Human Reproduction, Vol.27, No.2 pp. 303–313, 2012
Advanced Access publication on December 12, 2011 doi:10.1093/humrep/der414

human reproduction

OPINION

When and how should new technology be introduced into the IVF laboratory?

Joyce Harper[1,*], M. Cristina Magli[2], Kersti Lundin[3], Christopher L.R. Barratt[4], and Daniel Brison[5]

[1]UCL Centre for PG&D, Institute for Womens Health, UCL and Centre for Reproductive and Genetic Health, University College London Hospital, London, UK [2]SISMER, Reproductive Medicine Unit, Bologna, Italy [3]Reproductive Medicine, Sahlgrenska University Hospital, Institute for Clinical Sciences, University of Gothenburg, 413 45 Gothenburg, Sweden [4]Reproductive and Developmental Biology Group, Division of Medical Sciences, Ninewells Hospital, University of Dundee, Dundee, Scotland DD1 9SY, UK [5]Department of Reproductive Medicine, St Mary's Hospital, Central Manchester University Hospitals NHS Foundation Trust, Manchester Academic Health Sciences Centre, Oxford Road, Manchester M13 9WL, UK

*Correspondence address. joyce.harper@ucl.ac.uk

ABSTRACT: There are many examples in assisted reproduction technology, where new technology and methods have been introduced into the clinical setting without appropriate development and evidence-based medicine to show that the procedure is safe and beneficial to the patient. Examples include preimplantation genetic screening, assisted hatching, *in vitro* maturation, blastocyst transfer and vitrification. Changes to culture media composition, stimulation regimes and laboratory protocols are also often established internationally without adequate validation. More recently, novel equipment that needs to be validated before it enters routine clinical use is being developed for IVF. With technologies such as producing gametes from stem cells around the corner, it is vital to ensure that the necessary research and development is conducted before bringing new techniques into clinical practice. Ideally, this should include preliminary work on animal models, such as mice/rats/rabbits/larger mammals, followed by studies on human embryos donated for research and finally well-designed RCTs with a follow up of all children born from the procedure. If such preliminary studies are not performed and published, it is possible that technology bringing no clinical benefit or leading to adverse health outcomes in the children born by these practices may be introduced. All IVF clinics need to consider the safety and efficacy of new technologies before introducing them.

Key words: IVF / technology / embryology / embryo selection / safety

Introduction

Since the birth of Louise Brown in 1978 (Steptoe and Edwards, 1978), the field of IVF has led to huge advances in our knowledge of human embryology but it has also exploded into a multi-million dollar business. Compared with other related fields, such as prenatal diagnosis and clinical genetics, IVF has a huge commercial market as it is mainly conducted in the private sector. In addition, so-called 'cross-border IVF' is now becoming more common, where patients, sperm, oocytes or embryos cross borders so that treatments banned in one country can be undertaken in a country where it is legal (McKelvey *et al.*, 2009; Shenfield *et al.*, 2010). On the other hand, and from a more 'humanitarian' perspective, IVF is being developed in the developing world where female infertility also has severe social consequences (Cooke *et al.*, 2008).

The number one aim of the IVF clinic is to ensure the delivery of a healthy baby. In some countries, such as the UK, league tables of success rates are published and clinics may feel under pressure to ensure they have the latest technology to achieve the best results. But as time is money, many of these new techniques have not been shown to be safe, to have a clear clinical benefit and/or not been properly validated. This is a very worrying situation that may become even more common as new technologies are developed.

Every procedure involving application to the human body should be defined as experimental until adequate scientific evidence is provided regarding its safety and efficacy. The obtained results are expected to be reproducible in order to demonstrate the reliability of the proposed procedure. In this respect, appropriately designed studies should be published in peer reviewed journals by more than one research group. Nevertheless, there are several examples in science/medicine that are in contrast to the above statement, reproductive medicine being among them.

Ideally, hypothesis-driven basic research leading to clinical treatments in IVF should follow the model shown in Fig. 1.

New technologies should where possible be developed or first tried on small mammals, such as mouse or rats, and then moved onto larger animals, such as cow and pig. IVF should be no exception, but in some situations, such as preimplantation genetic screening (PGS) and to some extent ICSI, animal models will not be appropriate as there is really no suitable animal model for human development. Whether

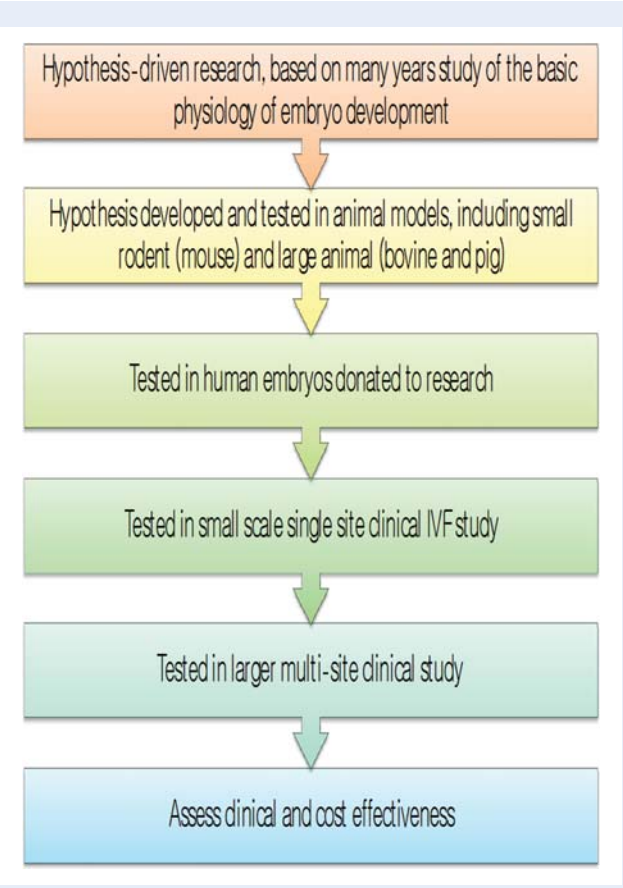

Figure 1 Ideal paradigm of hypothesis-driven basic research.

animal studies are appropriate or not, research should be performed on human eggs/sperm or embryos donated for research and with an appropriate risk assessment. In addition, sufficiently powered RCTs need to show that there is a clinical benefit and that the technique is safe, with paediatric follow ups if appropriate. A clinical RCT is the gold standard of evidence-based medicine as it is an unbiased evaluation of the technology and outcomes (delivery rates), which are compared with a control group without treatment (Barlow, 2003; Vail and Gardener, 2003). It is then possible to see whether it is the actual technology causing the effect (positive or negative) or other factors. For certain procedures, such as PGD and ICSI, RCTs are not appropriate because it would be impossible to have a non-treatment control group but for procedures that claim to increase delivery rates, such as PGS or blastocyst transfer, RCTs are essential. All deliveries need to be followed up to ensure that any risks are within an acceptable range.

