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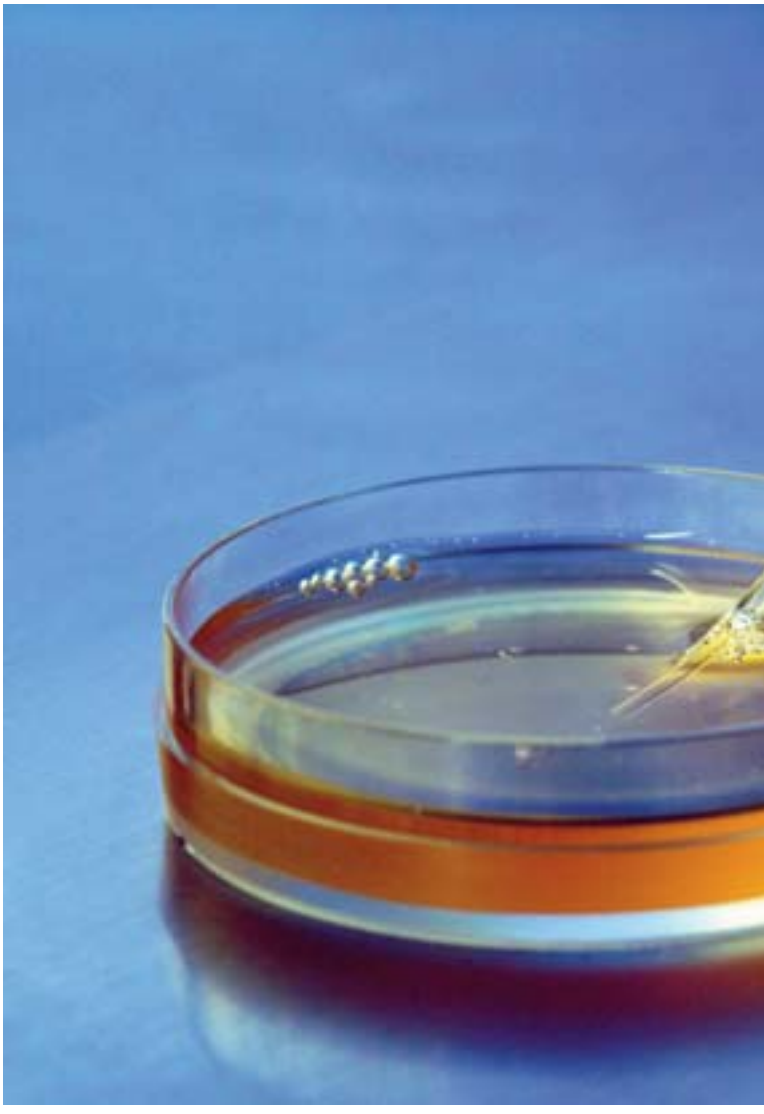
The problem with *culture media*



Last year ESHRE's Executive Committee agreed to the formation of a working group on culture media. The group works within the SIG Embryology (Kersti Lundin), with members from the SIGs Safety and Quality in ART (Petra De Sutter) and Genetics (Joyce Harper). Three embryologists were also appointed from the Executive Committee: Cristina Magli, Etienne Van den Abbeel and Miodrag Stojkovic. Arne Sunde, appointed as co-ordinator of the group, describes progress so far.

In some animal models ART will lead to changes in the phenotype of the offspring, such as 'large offspring syndrome', increased systolic blood pressure, and changes in metabolism.[1,2,3] Certain stages of early embryo development seem to be very susceptible to the environment, whether exposure is in vitro or in vivo.

Similarly in humans, ART is associated with small but significant changes in phenotype of the offspring. This includes bone length, fat distribution, blood pressure, blood lipids and time of entry into puberty (see Table 1).[4,5,6,7,8] The cause of these subtle changes in phenotype is not known, but factors such as infertility, ovarian hyperstimulation, in vitro fertilisation and in vitro culture may all contribute. In 2010 in a seminal paper Dumoulin et al presented data suggesting that in humans culture media may also have a profound influence on the phenotype of the offspring.[9] Just two or three days of in vitro culture in two different commercially available culture media resulted in a 200 g difference in average birth weight (see Figure 1).This difference in birth weight is paralleled by a difference in the kinetics and magnitude of hCG rise in early pregnancy and a 500 g difference in the body weight at two years of age.[10,11] In addition, Orasanu et al observed an effect of different culture media on the initial hCG rise in ART pregnancies.[12] One explanation for these observations is that different culture media lead to different methylation patterns in the placenta (see Figure 2).[13]

Such findings suggest that the media for human ART are not just an innocent vehicle providing a cosy environment for gametes and embryos, but may actually induce epigenetic changes with lifelong consequence for the health of the offspring. To ESHRE, this realisation was a strong argument for looking into the composition of the culture media we use. The introduction of new commercially available culture media containing growth

factors which might introduce further epigenetic changes added to the urgency.

