetal weight, decreased DNA methylation, and increased expression of certain imprinted genes (mouse; Shao et al., 2007), decreased apoptosis (bovine; Byrne et al., 2002), increased implantation rate (bovine; Block et al., 2011), and large offspring syndrome (LOS; bovine; Chen et al., 2013).

In order to establish whether or not supplementation of culture media used for human IVF with specific growth factors is beneficial, risk/benefit analysis is required in order to establish if "success rates" are indeed improved, and if so by how much; what is the precise nature of their effect, if any, on embryo development; and finally, what effect do they have on foetal development, birthweight and the long term health of offspring? A randomised clinical trial has explored these questions, examining the effect of supplementation of culture medium with GM-CSF (Ziebe et al., 2013). The findings from this large multi-centre trial suggest that GM-CSF increased ongoing implantation, clinical pregnancy and live birth rates in a subgroup of women who had experienced previous miscarriage. However, the authors point out that this interpretation must be confirmed in a well powered prospective randomised controlled study on this specific group of patients. Interestingly, the observed benefit of GM-CSF supplementation was only apparent in culture medium containing a low concentration human serum albumin (2 mg/mL HSA), and not when a higher concentration of HSA (5 mg/mL) was used, leading to the possibility that the effect of GM-CSF was to alleviate culture stress induced by suboptimal conditions

The study reported no effect of GM-CSF on the incidence of abnormal outcomes or birthweight. Any further possible risk, or indeed benefit in reducing the miscarriage rate, will require further evaluation.

Finally, the fact cannot be ignored that undefined growth factors may be present at unknown concentrations in the commercial serum supplements that are used routinely in IVF culture (Meintjes, 2012), and until these are defined, their effects cannot be evaluated.

John Huntriss *(University of Leeds)* discussed the potential sources of damage to embryos through *in vitro* culture, with particular reference to the potential for epigenetic alterations. Epigenetic effects are those where changes in phenotype or gene expression are caused by mechanisms other than the changes in the underlying DNA sequence. In normal development, the preimplantation embryo requires epigenetic information inherited from the sperm and oocyte, without which the embryo is non-viable. Significant reprogramming of epigenetic information occurs during gametogenesis and preimplantation development, coinciding precisely with the period of exposure to *in vitro* culture conditions during IVF, leading to the possibility that IVF may affect epigenetic reprogramming, causing disease.

Epigenetic marks, which are molecular modifications that regulate gene expression and genome function, can lead to heritable changes in gene expression without changes in DNA sequence. The major epigenetic marks are modifications of histone proteins (including methylation and acetylation) which alter the activity of the associated DNA, and DNA methylation (the addition of methyl marks to DNA bases) usually associated with repression of gene activity. These differ between the maternal and paternal genomes, and can be recognised by the early embryo.

Global remodelling of epigenetic marks is associated with major transitions of cellular identity (reviewed by Morgan et al., 2005). Throughout gametogenesis and embryogenesis, epigenetic marks may be erased (demethylation in primordial germ cells and of the paternal genome after fertilisation), maintained (methylation status of imprinted genes during preimplantation development) or established *de novo* (methylation of the gamete genomes, and differentiation of the ICM and TE cell lineages in the blastocyst). Epigenetic information regulates gene expression via mechanisms including genomic imprinting, whereby genes are expressed in a parent-of-origin manner, from either the maternal or the paternal alleles, but not both.

It has been postulated that there are up to 200 imprinted genes in the human genome (Luedi et al.,

2007), and these are known to have a role in growth or placental function. Imprinting disorders, by which genes are expressed from the inappropriate parental allele, may cause diseases, and of these, there have been reports of associations with assisted conception, in particular for Beckwith–Wiedemann (DeBaun et al., 2003), Angelman (Cox et al., 2002) and Silver–Russell (Hiura et al., 2012) syndromes. Indeed, there is mounting evidence that *in vitro* culture conditions and the use of certain assisted conception techniques may disrupt epigenetic programming. Possible causes of such effects include culture media used both for oocytes [including *in vitro* maturation (IVM)] and for embryos throughout preimplantation development; the use of controlled ovarian stimulation, and possibly immature gametes, for IVF; intracytoplasmic sperm injection (ICSI); and cryopreservation of gametes and embryos.