Unfortunately, developments of IVF treatments are often both money and patient-driven and the necessary research is not conducted or is conducted after the procedure has been introduced into the clinical setting. In addition, possible negative health outcomes in children may not be seen until many years later. In the absence of official guidelines regulating the introduction of experimental procedures in clinical practice, results are used to retrospectively validate the techniques according to the performance obtained directly from clinical application. This is of course not restricted to IVF as many other disciplines introduce treatments without evidence that it is of benefit.

The list of techniques that have been brought into the IVF clinic over the last 20 years is long and it continues to grow, with new technologies coming on stream recently. Here, we discuss some of these techniques to illustrate the importance of research and development; PGS, ICSI, oocyte cryopreservation, sperm DNA damage testing, non-invasive analysis of culture media and development of culture media (growth factor supplementation). We argue that any new technology should be evaluated for: (i) effectiveness, (ii) safety and (iii) cost-effectiveness. In other words, does the technology work sufficiently well to be worth considering, if so is it safe, and finally if the risk: benefit ratio is above a predetermined certain threshold, is the technology likely to be cost-effective, to clinics, patients and/or the state?

Preimplantation genetic screening

The main aim of PGS is to aid embryo selection for patients going through IVF by analysing embryonic chromosomes. This is in contrast to PGD that is specifically used for couples at high risk of transmitting a specific genetic or chromosomal abnormality to their children (Harper, 2009; Harper and SenGupta, 2011). In PGS, polar body or embryo biopsy is performed and analysis of the chromosomes has mainly been performed by fluorescence *in situ* hybridization (FISH) and, more recently, by arrays (Harper and Harton, 2010) (Table I). Since chromosome abnormalities increase with advanced maternal age (AMA), it seems logical that checking embryos from these patients for chromosome abnormalities may increase their chance of having a chromosomally normal embryo transferred. AMA has been the main indication for PGS but other indications include repeated implantation failure, repeated miscarriage (with normal karyotypes in the parents) and severe male factor infertility (Goossens *et al.*, 2009; Harper *et al.*, 2010a).

PGS was first reported by Verlinsky *et al.* (1995) and Munné *et al.* (1995a) on polar bodies and Munné *et al.* (1993, 1995b) on cleavage stage embryos. To date, there have been numerous papers on the use of PGS for IVF patients. The majority of these have been non-randomized comparative studies with the outcome measure being clinical pregnancy rate rather than delivery rate (Gianaroli *et al.*, 1999; Munné *et al.*, 2003, 2005, 2006; Colls *et al.*, 2007). The most obvious criticism of non-randomized studies is their poor experimental design and inadequate control groups. Few of these studies reported delivery rate as the end-point, some involved small numbers of patients, several used only historical controls, some used 2-cell biopsies and some used low numbers of FISH probes. Despite this, based on these studies, PGS was rapidly introduced into the IVF arena and is performed more than all other PGD indications added together (Harper *et al.*, 2010a).

There are now 11 RCTs applied to both good (Blockeel *et al.*, 2008; Jansen *et al.*, 2008; Mersereau *et al.*, 2008; Staessen *et al.*, 2008; Meyer *et al.*, 2009) and poor (Staessen *et al.*, 2004; Stevens *et al.*, 2004; Mastenbroek *et al.*, 2007; Hardarson *et al.*, 2008; Schoolcraft *et al.*, 2009; Debrock *et al.*, 2010) prognosis patients which have all shown that PGS has not improved the delivery rate compared with a control group, and some of these studies show that it has significantly

Table I Differences between PGD and PGS.

	PGD	PGS
Aims	Identify genetically normal embryos	Identify euploid embryos
	Achieve a genetically normal pregnancy/birth	Achieve a pregnancy/birth
Indication	Monogenic disorder X-linked disease known chromosome abnormality	Advanced maternal age Repeated implantation failure Repeated miscarriage Severe male factor
Fertility	Often fertile	Infertile or subfertile
Biopsy	Usually Day 3 (1–2 cells)	Usually Day 3 (1 cell), but also polar body. Recently blastocyst biopsy being used
Diagnosis	FISH for chromosome abnormalities and sexing. PCR for monogenic disorders	FISH with as many probes as possible
		Recently arrays being used
		Recently arrays being used
Undiagnosed or inconclusive results	Never transfer these embryos	Can transfer these embryos
Prenatal diagnosis	Indicated	Indicated for the same risk factors as natural conceptions

Adapted from Harper (2009). Copyright Cambridge University Press.
FISH, fluorescence *in situ* hybridization.

decreased the delivery rate. Almost all of these studies have been applied to cleavage stage embryos and used FISH to study 5–12 chromosomes, except Jansen *et al.* (2008) who performed trophectoderm biopsy. As a result of these studies, the British Fertility Society, American Society of Reproductive Medicine and American College of Obstetrics and Gynaecology have all issued statements that PGS should not be offered clinically.

Performing the biopsy at cleavage stages has a biological problem as at this stage human embryos show high levels of chromosome abnormality (Harper *et al.*, 1995; Munné *et al.*, 1995b) and analysis of one cell from these embryos is not representative of the rest of the embryo. Abnormal cells in mosaic embryos may well arrest or undergo apoptosis to give a chromosomally normal embryo. Therefore, work on PGS is currently concentrating on polar body (Geraedts *et al.*, 2010, 2011) or trophectoderm biopsy. However, recent reports have shown the blastocysts are also highly mosaic, which may also affect the possible success of PGS (Fragouli *et al.*, 2011).

Moving away from using FISH, recent work is concentrating on array (Hellani *et al.*, 2008; Alfarawati *et al.*, 2011) comparative genomic hybridization (CGH) that allows analysis of all of the chromosomes of a single cell. This has an advantage over FISH, a technique where a limited number of chromosomes can be analysed; however, for blastomere analysis the problem of embryo mosaicism will remain. The need to obtain data to demonstrate whether CGH techniques on single cells can be validated has to be addressed but, more importantly, RCTs need to be conducted to determine if using CGH for PGS will increase delivery rates (van Steirteghem, 2008; Harper *et al.*, 2010b). The European Society of Human Reproduction and Embryology (ESHRE) PGS task force have set up an RCT which will be performed for patients of AMA on the first and second polar bodies using array CGH (Geraedts *et al.*, 2010, 2011). Even though it will take several years to obtain the results of this trial, centres worldwide have already started to offer array CGH PGS routinely to IVF patients, with no data on whether it improves outcome (Hellani *et al.*, 2008).

Intracytoplasmic sperm injection

In 1962, Hiramoto microinjected sperm into sea urchin eggs. This approach was developed in rodents in the 1970s and 1980s but with a low number of pregnancies reported (Hiramoto, 1971; Thadani, 1979; Keefer, 1989). A similar outcome resulted when applied to larger animals such as the bovine (Goto, 1993). The technique was first applied in the human in 1988 (Lanzendorf *et al.*, 1988). Soon after the first reported ICSI pregnancy (Palermo, 1992), the vast majority of centres rapidly adopted this technique. According to the data from the European registry, since 2002 ICSI represents by far the most commonly used insemination technique (De Mouzon *et al.*, 2010).

