ESHRE Working group on Culture Media
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TIME TO TAKE HUMAN EMBRYO CULTURE SERIOUSLY

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Abstract

The safety of ART procedures, especially with respect to the health of the offspring, is of major importance. There are reports from the literature indicating a possible effect of culture conditions, including culture media, on embryo and fetal development.
Since the introduction of commercially available culture media, there has been a rapid development of different formulations, often not fully documented, disclosed, or justified.

There is now evidence that the environment the early embryo is exposed to can cause reprogramming of embryonic growth leading to alterations in fetal growth trajectory, birthweight, childhood growth and long term disease including type II diabetes and cardiovascular problems.

The mechanism for this is likely to be epigenetic changes during the preimplantation period of development. Thus, some evidence is there, but do we need to worry?

In the present paper the ESHRE working group on culture media aims to summarize the present knowledge of potential effects on embryo development related to culture media, and make recommendations to end-users of ART products.

Introduction

For a successful pregnancy to occur, good quality embryos that are able to implant are essential. Several studies have suggested that the composition of the media that embryos are cultured in may have an impact on the quality of embryos generated in IVF/ICSI cycles thereby influencing implantation and pregnancy rates (Mantikou, et al., 2013).

Looking back, the culture of human gametes and embryos has been performed with surprisingly little standardization and a lot of experimentation. In the first decade of IVF, the culture media ranged from fairly simple salt solutions such as Earle's or Tyrode's solutions to complex media intended for tissue culture such as HamF10. Even today commercially available culture media range from fairly simple with 8-10 different salts and sugars to media containing nearly 80 different component including amino acids, lipids, vitamins, trace ions, and bioactive molecules such as hormones and expression modulators (Chronopoulou and Harper, 2015; Dyrlund, et al., 2014; Gomes, et al., 2009; Morbeck, et al., 2014a;Sunde and Balaban, 2013).

Today, culture media for embryos can be used either as a sequential system, with different compositions for days 0-3 and 3-6, or as a single medium used for the whole culture period. Culture media should be used as intended by the manufacturer, however, in many laboratories combinations of media from different manufacturers are used (Biggers and Summers, 2008; Gardner and Lane, 1997), making the evaluation of potential impact more difficult.

It is well documented from epidemiological data that the nutritional status of the mother in early pregnancy may have lasting effects on the health of the offspring (Heijmans, et al., 2008; Huang,

et al., 2010; Roseboom, et al., 2006). In animal models, it is demonstrated that minor dietary changes that influence the composition of the intrauterine fluid at the time of implantation will have an effect on the cardiovascular system in the offspring (Kwong, et al., 2000; Watkins, et al., 2007). These observations have led to the formulations of the DOHaD (Developmental Origins of Health and Disease) hypothesis (Barker, 2007; *https://**dohad**soc.org*). This can be understood as a physiological response of the embryo to environmental conditions, most likely mediated through epigenetic changes that may persist throughout life and into subsequent generations.

Following this line of reasoning, it would not be surprising if different culture media may have different effects on the epigenome of the developing pre-embryo, and data suggests that in vitro culture of gamete and embryos may influence the phenotype of the offspring (El Hajj and Haaf, 2013; Gomes, et al., 2009; Katari, et al., 2009; Khosla, et al., 2001; Nelissen, et al., 2014). The consequences of this potential shift in phenotype after IVF is to a large extent unknown, and it is unclear whether this will have an effect on the long term health of the ART-offspring.

The following sections summarize the knowledge currently available on the main components of culture media and their possible effects on embryo and perinatal development and long-term outcome.

Media composition and significant changes made by manufacturers

Since the nineties the manufacturers have introduced numerous changes in culture media supplements, with the aim to improve success rates of IVF/ICSI. As shown in a recent study (Morbeck et al, 2014a), commercial IVF media vary widely in their nutrient composition, so to evaluate possible effects on embryo growth it is crucial to know which media the embryo has been exposed to. For example, amino acids (e.g. methionine) and vitamins taken up by the embryo have been shown to cause direct epigenetic changes via the 1 carbon cycle of the embryo (Xu and Sinclair, 2015, Menezo et al, 2013). The quantity of these vitamins and amino acids in culture media is variable and poorly defined.

Another important component are peptide growth factors, which are present in at least two commercially available IVF media. Growth factors have the ability to alter embryonic growth, cell

lineage allocation to inner cell mass (ICM) and trophectoderm (TE), and apoptosis, the mechanism by which developing organisms eliminates cells which contain genomic damage.

Another example of an important component in culture media formulations which is under debate is protein/albumin supplementation. HSA and protein preparations contain not only albumin, but also a range of potentially bioactive molecules and contaminants (Morbeck et al, 2014b). We still do not know to what extent different preparations of HSA will affect the development of human embryos in vitro. Several questions remain about culture media protein supplementation, such as: Can hyaluronan replace albumin? Is recombinant albumin as effective as sera-derived albumin?