There is a wealth of information from animal studies indicating that the composition of and the use of certain additives to culture media affect epigenetic mechanisms and thus the regulation of imprinted genes (Sasaki et al., 1995; reviewed by Khosla et al., 2001; Denomme & Mann, 2012; Table II), with possible long-term developmental consequences, including disease, deformity, behavioural changes and obesity.

One of the most severe epigenetic effects is large offspring syndrome (LOS), where animals grow abnormally large during gestation and pose a danger to the mother and the foetus (Young et al., 1998). Development of the syndrome has been shown to be associated with *in vitro*-induced epigenetic effects on the IGF-2 receptor (Young et al., 2001) and on the imprinting control region KvDMR1, which regulates the coordinated expression of certain growth control genes (Hori et al., 2010). It is probable that rather than targeting only a single imprinted gene, epigenetic disruption in these cases is more global. Thus, the consequences would depend upon the stage at which the effect is exerted, and the developmental role(s) of the affected genes.

Studies have compared the effects of different culture media that are available commercially for use in human IVF on development of and genomic imprinting in mouse embryos cultured *in vitro* with *in vivo* controls (Market-Velker et al., 2010; Schwarzer et al., 2012). These have demonstrated that media type significantly affects blastocyst and foetal development rates, and significantly affects imprinted gene expression, and cell lineage composition (ICM:TE), in blastocysts. (Market-Velker et al., 2010), All five commercial media were found to cause loss of imprinted gene methylation compared with *in vivo* controls, with the extent of loss varying between media, and the effect exacerbated in all by the use of superovulation (Market-Velker et al., 2010). In the bovine, a review of studies that have examined the effect of different culture conditions on the transcriptome profile of bovine embryos identified 54 genes that are implicated in the regulation of preimplantation development that may be influenced by *in vitro* culture (Gad et al., 2012).

In the human, studies are more limited; however, comparisons of IVF outcomes using two different culture media have drawn attention to the possibility of an association between the culture medium used for IVF and the birthweight and perinatal outcome of resulting infants (Dumoulin et al., 2010; Nelissen et al., 2012). Another retrospective comparison between birthweight and length of newborns following IVF using 2 other commercially available media showed no significant association between birthweight or length and the type of IVF culture medium used (Lin et al., 2013). Despite this, it was acknowledged by the authors that the effects of culture medium on epigenetic variation need further investigation (Lin et al., 2013).

Table II. IVC and epigenetic defects (animal studies).

ART technique	Species	Affected gene	Comments	References
Culture Media	Mouse	*H19, Igf2*	Aberrant expression due to presence of FCS in M16 culture medium.	Khosla et al. (2001)
Culture Media	Mouse	*H19*	Loss of *H19* methylation upon culture in Whitten's medium	Doherty et al. (2000)
Culture Media	Mouse	*Igf2*	Aberrant expression bias to maternal Allele in preimplantation embryo	Ohno et al. (2001)
Culture Media	Mouse	*H19*	High levels of ammonium causes aberrant expression of *H19*	Lane and Gardner (2003)
Culture Media	Mouse	*Igf2, Meg1 and Peg1*	Reduced expression of three imprinted genes after culture with FCS	Fernández-Gonzalez et al. (2004)
Culture Media	Mouse	*H19, Igf2*	Quinn's medium affects *H19* expression in embryos, and *H19* and *Igf2* in ES cells	Li et al. (2005)
In vitro development	Sheep	*Igf2r*	Aberrant expression and methylation of *Igf2r* in a Large Offspring Syndrome model	Young et al. (2001)
In vitro development	Cow	*Igf2, Igf2r*	Reduced expression in *in vitro* produced embryos compared to *in vivo* embryos	Gutiérrez-Adán et al. (2004)
In vitro culture	Mouse	*Dnmt1*	Increased *Dnmt1* expression in *in vitro* produced blastocysts	Wang et al. (2005)
In vitro development	Cow	*Dnmt1, Mash2*	Increased *Dnmt1* expression, decreased *Mash2* in *in vitro* produced blastocysts	Wrenzycki et al. (2001)
In vitro development	Mouse, rat	DNA methylation	Increased DNA methylation compared to *in vivo* embryos	Zaitseva et al. (2007)

Although currently less widely practised, *in vitro* maturation (IVM) or growth (IVG) of oocytes for assisted conception presents further scope for epigenetic disruption. Studies on the mouse human and rhesus monkey have shown loss, gain or abnormal methylation of imprinted genes following IVM or IVG (Kerjean et al., 2003; Borghol et al., 2006; reviewed by Denomme & Mann, 2012).