Hardly any experimental studies were performed to validate the ICSI technique before its clinical application and the only precaution, if any that was taken by the majority of centres was to prepare a specific informed consent to make patients aware of the lack of data supporting the safety of this micromanipulation technique. The closest experimental model for our species possibly is the rhesus macaque, for which fertilization rates and implantation rates after ICSI are similar to those in the human (Sotovsky *et al.*, 1996; Hewitson *et al.*, 1998). In most species, it is necessary to induce oocyte activation artificially by supplementing media with calcium chloride or calcium ionophore (Gómez *et al.*, 1998), a strategy that is taken with caution for a possible application in assisted reproduction technology (ART) owing to the many consequences associated with the massive release of calcium in the oocyte. On the other hand, other micromanipulation techniques, such as partial zona dissection and subzonal sperm microinjection, were shown to be successful in animal models but failed to have similar results when tried on human gametes (Gordon and Talansky, 1986; Lacham *et al.*, 1989). Although significant advances in our knowledge of oocyte activation and potential causes of male infertility have some roots in animal studies, these data at the same time demonstrate how animal models are hampered in the case of ICSI. Thus, more basic research and follow-up studies in the human embryo, such as monitoring development and analysis of aneuploidy, metabolism and methylation and paediatric follow-ups, should have been conducted before the widespread introduction of ICSI. Nowadays, some concerns have been raised from data on pregnancy follow-up and on the fertility of the boys born after ICSI (Basatemur *et al.*, 2010; Belva *et al.*, 2011), from which it is clear that exhaustive information must be given to couples.

We cannot deny that ICSI represents the most revolutionary innovation in ART and that its discovery would have probably not been achieved by following the classical steps of looking for new technologies to be applied to humans.

Oocyte cryopreservation

The more recent large-scale introduction of oocyte cryopreservation has similarities to that of ICSI. A few sporadic pregnancies were reported in the past (Chen, 1986) with some improvements in the following years as a result of technical modifications (Porcu *et al*., 2000) culminating in the introduction of vitrification. After the report of the first pregnancy from vitrified oocytes (Kuleshova *et al*., 1999), more interest has been dedicated to the vitrification method. According to the most recent publications, delivery rates using vitrified oocytes are now similar to those obtained with fresh oocytes (Cobo *et al*., 2008; Rienzi *et al*., 2010; Ubaldi *et al*., 2010).

As for ICSI, concerns have been raised regarding the safety of oocyte cryopreservation. Central outcome registries have not addressed these concerns but by the personal initiative of particularly active groups the obstetric outcome of pregnancies from cryopreserved oocytes has been evaluated. Also in the case of oocyte cryopreservation, and vitrification especially, the experimental phase was mostly performed in the human directly (in some cases, as for Italy, brought on by the national regulation prohibiting embryo cryopreservation and limiting to three the number of oocytes to be inseminated (Benagiano and Gianaroli, 2004). The protocols successful with animal oocytes, for example in the mouse and in the rabbit, failed with human oocytes (Cai *et al*., 2005) implying that also in this case the results from the experimental models did not support the clinical application in humans, owing to the great diversity in the oocyte structure and physiology in the different species. However, as for ICSI, basic research in the human and follow-up studies should have been conducted before its widespread clinical application.

Sperm DNA damage testing

The assessment, origin, dynamics and consequences of damage to the paternal genome are receiving ever increasing attention. In animals, where experiments can be performed to induce DNA damage to the paternal germ line, there have been clear associations between damage to the paternal genome, adversely affected embryo development and subsequent negative effects on the new born and subsequent generation (e.g. see Fernandez-Gonzalez *et al*., 2008). In humans, with the urgent need to provide new assessments of male reproductive potential, the testing of DNA and its packaging in the human spermatozoon is likely to be an important diagnostic/prognostic tool (Barratt *et al*., 2010 and references within; Barratt and De Jonge, 2010; Aitken *et al*., 2010).

However, although the assessment of DNA integrity was suggested in 1980 as a potential useful and independent marker of fertility (Evenson and Darzynkiewicz , 1980), there is still considerable controversy over clinical relevance. This was highlighted by a recent meta-analysis (Collins *et al*., 2008) which concluded that the clinical relevance was not clear, a statement that is supported by additional reviews of the literature (Sakkas and Alvarez, 2010). In view of the ever increasing number of commercially available assays for the assessment of DNA damage in sperm, combined with the rapid progress in basic research in the arena, ESHRE organized an expert panel to report on the progress in the field. Their report 'Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications – a position report' (Barratt *et al*., 2010) made five key recommendations. Two recommendations are specifically relevant to the introduction of new technology into ART namely (i) standardization of any current or proposed clinical assay is essential and (ii) high quality comparative clinical data are currently lacking and urgently required. The impact of these recommendations for determining the clinical relevance of sperm DNA assessment has been discussed in detail elsewhere (Barratt and De Jonge, 2010). Yet, it is worthwhile reiterating that methods of assessment of any assay must be robust, repeatable and reliable. Without this, any assay will have fundamental flaws.

The assessment of DNA damage in spermatozoa is clearly not as simple as initially suggested and very strict quality control and quality assurance procedures are necessary for the assays to have meaning. Undoubtedly, poor methods have led to erroneous and misleading data (Mitchell *et al*., 2011). Regarding the second recommendation from ESHRE, there is a plethora of publications in the literature; however, with few adequately powered, large, prospective clinical trials. For example, at the time of publication of the report (2010) there was only one large trial involving patients undergoing intrauterine insemination. Additionally, the clinical data that were available suggested that the greatest clinical utility of sperm DNA assessments was for predicting pregnancy loss after ICSI and IVF as opposed to markers for embryo development and fertilization success.

Herein lies the problem. It is unrealistic to wait for the perfect study as it will of course never appear. As such there needs to be a clinical and scientific judgement as to what is the best course of action (see Barratt and De Jonge, 2010). For DNA assessment the recommendations should be clear. In view of the almost overwhelming animal data, the biological basis of assessing DNA integrity in humans is logical, i.e. it should be useful. However, what is missing is the most appropriate method for the assessment of DNA integrity and a robust interpretation of the clinical data. It is very likely that both of these will be addressed in the very near future. For example, robust methods are now being developed (Mitchell *et al*., 2011) and clinical data are appearing which suggest potential pathological/normal values (Aitken *et al*., 2010). There are well-defined pathways for the routine use of assays in clinical practice that need to be followed (Gluud and Gluud, 2005). Additionally, there needs to be a critical determination of where these assays fit within the patient pathway. For example, diagnostic tests can be used as replacement, triage or add-on with their usefulness being dependent on a large number of factors (Bossuyt *et al*., 2006). To date, DNA damage assays are just starting to be evaluated in this critical manner but we are doing this far too late in the development process. An earlier detailed analysis would have allowed the introduction of effective testing into clinical practice 10 years ago—an important lesson to learn.

Non-invasive analysis of culture media

Analysis of embryo culture medium is a non-invasive technique, which offers little or no risk to the embryo and therefore might be considered a relatively safe technological application. In other words, the risk:benefit ratio is favourable (Brison *et al*., 2007).