It is still unclear how the composition of the media affects embryo quality and IVF/ICSI success rates and which – if any - culture medium leads to the highest IVF/ICSI success rates. Mantikou et al (2013) performed a systematic review including 22 studies of 20 different culture media from 11 commercial companies, but was unable to find a superior culture medium from the pooled data.

The potential impact that varying concentrations of components (such as energy substrates, amino acids, EDTA, macromolecules) and other variables (such as growth factors and osmolality) may have on early embryo development and on longer term health implications in the newborn or adult is not fully known. When comparing the potential impact of culture media formulations on embryo quality as well as on the epigenome, two additional questions must be considered (1) How long the embryo is cultured in vitro; and (2) What culture environment is provided by the laboratory.

For quality control, culture media companies use the mouse embryonic assay (MEA)(Ackerman et al., 1984; 1985; Byers et al., 2006). A specific culture medium is considered to pass the MEA if it supports development of ≥ 80% of mouse embryos to the blastocyst stage. Unfortunately there is no standardization of the day of development the culture should start, the mouse strain to be used, the minimum number of embryos to be cultured, the presence of oil overlay, the supplementation with proteins, osmolarity etc.
This raises concerns regarding the reliability of the current MEA as a proof of the suitability and safety of culture media for human embryos. No alternative is currently available.

Impact of culture medium on embryonic development

The genome and epigenome of the embryo are completely remodelled (integration of the paternal genome, transition from maternal to zygotic transcripts, global genome demethylation and remethylation, maintenance of genetic imprints marking parent of origin alleles) between fertilisation and implantation, making the embryo uniquely vulnerable at precisely the stage of *in vitro* manipulation and culture during IVF. At the blastocyst stage, changes in the allocation of cells to the ICM and TE lineages may also occur? This can alter ICM/fetal size directly, or indirectly via supply of nutrients by altering TE and hence placental size and function, and thereby altering fetal growth trajectory and birthweight.

Culture media contain a number of components which might affect epigenetic or other reprogramming events, leading to detrimental effects on the embryo and the newborn and potentially to long term health problems in the adult. A main component is nutrients. There is evidence that cells - including early embryos - undergo "nutrient sensing" in order to determine the environment in which they are developing. Glucose and amino acids are particularly important, triggering responses via the AMPK and mTOR pathways, both of which regulate growth and development including via epigenetic changes. Embryonic sensing of a low nutrient environment in IVF culture media can therefore programme the embryo to be "thrifty" in its metabolism, programming a long-term thrifty phenotype. When the embryo is then replaced in the maternal environment, it encounters a high nutrition environment and experiences overgrowth (Eckert et al., 2015; Pantaleon, 2015). The reverse would also be true, in a high nutrient culture medium environment.

The background literature in animals is extensive, showing that nutrition during preimplantation development, either via the maternal tract or in culture, can cause long term reprogramming of development and health (Kwong et al., 2000; Eckert et al., 2015). This is likely mediated by epigenetic changes, and there is good evidence that embryo culture particularly in suboptimal conditions disrupts the maintenance of the embryonic epigenome in the mouse and potentially human in preimplantation embryos (Market-Velker et al., 2010; Velker et al., 2012; White et al., 2015).

The impact of culture medium can be captured by global gene expression profiling (transcriptomics) of individual embryos (e.g. mouse: Rinauldo and Schultz, 2004; bovine: Urrego et al., 2014). Human embryos show intrinsic heterogeneity in their transcriptomes (Vassena et al., 2011; Shaw et al., 2013) with evidence of an impact of culture medium (Kleijkers et al., 2015a). Cryopreserved human embryos also show a different gene expression pattern to fresh embryos, including genes regulating formation of ICM and TE, suggesting a possible mechanism for altered fetal growth and placental function in children arising from cryopreserved embryos (Shaw et al., 2012).

Impact of culture media on children

It has been shown in several systematic reviews and meta-analyses that singletons born after IVF or ICSI are at increased risk of adverse perinatal outcome when compared with naturally conceived children with respect to preterm birth, lower birth weight, perinatal mortality and congenital abnormalities (Helmerhorst et al., 2004; Jackson et al., 2004; McDonald et al., 2009; Pandey et al., 2012; Wen et al., 2012; Feuer et al., 2013; Pinborg et al., 2013; Albertini et al., 2014; Lazaraviciute et al., 2014; Marino et al., 2014; Simpson, 2014; Declerq et al., 2015).