Are culture media for human ART optimal?

Until end of the 1980s most ART clinics prepared their own culture media based on fairly simple formulations - and only then did commercially available culture media gradually replace the homebrew. ART culture media can broadly be divided in three categories: relatively simple solutions with a few basic salts and energy sources; very complex culture media similar to those used for continuous culture of mammalian cells; and culture media approximating to the physiological conditions in the fallopian tube and the uterus.

Using a simplex optimisation, Lawitts and Biggers in 1991 were able to formulate optimal culture media for mouse embryos.[14] But even in this ideal situation, using a genetically defined mouse strain and only a handful of ingredients in the media, a large number of zygotes were necessary to complete their experiments. A similarly systematic approach to the optimisation of human ART media is thus unthinkable. Humans are out-bred, not in-bred, and human ART media typically contain anything from 20 to 80 ingredients, in addition to human serum albumin, which is a chemically undefined blood product. This realisation - that we will never have a truly optimised human ART culture medium - may well present a case for making our current media less complex and more focused on good documentation, excellent quality control and increased batch-to-batch consistency.

Currently, there is a variety of commercial ART culture media available. These media differ in composition and intended use. Published studies comparing clinical outcome with the different formulations are generally too small or have other design flaws which make it impossible to know which of those currently available are 'the best'. It is. therefore, my own view that the ART community should collaborate to organise sufficiently large and properly designed clinical studies to evaluate our current ART culture media. Endpoints should include traditional parameters such as implantation rates, as well as data on the health of children. The ongoing large multicentre trial in the Netherlands comparing two commercially available culture media is exemplary in this respect.

The Working group's goals and activities

Increase transparency

In the working group's opinion, the full composition of any ART culture medium must be available to customers. Formulations should have a scientific backing with documentation which includes a rationale for adding supplements and growth factors. Details of the methods

Table 1. Physical/biochemical parameters influenced by ART

Parameter	Effect	Reference
Birth weight	Higher*	Dumoulin[9]
Early hCG rise in preg'cy	Earlier, higher*	Orasanu[12]
Blood pressure	Higher**	Ceelen[5]
Fasting glucose	Higher**	Ceelen[5]
Peripheral skinfolds	Higher**	Ceelen[4]
Serum LH and DHEAS in pubertal girls	Higher**	Ceelen6
Late infancy growth rate	Increased**	Ceelen[7]
Bone age/chronological age	Increased*	Ceelen[6]
Height for age	Increased**	Miles[8]
Serum IGF-II	Increased**	Miles[8]
Serum IGF-I/IGF-I binding protein ratio	Higher**	Miles8
Serum HDL	Higher**	Miles[8]
Serum triglycerides	Lower**	Miles[8]
HDL/cholesterol ratio	Lower**	Miles[8]
Cancer incidence	Higher**	Källen[21]

*Difference in outcome when embryos were cultured in different culture media

**Compared to a control group of children born after natural conception

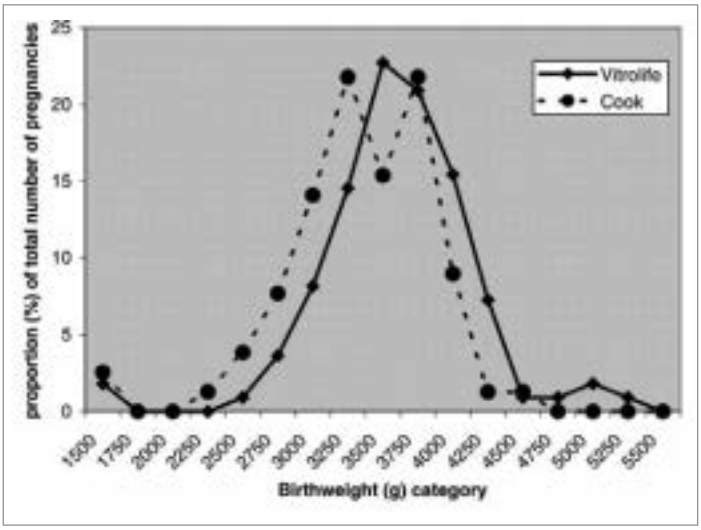


Figure 1. Birthweight distributions of live born singletons resulting from embryo culture in either Vitrolife or Cook sequential media.The graph depicts the percentage of newborns per birthweight category. From Dumoulin et al.[9]

used for production quality control (QC) and standardised QC certificates should be easily available