Non-invasive analysis of embryo culture media originated in principle in the late 1980s when microanalytical techniques became

sensitive enough to be applied to the culture media of single animal or human embryos (Leese, 1987). At first, research was restricted to analysis of single metabolites, such as pyruvate, glucose and lactate, in animal models such as mouse and rat (Brison and Leese, 1991), then extended to spare human embryos for research (Gott *et al.*, 1990) and finally to initial ground-breaking clinical IVF studies (Conaghan *et al.*, 1993).

Based on studies of the composition of human Fallopian tubal fluid, Leese and co-workers found that the turnover of a number of amino acids correlated with the development of early cleavage embryos to the blastocyst stage in animal models including mouse, bovine and pig (Lamb and Leese, 1994; Partridge and Leese, 1996; Booth *et al.*, 2005). The hypothesis was then tested on human embryos donated to research, where it was shown that amino acid turnover could predict the formation of blastocysts from Day 2/3 embryos (Houghton *et al.*, 2002). Subsequently a small single site clinical IVF study showed that amino acid turnover by Day 1/2 embryos could also predict the likelihood of an individual embryo giving rise to a live birth (Brison *et al.*, 2004), and this was confirmed in a further multicentre clinical IVF study (unpublished data). Further basic research has suggested that amino acid turnover by human embryos, and again in an animal model, the bovine, is correlated with and may be a marker of structural DNA damage in the embryo (Sturmey *et al.*, 2009). This interesting possibility is consistent with Leese's Quiet Embryo hypothesis (Leese, 2002; Leese *et al.*, 2008) which holds that embryos which show a low or quiet metabolic rate are more viable, possibly because this is a marker of reduced stress and/or less need for genome repair. To date, the development of amino acid profiling for use in clinical IVF practice has followed the paradigm of traditional, reductionist, hypothesis-driven basic research (Fig. 1).

Metabolomics is defined as the measurement of species of molecular weight <1 kDa. The metabolomic footprint of an embryo can be measured using infrared (IR) spectroscopy, mass spectroscopy and, arguably, nuclear magnetic resonance among other techniques. IR spectroscopy is often adapted as a first approach in order to capture significant biological correlations which can then be followed up using other techniques. Hollywood *et al.* (2006) and Brison *et al.* (2007) used the IR spectroscopy approach to demonstrate proof of principle that an IR metabolic footprint could identify human embryos which would go on to implant in a clinical IVF cycle, compared with those which failed to implant. IR spectroscopy has been used in a number of clinical IVF studies (Vergouw *et al.*, 2008; Seli *et al.*, 2010) to demonstrate retrospective correlations with embryo implantation but recent data from the interim analysis of a prospective RCT have shown that it did not increase pregnancy rates (Hardarson *et al.*, 2012).

Development of culture media; growth factor supplementation

The media used for the culture of human embryos in IVF is largely based on those designed over the last four decades for the culture of animal embryos. These animal models in turn have been largely restricted to a few which are experimentally amenable, such as mouse or to a lesser extent rabbit and hamster, or to those with their own inherent commercial value, such as the bovine. It is noteworthy that these animal species were not necessarily selected for their value as models for human embryo development, and yet almost all of the baseline data, which we use for the design of human media, are based on them.

It remains true in 2011 that there is no culture medium available that is truly optimized for human embryo development. Should we be surprised by this? No. One has only to read the landmark papers by Ralph Brinster in the 1960s to appreciate that 1000 of mouse embryos were required to perform systematic multifactorial experiments in order to optimize the basic components of medium, such as salt concentration, osmolarity, pH, concentrations of energy substrates etc., to produce a semi-defined medium which could reproducibly support the development of 2-cell stage mouse embryos to blastocyst (Brinster, 1963, 1965). Even employing a more sophisticated experimental design drawn from engineering processes, Lawitts and Biggers (1991) still required large numbers of mouse embryos to design their SOM (simplex optimization medium) and later KSOM (potassium-SOM) formulations, which supported strains of mouse embryo that otherwise 'blocked' at the 2-cell stage through to blastocyst.

It is obvious that unless the approach to the design of human embryo culture media is to alter radically, with large numbers of embryos becoming available for research, then we will never be able to replicate in clinical IVF the optimization of Brinster's or KSOM medium for the mouse. The 'first phase' of an ideal approach would be to form a worldwide consortium to develop and test media formulations and the 'second phase' would of course be the testing of these phase 1 formulations in a prospective clinical RCT.

In the meantime, human embryo culture media cannot be considered optimized for human embryo development. For example, osmolarity is a crucial parameter in embryo culture media. Culture media are commonly designed in the range of 270–80 mOsmols of osmotic pressure. There is no basis in physiology for this: 280 is likely far below the level the embryo would experience *in vivo*, where blood plasma is of the order of 300–310 mOsmols. The justification for a level of 270–280 mOsmol can be traced back nearly half a century to Brinster (1965), where the optimal development of 2-cell mouse embryos to blastocyst was estimated to occur at 276 mOsmols. We have no idea if this level is optimal for human embryo development, for development *in vitro* to blastocyst or indeed to produce a live healthy baby. Most other aspects of culture media design have not been systematically optimized for the human embryo, including: the concentrations of essential energy substrates, macromolecules, concentrations of amino acids and growth factor supplementation (see below).

Does this matter? In a sense no, as in the absence of a radical rethink in culture media design, we have little choice but to proceed with media based on animal studies, tweaked for human application with such data as are available. However, if we accept that human embryo culture media are not optimized, then there follow a number of important corollaries:

(i) There is an implied risk to the culture of human embryos in the laboratory.
(ii) The longer the period of culture, the greater the risk, at least in principle. Logically, if the *in vitro* environment has not been optimized for human embryo development, then keeping embryos in

that environment for 5–6 days to the blastocyst stage, rather than 2–3 days, must incur a greater risk in principle. In the absence of data on safety or otherwise, the risks in principle of *in vitro* culture should be explained carefully to patients.

(iii) We must inform patients fully of potential risks. A number of potential areas of risk have started to emerge recently, including studies on imprinting, large offspring syndrome (LOS) in sheep and cattle (McEvoy *et al.*, 2000; Odom and Segars, 2010); and in the area of the Developmental Origins of Health and Adult Disease (Dohad). The latter is based on Barker's original hypothesis (Barker, 1994) suggesting that birthweight is linked to an increased risk of early onset of adult diseases, including diabetes, hypertension and cardiovascular disease. Some evidence now suggests that the peri-implantation period of development may be particularly sensitive and that culture *in vitro* may be a risk factor (Kwong *et al.*, 2000; Watkins and Fleming, 2009) and the first studies of IVF children (Ceelen *et al.*, 2009) suggest early signs of altered cardiovascular function.

(iv) The composition of culture medium may be important. Evidence that culture media may be important in human IVF comes from a recent quasi-RCT comparing two commercially available and widely used culture media. One of the media gave rise to higher birthweight in the resulting babies (Dumoulin *et al.*, 2010). This raises the possibility that the composition of culture medium could be linked to Dohad effects.

(v) If we aim to change the design of culture media in significant ways, we must carefully assess the risk of those changes, as well as the potential benefits.