Thus, epigenetic programming is a key mechanism that dictates the consequences of assisted conception in the short term (affecting blastocyst cell number and lineages, embryo metabolism and viability, and patterns of gene expression) and long term (affecting foetal growth and infant development and behaviour) and with implications for late-onset effects (obesity, cardiometabolic effects and transgenerational inheritance). Although concerns have been expressed (Maher et al., 2013; Palermo et al., 2008; Grace & Sinclair, 2009; reviewed by Huntriss & Picton, 2008; Batcheller et al., 2011; Hart & Norman, 2013), it is difficult to be certain what to advise patients without focussed research examining the effects of different culture media, and of specific assisted conception procedures.

Thomas Ebner examined how best to optimise individual elements of the environment in which human embryos are cultured, starting with the widespread use of mineral oil to overlay drops of culture medium. This practice protects the culture medium from evaporation, contamination, and fluctuations in pH and osmotic pressure. Commercially available products are routinely tested for microbial contamination and endotoxins, and *in vitro* efficacy using mouse embryos, yet toxic effects have been reported (Otsuki et al., 2007), highlighting the importance of protecting oil from oxidative effects including UV irradiation and atmospheric oxygen.

Levels of oxidative stress and the production of reactive oxygen species (ROS) are influenced by the culture environment, and have been shown to impact negatively on embryo development (Guérin et al., 2001). Endogenous cellular antioxidants which have a protective role include superoxide dismutase, glutathione peroxidase and gamma-glutamylcysteine synthetase enzymes, and non-enzymic hypotaurine, transcripts for which are stored in the oocyte during maturation. Despite their presence, and in the absence of full understanding of the complexity of the pro-oxidant-antioxidant equilibrium in embryos, it has become a common practice to supplement culture media with exogenous antioxidants, such as taurine and ascorbic acid. Further reduction in the formation of detrimental ROS is achieved through culture in an atmosphere of low oxygen, which has been shown in prospective randomised trials to enhance development of human embryos to the 8-cell stage *in vitro* (Kirkegaard et al., 2013), influence embryo metabolism (Gardner & Wale, 2013) and improve IVF outcome after embryo transfer at the blastocyst stage (Meintjes et al., 2009; Kovacic et al., 2010).

Whether or not it is necessary, and if so how frequently, to "refresh" culture medium during culture, in order to reduce accumulation of ROS as well as toxic by-products of metabolism such as ammonium (see above), remains controversial. The benefits of removing accumulating ROS or metabolic waste, which remain unproven, must be weighed against the potential detrimental effects of removing any accumulated embryo-derived autocrine or paracrine factors (Paria & Dey, 1990; reviewed by O'Neill, 2008). Accumulation of such factors will be time dependent, and as their effect is presumably concentration dependent, this will be influenced by the volume of culture drops and the number of embryos cultured per drop, as well as by whether or not the medium is "refreshed". However, there are very few studies that have examined the significance of these specific aspects of human embryo culture. Two retrospective studies demonstrated beneficial effects of cleavage stage group culture on embryo quality (Moessner & Dodson, 1995) and IVF outcome (Almagor et al., 1996); two prospective studies showed no effect, either up to day 2 of development (Spyropoulou et al., 1999) or when group culture was investigated between days 3 and 5 of culture (Rijnders & Jansen, 1999). However, the culture volumes used were relatively large, and the study periods possibly too short for sufficient accumulation of embryo-derived factors to have an effect.