In January the working group spent two days in a series of one-to-one meetings with the largest ART culture media producers. From our perspective, the agenda was to encourage constructive co-operation with the industry over transparency, composition and QC parameters. And much to our delight, the majority of the companies agreed to be open on composition - and even on the formulation of the culture medium if this became the industry standard. So the working group will now propose to ESHRE's Executive Committee that it should advise members to source culture media only from manufacturers who disclose all relevant information on the composition and QC of their media.

However, an almost unanimous feedback from the companies was frustration that their customers did not always use the products according to the manufacturers' recommendations. They reported that their carefully produced and meticulously quality controlled products were often used sub-optimally. We have therefore suggested to ESHRE that the SIG Embryology might also offer basic training courses in the handling and culture of gametes and embryos.

A second meeting between the ART media industry and ESHRE is already planned. This will be organised as a workshop where representatives from the companies and experts invited by ESHRE will meet. The objective is to agree upon a common minimum quality control certificate with standardised procedures and endpoints

One important topic in the workshop will be relevant biological test methods for toxicity. Currently, the mouse embryo assay (MEA) is the standard test. However, the way the MEA is performed is not standardised. The sensitivity of the test is dependent on the mouse strains used, the starting points (zygote or 2-cell) and the endpoints chosen (blastocyst rate). One of the aims of this meeting is thus to agree upon a standard protocol for the MEA, but we must also discuss the relevance of the MEA for testing culture media in general and any possible alternative assays which might be more relevant.

Epigenetic effects of human ART?

In animal models changes in the phenotypes associated with ART are mediated by changes in the expression pattern of key regulatory genes. Different culture conditions and different culture media have been shown to cause changes in the expression of genes involved in regulating cellular function. Culture media containing growth factors will have a direct influence on the epigenome of the embryo and possibly on the phenotype.[15] Large offspring syndrome in ruminants is associated with dysregulation of the imprinted genes H19 and insulin growth factor 2 (Igf2).[16] In the mouse the methylation pattern of a range of imprinted genes has also been shown to be affected by ART.[17]

The situation is less clear on the effects in humans. In general there are few data available and results tend to be conflicting. However, to discuss the data that are currently available ESHRE staged a meeting of experts in epigenetics and early embryology in London in February. A report from that meeting will be published separately but a short summary follows. On the subject of classical imprinting disorders, reports indicate that there may be an increased frequency of the imprinting-related Beckwith–Wiedemann syndrome following ART in which there is culture of gametes and embryos (see Figure 3).[18] For other

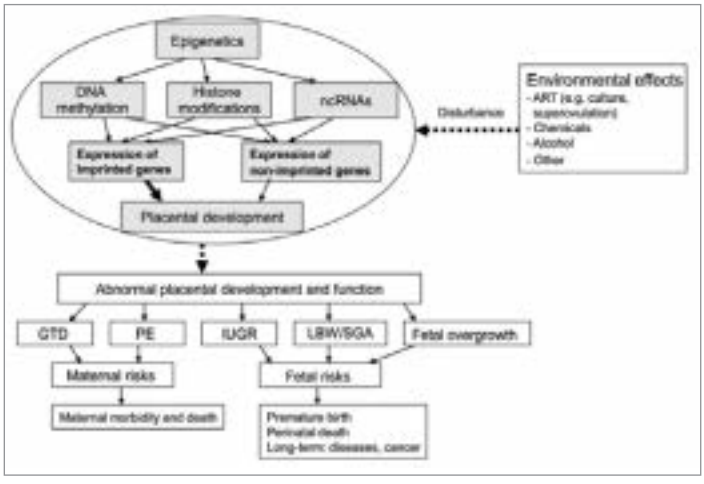


Figure 2. The role of epigenetics in placental development and the possible consequences of its disturbance which can be caused by environmental effects. Arrows indicate relationships between the different steps. Bold arrow indicates the importance of imprinted genes for placental development. Dashed arrows indicate the effect of environment on placental disturbance; ncRNAs, non-coding RNAs; ART, assisted reproduction technologies; GTD, gestational trophoblastic disease; PE, pre-eclampsia; IUGR, intrauterine growth restriction; LBW, low birthweight; SGA, small for gestational age. From Nelissen et al.[13]