Addition of growth factors to human embryo culture medium

It has long been observed in animals that the development of embryos *in vitro* is impaired relative to that *in vivo*, with reduced cell proliferation (Bowman and McLaren, 1970), increased cell death (Brison and Schultz, 1997) and reduced viability. As peptide growth factors are known to act as mitogens, regulating cell proliferation and as survival factors, regulating cell death by apoptosis, there have been a number of studies suggesting that addition of these to human embryo culture media might be beneficial. The prima facie case for this is straightforward as human embryos, in common with animal counterparts, express receptors for a number of growth factors. This field has been well reviewed, notably by Kane *et al.* (1997), and the clinical implications have been covered recently by Richter (2008), and this most recent review raised the question of whether growth factors should now be added to clinical IVF culture media.

Early work using research embryos showed that several growth factors could indeed stimulate the development of human embryos *in vitro*, with insulin-like growth factor-1 (IGF-1), leukaemia inhibitory factor (LIF) and heparin-binding epidermal growth factor (HB-EGF) variously increasing development to blastocyst and cell number, and reducing apoptosis (Dunglison *et al.*, 1996; Lighten *et al.*, 1998; Martin *et al.*, 1998). More recently, granulocyte-macrophage colony stimulating factor has been shown to promote the development of human embryos in culture, including effects on blastocyst formation, cell number and apoptosis (Sjoblom *et al.*, 2005).

What are the implications of adding growth factors to culture medium? While they may well have beneficial effects as outlined above, there are also risks to be considered.

Growth factors generally act on cells in what is termed a pleiotropic manner, influencing all aspects of cellular function including mitogenesis, differentiation, apoptosis, metabolism, ion transport, gene expression etc. In an attempt to understand the potential mechanisms by which growth factors might stimulate embryo development, Kimber and colleagues repeated the experiments described above, adding IGF-1, HB-EGF and LIF to human embryos in culture, and examined the impact on gene expression at the blastocyst stage (Kimber *et al.*, 2008). They found that the growth factors exerted effects on gene expression that could not have been predicted, including altering the expression of cell fate-genes, such as Sox2 and Taube Nuss. Interestingly, LIF and HB-EGF also increased expression of not only their own receptors, LIFR and ErbB4, respectively, but also the receptors of the other ligand, i.e. LIF up-regulated expression of ErbB4, and HB-EGF up-regulated LIFR. This gives rise to the possibility of cross talk between these two pathways in response to growth factor addition, increasing the chance of an unexpected response if one or other factor was added to medium.

Growth factors also have known actions that can be predicted, and one example common to most of the growth factors under consideration is that they block apoptosis (Brison and Schultz, 1997). In common with many other developing organisms, apoptosis occurs at quite high levels in human embryos (Hardy *et al.*, 1989), including in the inner cell mass of the blastocyst. The purpose of apoptosis in development may be to eliminate cells that are abnormal, in the wrong place or are genetically abnormal in terms of chromosomal constitution or structural DNA damage. Therefore blocking this pathway, particularly in embryos exposed to high levels of insult *in vitro*, could potentially result in the accumulation of damage.

Agencies around the world have been slow to recognize the importance of regulating human embryo culture media, which is surprising considering the potential impact on human life and health. In the UK, the Medicines and Healthcare Regulatory Agency is now responsible for IVF culture media, and have classified them as class III medical devices. This means that they must be CE (European Conformity) marked, and manufacturers must disclose the composition of culture media, they must conduct safety assessments and they should carry out post-market surveillance programmes to monitor the long-term safety of these products. While this will be difficult for manufacturers to achieve in practice, the movement towards tighter regulation of IVF culture media is to be welcomed. One remaining loophole is that manufacturers are not obliged, for reasons of intellectual property, to disclose the exact composition of media, including concentrations of components, to customers. This needs to change in the future, as without knowing the concentration of potentially highly bioactive compounds, it is difficult for IVF practitioners to assess risk and to pass this information on to patients as part of fully informed consent.

Experience from animal studies

It should not be forgotten that most of the techniques used in human IVF originate from animal studies, having been used, sometimes for many years, for breeding and/or research purposes. As an example, in cattle high pregnancy rates from IVF transfers were reached in

the early 1980s, and cryopreservation techniques, both slow freeze and vitrification, were already quite efficient. These results from highly fertile animals, although not immediately comparable with those from a subfertile human population, constitute a useful model for methodological development. Animal IVF is used not only to obtain offspring but also to study fertilization and embryo development mechanisms, acquiring knowledge that has been also used in human IVF. Embryo and sperm freezing technologies are also important for maintenance of various animals, for example endangered species and laboratory animals.

Cryopreservation of human embryos using the slow freezing method was introduced shortly after the first IVF pregnancies (Trounson and Mohr, 1983). However, it took many years before the technique of vitrification gained widespread use in clinical human IVF, despite having been used for many years in animals (Rall and Fahey, 1985). During these years, considerable development and optimization were being performed in animals. Timing and concentration of different vitrification cryoprotectants were studied, with embryo and fetal development as end-points. Thus, more optimized protocols could be used as a starting point when the technique was transferred to human IVF.

Blastocyst transfer is routinely used in animal breeding programmes, with high efficiency (e.g. Iritani, 1988). It is known, however, that blastocysts produced *in vitro* are morphologically of lower quality and result in lower birth rates than embryos produced *in vivo* (Hasler *et al*., 1995). In humans, blastocyst transfers have shown to result in higher pregnancy and birth rates than transfer of cleavage stage embryos (Blake *et al*., 2007). However, it is still an ongoing discussion whether the cumulative live births for all patients entering an IVF programme is increased after prolonged embryo culture. New data have recently been added to the debate; a Swedish study found that singletons born from blastocyst transfer ($n = 1311$) had a significantly increased risk of preterm birth and of congenital malformations than after cleavage-stage transfer (Källén *et al*., 2010). The authors emphasize that more studies have to be performed to verify or refute the findings.

In vitro maturation (IVM) is another technique that has been developed in animals. Today IVM is frequently used in animal production (e.g. Gordon, 2004), although showing lower success rates than the use of *in vivo* matured oocytes. In humans, IVM has a much lower efficiency than ordinary IVF and concerns have also been raised from animal studies showing that *in vitro* production of embryos, including IVM, IVF and *in vitro* culture, may affect embryonic gene expression and result in LOS in cattle and sheep (Sinclair *et al*., 2005). This, coupled with the lower efficiency, is probably one reason why this method is not so widely applied in human IVF. There is much ongoing research in animals on the possible influence on epigenetics of *in vitro* culture, and perhaps more data should be collected before introducing this technique on a wider scale for human IVF.

Sorting of X and Y-bearing sperm by fluorescent labelling and irradiation by UV laser have been used commercially in animal breeding for many years. It is, however, shown in cattle that semen which has been sexed (labelled and irradiated) results in fewer blastocysts than semen which has not undergone this procedure (Palma *et al*., 2008; Blondin *et al*., 2009). The sorting technique has been applied to human ART (review Karabinus, 2009) with over 1000 babies born as part of a Federal Drug Agency trial. The sorting of X-bearing sperm resulted in 92.0% females born and of Y-bearing sperm in 81.5% males born.