A more recent study used culture dishes that allow the culture of individual embryos or groups of embryos, whilst leaving them able to communicate with sibling embryos through diffusion of embryo-derived factors (Ebner et al., 2010). This study found that embryos cultured in close proximity to each other showed better outcomes than those that were cultured separately, but still in communication with sibling embryos in the dish, implying that there may be an optimum minimum distance for proximity of embryos in culture if the positive co-operation effect is to be exerted, as has been suggested for the bovine and porcine embryos (Gopichandran & Leese, 2006; Stokes et al. 2005). However, embryo-to-embryo communication may not always be positive, as suggested by a study where poor-quality embryos appeared to exert a deleterious influence on development of good-quality embryos in co-culture (Tao et al., 2013).

Thus, while changing or "refreshing" medium during culture would be of benefit should it prove necessary to remove harmful waste products and replenish diminished substrates, such steps might eliminate any positive effects of accumulated embryo-derived development-enhancing factors. Alternatives (reviewed by Swain & Smith, 2011) include dynamic culture systems such as tilting (Matsuura et al., 2010; Hara et al., 2013) and micro-vibrating (Hur et al., 2013) culture systems, and microfluidics (reviewed by da Rocha & Smith, 2012), all of which have yet to be fully developed and evaluated.

Survey of UK culture practice

Before the meeting, 39 participating clinics provided information concerning the culture media and culture systems they used, as well as the implantation (presence

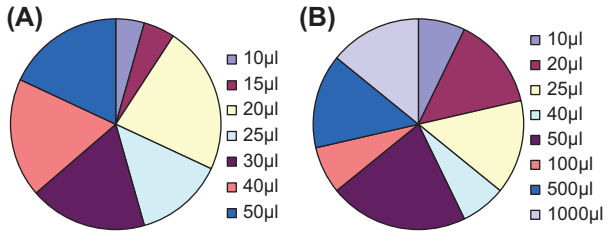

Figure 1. Proportion of participating clinics using different drop sizes for: **A** – Individual and **B** – Group embryo culture.

of foetal heart) rate per embryo transferred using that system. In total, 7 different products were used, either singly or in combination. Almost half the centres ($n = 18$) used one of only 2 commercial products ($n = 14$), while the remainder ($n = 19$) used one of 4 other products, or a combination of 2 different products ($n = 2$). The mode of utilisation of the different commercial products varied widely between centres: while 15 centres used culture medium and oil from the same manufacturer, the others used culture medium from one and mineral oil from another, or used a different culture medium at different stages of the IVF process. The majority of centres ($n = 25$) favoured individual culture of embryos, but the drop size varied between 10 and 50 µl, while centres culturing embryos in groups used drops varying in size between 10 and 1000 µl (Figure 1). The types of incubators used varied, with most ($n = 38$) centres using chest incubators and16 using bench-top incubators; 18 of the centres used more than one make and/or type of incubator. Although 7 centres used Embryoscope® incubators, none used these exclusively. Regarding low (5% O_2) oxygen culture, this was used by all but 6 centres, some culturing in low oxygen exclusively ($n = 19$), others only at specific stages of development ($n = 5$), and yet others using it randomly, according to available incubator space ($n = 9$).

Implantation rates reported by the centres ranged from 15–27% for one of the media used, to 18–38% for another, with no one medium obviously outperforming the others. Any useful interpretation of centres' outcome data, in terms of the performance of different culture media and conditions, is not possible due to the lack of any control for the many variables.

Summary: Discussion points arising from the meeting

At the end of the meeting, the following consensus discussion points were acknowledged as requiring further consideration:

(1) There is strong evidence for beneficial effects of low oxygen during culture.
(2) No individual commercially available culture system has been definitively shown to be better than others in terms of embryo viability.
(3) Concerns exist regarding possible epigenetic and long-term culture effects and require further investigation.
(4) Concerns over depletion of nutrients and accumulation of waste products during prolonged culture may be exaggerated, particularly considering the relatively large volumes of medium used routinely
(5) Large multicentred randomised controlled trials are needed to validate different commercial products.
(6) Commercial companies should provide explicit information concerning the precise concentration of each media product.

Acknowledgements

ACE would like to thank all speakers and delegates for sharing data and experience.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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