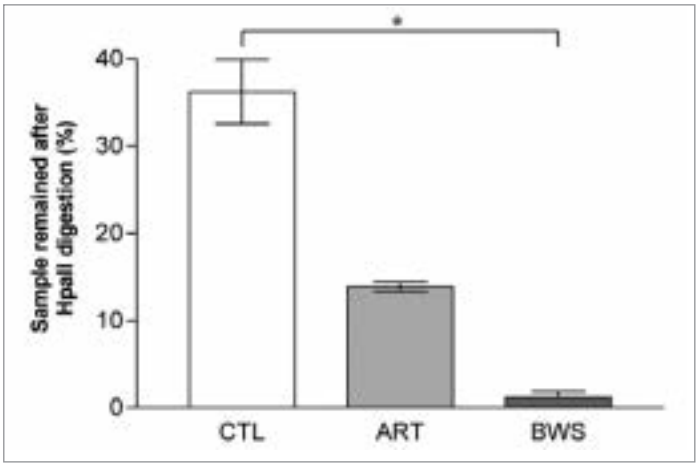


Figure 3. Graphic representation of methylation pattern at KvDMR ICR obtained by MSED-qPCR. The results were expressed as mean methylation and SEM. Asterisk represents the statistically significant differences between CTL and BWS groups (P <0.01). CTL, negative control group (clinically normal naturally conceived children); ART, group of children conceived by assisted reproductive technologies in which hypomethylation was previously detected by MS-PCR; BWS, group of naturally conceived patients with BWS. From Gomes et al.[18]

conditions, such as Angelman syndrome or retinoblastoma, the available data do not yet warrant a conclusion.

Studies on global DNA methylation patterns indicate that ART leads to a change in the methylation pattern of many genes.[19] IVF and ICSI do not seem to produce different methylation patterns, but there is evidence that in vitro culture may induce methylation defects.[20] The observed methylation disturbances seen in offspring after ART may be causally linked to the slight differences in phenotype of the ART children seen in some studies.[4,5,6]

One general comment from the experts at the London meeting was that the observed changes in the epigenome in vitro might not necessarily lead to a phenotype which is considered 'bad', or vice versa

The experts were also challenged with a second question: Can we find a suitable model to test the epigenetic effects of specific culture media in human ART? Technically, it is possible to devise a test using embryonic stem cells or animal models, but the limitation now is that we do not understand how to interpret a test result. Embryonic stem cells may be cultured in the different commercially available culture media and induce different epigenomes, but we do not yet understand how to rank these media - from 'bad' to 'good' - based on the different epigenomes. We have a substantial lack of understanding of the relationship between the regulation of gene expression in the early embryo and any phenotypic variations in the offspring

Regulation of ART media

In Europe the EU Tissue and Cells Directive recommends that utensils, disposables and culture media in direct contact with gametes, zygotes or embryos should preferably have a CE mark. However, this CE mark does not say anything about the quality of the medium in terms of, for example, pregnancy rates. CE is only a certificate of conformity and documentation. It is similar to an ISO certificate; a clinic which has poor clinical results may still obtain an ISO certificate provided it runs the clinic with 'quality management'. CE marks are issued by 'notifying bodies'; commercial organisations are certified for this job. ESHRE has received feedback from the media industry indicating that different notifying bodies operate differently, which may suggest that CE marks do not necessarily mean the same.

The European Commission has recently defined ART media and utensils as 'medical devices', but the regulatory and legal implications of this are still unclear.

Epidemiology

Is it possible to organise large retrospective epidemiological studies which link parameters from the ART laboratory with the phenotype of the offspring? In order to generate meaningful data, such studies must be conducted in clinics and countries where complete datasets on ART parameters are available, which might then be linked to comprehensive national registries containing relevant information on the health of the ART children. To be useful, the study would have to be very large and require substantial human and financial resources. So we are also meeting experts in conducting such studies. Our aim is an evaluation of the feasibility of this approach, which will include estimates of the resources needed and the time frame for conducting such a very large study.

Arne Sunde is Professor and head of the Fertility Clinic of St Olav's University Hospital, Trondheim, Norway. He is a previous Chairman of ESHRE and presently co-ordinator of the ESHRE working group on culture media.

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