Summary/conclusions

A recent collaborative study between ESHRE and Alpha highlights the importance of going back to basics and examining oocyte and embryo morphology (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group in Embryology, 2011). However, the field of IVF continues to develop new technologies to try to improve treatments and delivery rates. Sadly, many poorly designed experiments are published and reported in the media and desperate patients then demand these novel, unproven treatments. Perhaps, it is time to put the brakes on and examine the scientific work that has been performed on new technology and ask the questions; has the technique been validated and is there evidence to show that it has clinical significance (van Steirteghem, 2008)? Some may argue that since it takes years to do the necessary research and development and obtain the results of RCTs, such an approach will dampen the development of the field. However, with so many examples where data now show that the techniques that have been applied to thousands of patients have no clinical significance, for example PGS, we have to be fair to the patients.

The introduction of ICSI was a revolution in reproductive medicine but was introduced into clinical practice without any proof of safety. As some concerns have been raised from data on pregnancy follow-up, the main issue here (except in extreme cases such as spermatid ICSI) is giving the patients appropriate high quality information on the risks.

More important is that technology is now being developed, such as *in vitro* matured gametes (Albuz *et al*., 2010; Ata *et al*., 2010) and artificial gametes (Surani, 2004; Park *et al*., 2009), which may alter the genomic constitution of the embryos we create. This could lead to the birth of children with epigenetic, genetic or chromosomal errors if we do not ensure that these procedures are safe. For example, data on reproductive cloning have shown that the technique is medically unsafe (Wells, 2005) but there are those who claim to be offering this technology to their patients (Elsner, 2006).

Of course, we all want the field of IVF to advance and to be able to offer the best possible treatment to our patients but most of all we must perform good medicine and do the necessary studies before bringing new techniques into routine clinical practice.

Authors' roles

J.H. contributed to paper design, conception, analysis of data, drafting the article and final approval of the version to be published. C.M., K.L. and C.B. involved in analysis of data, drafting the article and final approval of the version to be published. D.B. involved in conception of article, analysis of data, drafting the article and final approval of the version to be published.

Funding

None of the authors had any sponsorships that biased this manuscript.

Conflict of interest

D.B. is a minor shareholder in Novocellus Ltd. involved in amino acid profiling and advises Irvine Scientific on design of culture media.

References

Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: development of diagnostic criteria. *Hum Reprod* 2010;**25**:2415–2426 Epub 2010 August 17.

Albuz FK, Sasseville M, Lane M, Armstrong DT, Thompson JG, Gilchrist RB. Simulated physiological oocyte maturation (SPOM): a novel *in vitro* maturation system that substantially improves embryo yield and pregnancy outcomes. *Hum Reprod* 2010;**25**:2999–3011.

Alfarawati S, Fragouli E, Colls P, Wells D. First births after preimplantation genetic diagnosis of structural chromosome abnormalities using comparative genomic hybridization and microarray analysis. *Hum Reprod* 2011;**26**:1560–1574.

Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group in Embryology. The Istanbul Consensus workshop on embryo assessment: proceedings of an expert meeting. *Hum Reprod* 2011; **26**:1270–1283.

Ata B, Shalom-Paz E, Chian RC, Tan SL. *In vitro* maturation of oocytes as a strategy for fertility preservation. *Clin Obstet Gynaecol* 2010;**53**:775–786.

Barker DJ. *Mothers, Babies and Disease in Later Life*. London: BMJ Publishing, 1994.

Barlow DH. The design, publication and interpretation of research in subfertility medicine: uncomfortable issues and challenges to be faced. *Hum Reprod* 2003;**18**:899–901.

Barratt CL, De Jonge CJ. Clinical relevance of sperm DNA assessment: an update. *Fertil Steril* 2010;**94**:1958–1959 Epub 2010 August 19.

Barratt CL, Aitken RJ, Björndahl L, Carrell DT, de Boer P, Kvist U, Lewis SE, Perreault SD, Perry MJ, Ramos L *et al.* Sperm DNA: organization, protection and vulnerability: from basic science to clinical applications—a position report. *Hum Reprod* 2010;**25**:824–838.

Basatemur E, Shevlin M, Sutcliffe A. Growth of children conceived by IVF and ICSI up to 12 years of age. *Reprod Biomed Online* 2010;**20**:144–149.

Belva F, Bonduelle M, Schiettecatte J, Tournaye H, Painter RC, Devroey P, De Schepper J. Salivary testosterone concentrations in pubertal ICSI boys compared with spontaneously conceived boys. *Hum Reprod* 2011;**26**:438–441.

Benagiano G, Gianaroli L. The new Italian IVF legislation. *Reprod Biomed Online* 2004;**9**:117–125.

Blake D, Farquhar C, Johnson N, Proctor M. Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev* 2007;**17**:CD002118.

Blockeel C, Schutyser V, De Vos A, Verpoest W, De Vos M, Staessen C, Haentjens P, Van der Elst J, Devroey P. Prospectively randomized controlled trial of PGS in IVF/ICSI patients with poor implantation. *Reprod Biomed Online* 2008;**17**:848–854.

Blondin P, Beaulieu M, Fournier V, Morin N, Crawford L, Madan P, King WA. Analysis of bovine sexed sperm for IVF from sorting to the embryo. *Theriogenology* 2009;**71**:30–38.

Booth PJ, Humpherson PG, Watson TJ, Leese HJ. Amino acid depletion and appearance during porcine preimplantation embryo development *in vitro*. *Reproduction* 2005;**130**:655–668.

Bossuyt PM, Irwig L, Craig J, Glasziou P. Comparative accuracy: assessing new tests against existing diagnostic pathways. *Br Med J* 2006;**332**:1089–1092.

Bowman P, McLaren A. Cleavage rate of mouse embryos *in vivo* and *in vitro*. *J Embryol Exp Morphol* 1970;**24**:203–207.

Brinster RL. A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst Exp. *Cell Res* 1963;**32**:205–208.

Brinster RL. Studies on the development of mouse embryos *in vitro*. IV. Interaction of energy sources. *J Reprod Fertil* 1965;**10**:227–240.

Brison DR, Leese HJ. Energy metabolism in late preimplantation rat embryos. *J Reprod Fertil* 1991;**93**:245–251.

Brison DR, Schultz RM. Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. *Biol Reprod* 1997;**56**:1088–1096.

Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 2004;**19**:2319–2324.

Brison DR, Hollywood K, Arnesen R, Goodacre R. Predicting human embryo viability: the road to non-invasive analysis of the secretome using metabolic footprinting. *Reprod Biomed Online* 2007;**15**:296–302.

Cai XY, Chen GA, Lian Y, Zheng XY, Peng HM. Cryoloop vitrification of rabbit oocytes. *Hum Reprod* 2005;**20**:1969–1974.