Besides these perinatal differences, a growing number of studies have shown distinct phenotypic differences between ART children and naturally conceived children from birth until young adult age with respect to anthropometric parameters and adiposity (Green et al., 2013), cardiometabolic characteristics such as blood pressure and cardiovascular remodelling (Ceelen et al., 2009; Scherrer et al., 2012; Yeung and Druschel, 2013; Valenzuela-Alcaraz et al., 2013; Chen et al., 2014; Zhou et al., 2014; Pontessilli et al., 2015) and metabolites linked to obesity, insulin resistance and metabolic syndrome (Green et al., 2013; Gkourogianni et al., 2014; Lou et al., 2014).

Although this growing list of studies reporting adverse perinatal and long term outcome parameters of ART children shows compelling evidence that there could be an association between the mode of conception and outcome, there are several important points to consider.

First, it must be recognized that this association may be affected by bias and confounding factors, in particular in the selection of control groups. ART offspring need careful surveillance and follow up in order to record and evaluate possible adverse effects of the treatments (Barnhart et al., 2013). Second, it must be realized that some reported phenotypic differences are in the physiological range and, most importantly, that the implications are unknown. There are a number of publications that demonstrate that long-term health of ART children is comparable to that of spontaneously conceived children (see the extensive reviews of Bay et al., 2013, 2014; Hart and Norman, 2013a, b; Hediger et al. 2013, Fauser et al., 2014; Halliday et al, 2014). Third, even if the assumption of an association of ART and adverse outcomes in the children is true, there is no evidence that the origin of the problem is the ART treatment itself. Patient-related factors such as the subfertility of the progenitors and associated genetic factors may be one reason for adverse perinatal and postnatal outcomes in singletons after ART. There are reports showing that patient-related factors may be responsible for the differences observed in birthweight and other perinatal outcomes in ART children (Romundstad et al., 2008; Raatikainen et al., 2012; Messerlian et al., 2013; Declerq et al., 2015; Wise et al., 2015). For certain outcome parameters such as the rate of congenital abnormalities, patient-related factors are considered to be the most important (Simpson, 2014).

However, several studies show that specific ART treatment related factors are at least in part responsible for the observed differences in pregnancy outcome (see extensive review by Pinborg et al., 2013). For instance, in several studies that compare children born after fresh transfer with those of children born after transfer of frozen/thawed embryos, it was shown that singletons born after frozen embryo transfer (FET) have a significantly higher mean birthweight and higher risk of LGA (Large for Gestational Age), macrosomia and perinatal mortality when compared with singletons conceived after fresh transfer (Henningsen et al., 2011; Nakashima et al., 2013; Wennerholm et al., 2013; Marino et al., 2014). Here, subfertility could be ruled out as determining factor for these differences, since children from both groups were born from the same population of subfertile women. It is not clear from these studies which aspects of the ART technology (hormonal stimulation or cryopreservation) are responsible for the observed differences. Interestingly it was found that human embryos surviving the cryopreservation and thawing procedure exhibit a different gene expression compared with fresh embryos matched for morphological grade (Shaw et al., 2012).

Whether or not the in vitro culture of embryos, and more specifically, the culture medium used (or certain components of media), is also a determining factor for the observed adverse pregnancy outcome after ART was recently discussed in a review by Zandstra et al. (2015). It was concluded that from the 11 studies that investigated the association between birthweight after human IVF and the type of culture medium used, five found evidence of such an association while the other six studies did not. From these findings, together with the fact that the number of human studies is still limited, and that most studies were retrospective with consecutive use of different culture media and limited sample sizes, making bias of results likely, it can be concluded that it is still unclear whether or not culture media (or components in these media) can affect outcome parameters such as birthweight. Furthermore, it is unknown whether or not any possible relationship with birthweight is restricted to certain media or specific components. Interestingly, it was demonstrated in a recent study that birthweight of singletons born after IVF showed an inverse association with the storage time of the embryo culture medium used, indicating a possible accumulation of deterioration of certain medium components (Kleijkers et al., 2015b).

If the relationship between the culture medium used and certain perinatal outcome parameters would be found to be true, it is still far too early to speculate on any long-term effect. In one recent study, it was found that the differences in birthweight as a result from the type of culture medium used persisted during the first 2 years of life (Kleijkers et al., 2014). Whether or not this is an indication of possible health problems later in life is unknown.

ART children have also shown alterations in DNA methylation and transcript level of a number of genes such as those controlling growth (IGF2/H19 and IGF2R) in cord blood and placentae (Katari et al., 2009; Turan et al., 2010; Zhang et al., 2010; Song et al., 2015). The most recent of these studies strongly suggests that the causal effect is from ART procedures, rather than from parental infertility and may provide insight into the reported increases in imprinting disorders in ART children (Song et al., 2015).

Regulatory aspects of ART culture media

In 2009 it was agreed that the EU would regulate ART culture media as medical devices (class III) (Medical Device Directive 93/42/EEC, 2008, MEDDEV2.2/4 Jan 2012).