Ceelen M, van Weissenbruch MM, Prein J, Smit JJ, Vermeiden JP, Spreeuwenberg M, van Leeuwen FE, Delemarre-van de Waal HA. Growth during infancy and early childhood in relation to blood pressure and body fat measures at age 8–18 years of IVF children and spontaneously conceived controls born to subfertile parents. *Hum Reprod* 2009;**24**:2788–2795.

Chen C. Pregnancy after human oocyte cryopreservation. *Lancet* 1986; **19**:1884–1886.

Cobo A, Kuwayama M, Perez S, Ruiz A, Pellicer A, Remohi J. Comparison of concomitant outcome achieved with fresh and cryopreserved donor oocytes vitrified by the CryoTop method. *Fertil Steril* 2008; **89**:1657–1664.

Collins JA, Barnhart KT, Schlegel PN. Do sperm DNA integrity tests predict pregnancy with *in vitro* fertilization?. *Fertil Steril* 2008; **89**:823–831.

Colls P, Escudero T, Cekleniak N, Sadowy S, Cohen J, Munné S. Increased efficiency of preimplantation genetic diagnosis for infertility using no result rescue. *Fertil Steril* 2007;**88**:53–61 Epub 2007 February 12.

Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. *J Assist Reprod Genet* 1993;**10**:21–30.

Cooke ID, Gianaroli L, Hovatta O, Trounson AO. on behalf of the Low Cost IVF Foundation. Affordable ART and the third world: difficulties to overcome. *Hum Reprod* 2008; ESHRE Monogr 93–96.

De Mouzon J, Goossens V, Bhattacharya S, Castilla JA, Ferraretti AP, Korsak V, Kupka M, Nygren KG, Nyboe Andersen A, The European IVF-monitoring (EIM) Consortium, for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE. *Hum Reprod* 2010;**25**:1851–1862.

Debrock S, Melotte C, Spiessens C, Peeraer K, Vanneste E, Meeuwis L, Meuleman C, Frijns JP, Vermeesch JR, D'Hooghe TM. Preimplantation genetic screening for aneuploidy of embryos after *in vitro* fertilization in women aged at least 35 years: a prospective randomized trial. *Fertil Steril* 2010;**93**:364–373.

Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, Schreurs IL, Dunselman GA, Kester AD, Geraedts JP *et al.* Effect of *in vitro* culture of human embryos on birthweight of newborns. *Hum Reprod* 2010;**25**:605–612.

Dunglison GF, Barlow DH, Sargent IL. Leukaemia inhibitory factor significantly enhances the blastocyst formation rates of human embryos cultured in serum-free medium. *Hum Reprod* 1996; **11**:191–196.

Elsner D. Just another reproductive technology? The ethics of human reproductive cloning as an experimental medical procedure. *J Med Ethics* 2006;**32**:596–600.

Evenson DP, Darzynkiewicz Z. Melamed MR relation of mammalian sperm chromatin heterogeneity to fertility. *Science* 1980;**210**:1131–1133.

Fernandez-Gonzalez R, Moreira PN, Perez-Crespo M, Sanchez-Martin M, Ramirez MA, Pericuesta E, Bilbao A, Bermejo-Alvarez P, De Dios

Hourcade J, De Fonseca FR *et al*. Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biol Reprod* 2008;**78**:761–772.

Fragouli E, Alfarawati S, Daphnis DD, Goodall NN, Mania A, Griffiths T, Gordon A, Wells D. Cytogenetic analysis of human blastocysts with the use of FISH, CGH and aCGH: scientific data and technical evaluation. *Hum Reprod* 2011;**26**:480–490 Epub 2010 December 8.

Geraedts J, Collins J, Gianaroli L, Goossens V, Handyside A, Harper J, Montag M, Repping S, Schmutzler A. What next for preimplantation genetic screening? A polar body approach! *Hum Reprod* 2010; **25**:575–577.

Geraedts J, Montag M, Magli MC, Repping S, Handyside A, Staessens C, Harper J, Schmutzler A, Collins J, Goossens V *et al*. Polar body array CGH for prediction of the status of the corresponding oocyte. Part I: clinical results. *Hum Reprod* 2011;**26**:3173–3180.

Gianaroli L, Magli C, Ferraretti AP, Munné S. Preimplantation diagnosis for aneuploidies in patients undergoing *in vitro* fertilization with a poor prognosis: identification of the categories for which it should be proposed. *Fertil Steril* 1999;**72**:837–844.

Gluud C, Gluud LL. Evidence based diagnostics. *Br Med J* 2005; **330**:724–726.

Goossens V, Harton G, Moutou C, Traeger-Synodinos J, Van Rij M, Harper JC. ESHRE PGD Consortium data collection IX: cycles from January to December 2006 with pregnancy follow-up to October 2007. *Hum Reprod* 2009;**24**:1786–1810.

Gordon IR. *Reproductive Technologies in Farm Animals*. Oxfordshire, UK: CABI Publishing, 2004.

Gordon JW, Talansky BE. Assisted fertilization by zona drilling: a mouse model for correction of oligospermia. *J Exp Zool* 1986;**239**:347–354.

Goto K. Bovine microfertilization and embryo transfer. *Mol Reprod Dev* 1993;**36**:288–290.

Gott AL, Hardy K, Winston RM, Leese HJ. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod* 1990;**5**:104–108.

Gómez MC, Catt JW, Evans G, Maxwell WM. Sheep oocyte activation after intracytoplasmic sperm injection (ICSI). *Reprod Fertil Dev* 1998; **10**:197–205.

Hardarson T, Hanson C, Lundin K, Hillensjö T, Nilsson L, Stevic J, Reismer E, Borg K, Wikland M, Bergh C. Preimplantation genetic screening in women of advance maternal age decrease in clinical pregnancy rate: a randomized controlled trial. *Hum Reprod* 2008; **23**:2806–2812.

Hardarson T, Ahström A, Rogberg L, Botros L, Hellensjö T, Westlander G, Sakkas D, Wikland M. Non-invasive metabolomic profiling of day 2 and 5 embryo culture medium: a prospective randomized trial. *Hum Reprod* 2012;**27**:Epub 2011 November 7.

Hardy K, Handyside AH, Winston RM. The human blastocyst: cell number, death and allocation during late preimplantation development *in vitro*. *Development* 1989;**107**:597–604.

Harper JC. *Preimplantation Genetic Diagnosis*. Cambridge: Cambridge University Press, 2009.

Harper JC, Harton G. The use of arrays in PGD/PGS. *Fertil Steril* 2010; **94**:1173–1177.

Harper JC, SenGupta S. Preimplantation genetics diagnosis: state of the ART 2011. *Hum Genet* 2011;**11** Epub ahead of print.

Harper JC, Coonen E, Handyside AH, Winston RM, Hopman AH, Delhanty JD. Mosaicism of autosomes and sex chromosomes in morphologically normal, monospermic preimplantation human embryos. *Prenat Diagn* 1995;**15**:41–49.

Harper JC, Coonen E, De Rycke M, Harton G, Moutou C, Pehlivan T, Traeger-Synodinos J, Van Rij M, Goossens V. ESHRE PGD consortium data collection X: cycles from January to December 2007 with pregnancy follow-up to October 2008. *Hum Reprod* 2010a;**25**:2685–2707.