CE (Conformité Européenne) marking is a regulatory requirement to show a product is safe, effective, fit for purpose and meets EU regulations. To comply with European Directive 2001/83/EC, culture medium should be preferably CE marked ("Wherever possible, only CE marked medical devices must be used and all concerned staff must have received appropriate training on the use of such devices" Commission Directive 2006/17). To apply for CE marking, the manufacturers have to submit a technical file to their Notified Body (an organization that has been accredited by a Member State to assess whether a product meets certain preordained standards) which should include: the grounds for incorporating each component in the media, the risk of each of these components, manufacturing processes, design, validation of design, testing of product, toxicology, scientific data, what international standards have been used, pre-clinical and post clinical evaluation, ensure the product will not compromise safety of patients and errors and a risk benefit analysis. The clinical evaluation must show that there is an overall positive risk/benefit ratio of the product and take into account the safety to the gametes, embryos and the mother to be (in accordance with MEDDEV 2.7-1). The notified body will also do a physical audit of the production site and look at the quality management system and manufacturing process, post market surveillance and follow up. Manufacturers should also have a plan for post market surveillance including clinical follow-up (in accordance with MEDDEV 2.12/1).

If the medium contains a medicinal product or human derived blood product, such as human serum albumin or antibiotics, the notified body also has to undertake a consultation with a Medicines Competent Authority and/or European Medicines Agency (EMA). In both cases they will look at safety, performance and usefulness of the element within the media.

If the manufacturer makes any changes to a medium, they would need to have these changes validated and added to their CE certificate after review by their Notified Body and completion of all relevant consultations (MED 93/42/EEC, rule 13 of Annex IX).

Currently, it is not clear how the notified body system works. As far as we are aware it is not possible to find out who the notified bodies are, their membership, details of advisors or relevant

expertise. It is also currently unclear how manufacturers carry out post-market surveillance and clinical follow-up arising from use of culture medium in IVF.

Recommendations for end users

For IVF laboratories, the use of devices and equipment, including culture media, is a constant decision making process which has to be made on the basis of a number of criteria such as availability, user friendliness and clinical results, as well as cost/benefit. In addition, since culture media manufacturers often make changes (minor or major) in their products, decisions have to be re-evaluated and re-validated. The clinics have responsibility for the products that they use and decision-making processes have to be based on available information regarding the characteristics of the product itself and its suitability, including possible changes.

This of course requires that the end users are aware of when changes are being made. It is therefore extremely important that culture media manufacturers notify clinics of all changes being made to their products. Changes in the formulation and other characteristics of the culture medium have to be declared, and the scientific basis of the change has to be disclosed, including validation in experimental models (animal models) as well as clinical results, if available. The expected consequences of the medium change have to be discussed, especially with respect to safety concerns.

In parallel, the clinics should have a system to validate the (changed) product in their own laboratory. The results obtained with new media or other methodological changes must be carefully monitored and assessed. Multicentre studies that will allow a faster validation process of a specific product change should be encouraged. Biovigilance systems put in place by the competent authorities should assist in the detection of possible adverse events or reactions that may arise.

A quality management system must be in place to ensure the correct methodology of storage and use of a specific culture medium by all personnel involved according to the manufacturer instructions. For example an incorrect pH or temperature, caused by an non-optimal delivery

chain, faulty incubators or slow handling of the dishes outside the incubator, results in a poorer clinical outcome. It is mandatory that traceability, including type of culture media and lot number, is maintained for all ART products. In case incidents occur, they must be reported as notification of serious adverse reactions and events (Commission Directive 2006/86/EC).

Results regarding fertilisation rate and embryo quality as well as pregnancy, implantation and live born baby rates have to be carefully recorded and analysed. The follow-up of children needs to be performed periodically, preferably through national registries. In light of the large and emerging body of evidence on the importance of the nutritional environment during early development, national registries should include the type of culture medium embryos have been exposed to as well as unique identifiers for patients and babies on the register to enable large scale outcome studies to be performed routinely using data linkage.

Conclusions

The ultimate goal of any ART treatment must be to secure the health of the patients and the children. This can only be fulfilled by continuous follow-ups and by documentation and traceability of all methods performed and all materials used. As shown in this summary, culture media show a high degree of variability, and in general their quantitative composition is not known by the end users, which limits the possibility to correlate culture media type and culture media composition to outcome.

Existing data regarding the influence of culture media on embryonic, obstetrical, perinatal and postnatal parameters are conflicting. However, the fact that some studies do find such an association, particularly with birthweight, highlights the importance of further investigation.

Therefore clinical data related to culture media should be documented by ART centers in such a way that they can be used for biovigilance purposes and these data should preferably be collected in national registries, where available.

In addition, the complete formulation of culture media should be disclosed and all changes should be justified, validated and communicated to end-users by manufacturers. Changes should be made to the existing regulatory system to achieve transparency and improve monitoring of outcomes to the long term benefit of ART children and society.

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