Harper J, Coonen E, De Rycke M, Fiorentino F, Geraedts J, Goossens V, Harton G, Pehlivan Budak T, Renwick P, Sengupta S *et al*. What next for preimplantation genetic screening (PGS)? A position statement from the ESHRE PGD Consortium steering committee. *Hum Reprod* 2010b; **25**:821–823.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* 1995;**43**:141–152.

Hellani A, Abu-Amero K, Azouri J, El-Akoum S. Successful pregnancies after application of array-comparative genomic hybridization in PGS-aneuploidy screening. *Reprod Biomed Online* 2008;**17**:841–847.

Hewitson L, Takahashi D, Dominko T, Simerly C, Schatten G. Fertilization and embryo development to blastocysts after intracytoplasmic sperm injection in the rhesus monkey. *Hum Reprod* 1998;**13**: 3449–3455.

Hiramoto Y. Microinjection of the live spermatozoa into sea urchin eggs. *Exp Cell Res* 1962;**27**:416–426.

Hiramoto Y. Analysis of cleavage stimulus by means of micromanipulation of sea urchin eggs. *Exp Cell Res* 1971;**68**:291–298.

Hollywood K, Brison DR, Goodacre R. Metabolomics: current technologies and future trends. *Proteomics* 2006;**17**:4716–4723.

Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 2002;**17**: 999–1005.

Iritani A. Current status of biotechnological studies in mammalian reproduction. *Fertil Steril* 1988;**50**:543–551.

Jansen RP, Bowman MC, de Boer KA, Leigh DA, Lieberman DB, McArthur SJ. What next for preimplantation screening (PGS)? Experience with blastocyst biopsy and testing for aneuploidy. *Hum Reprod* 2008;**23**:1476–1478.

Källén B, Finnström O, Lindam A, Nilsson E, Nygren KG, Olausson PO. Blastocyst versus cleavage stage transfer in *in vitro* fertilization: differences in neonatal outcome? *Fertil Steril* 2010;**94**:1680–1683 Epub 2010 February 4.

Kane MT, Morgan PM, Coonan C. Peptide growth factors and preimplantation development. *Hum Reprod Update* 1997;**3**:137–157.

Karabinus DS. Flow cytometric sorting of human sperm: MicroSort clinical trial update. *Theriogenology* 2009;**71**:74–79 Epub 2008 October 26.

Keefer CL. Fertilization by sperm injection in the rabbit. *Gamete Res* 1989; **22**:59–69.

Kimber SJ, Sneddon SF, Bloor DJ, El-Bareg AM, Hawkhead JA, Metcalfe AD, Houghton FD, Leese HJ, Rutherford A, Lieberman BA *et al*. Expression of genes involved in early cell fate decisions in human embryos and their regulation by growth factors. *Reproduction* 2008;**135**:635–647.

Kuleshova L, Gianaroli L, Magli MC, Ferraretti AP, Trounson AO. Birth following vitrification of a small number of human oocytes. *Hum Reprod* 1999;**14**:3077–3079.

Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* 2000;**127**:4195–4202.

Lacham O, Trounson A, Holden C, Mann J, Sathanathan H. Fertilization and development of mouse eggs injected under the zona pellucida with single spermatozoa treated to induce acrosome reaction. *Gamete Res* 1989;**23**:233–243.

Lamb VK, Leese HJ. Uptake of a mixture of amino acids by mouse blastocysts. *J Reprod Fertil* 1994;**102**:169–175.

Lanzendorf SE, Maloney MK, Veeck LL, Slusser J, Hodgen GD, Rosenwaks Z. A preclinical evaluation of pronuclear formation by microinjection of human spermatozoa into human oocytes. *Fertil Steril* 1988;**49**:835–842.

Lawitts JA, Biggers JD. Optimization of mouse embryo culture media using simplex methods. *J Reprod Fertil* 1991;**91**:543–556.

Leese HJ. Analysis of embryos by non-invasive methods. *Hum Reprod* 1987;**2**:37–40.

Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* 2002;**24**:845–849.

Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. Metabolism of the viable mammalian embryo: quietness revisited. *Mol Hum Reprod* 2008;**14**:667–672.

Lighten AD, Moore GE, Winston RM, Hardy K. Routine addition of human insulin-like growth factor-I ligand could benefit clinical *in-vitro* fertilization culture. *Hum Reprod* 1998;**13**:3144–3150.

Martin KL, Barlow DH, Sargent IL. Heparin-binding epidermal growth factor significantly improves human blastocyst development and hatching in serum-free medium. *Hum Reprod* 1998;**13**:1645–1652.

Mastenbroek S, Twisk M, Van Echten-arends J, Sikkema-Raddatz B, Korevaar JC, Verhoeve HR, Vogel NEA, Arts EGJM, De Vries JWA, Bossuyt PM *et al*. *In vitro* fertilization with preimplantation genetic screening. *N Engl J Med* 2007;**357**:9–17.

McEvoy TG, Sinclair KD, Young LE, Wilmut I, Robinson JJ. Large offspring syndrome and other consequences of ruminant embryo culture *in vitro*: relevance to blastocyst culture in human ART. *Hum Fertil (Camb)* 2000; **3**:238–246.

McKelvey A, David AL, Shenfield F, Jauniaux ER. The impact of cross-border reproductive care or 'fertility tourism' on NHS maternity services. *BJOG* 2009;**116**:1520–1523 Epub 2009 July 15.

Mersereau JE, Pergament E, Zhang X, Milad MP. Preimplantation genetic screening to improve *in vitro* fertilization pregnancy rates: a prospective randomized controlled trial. *Fertil Steril* 2008;**90**:1287–1289.

Meyer LR, Klipstein S, Hazlett WD, Nasta T, Mangan P, Karande VC. A prospective randomized controlled trial of preimplantation genetic screening in the 'good prognosis' patient. *Fertil Steril* 2009; **91**:1731–1738.

Mitchell LA, De Iuliis GN, Aitken RJ. The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *Int J Androl* 2011;**34**:2–13.

Munné S, Lee A, Rosenwaks Z, Grifo J, Cohen J. Diagnosis of major chromosomeaneuploidies in human preimplantation embryos. *Hum Reprod* 1993;**8**:2185–2191.

Munné S, Dailey T, Sultan KM, Grifo J, Cohen J. The use of first polar bodies for preimplantation diagnosis of aneuploidy. *Hum Reprod* 1995a;**10**:1015–1021.

Munné S, Sultan KM, Weier HU, Grifo JA, Cohen J, Rosenwaks Z. Assessment of numeric abnormalities of X, Y, 18, and 16 chromosomes in preimplantation human embryos before transfer. *Am J Obstet Gynecol* 1995b;**172**(4 Pt 1):1191–1199.

Munné S, Sandalinas M, Escudero T, Velilla E, Walmsley R, Sadowy S, Cohen J, Sable D. Improved implantation after preimplantation genetic diagnosis of aneuploidy. *Reprod Biomed Online* 2003;**7**:91–97.

Munné S, Chen S, Fischer J, Colls P, Zheng X, Stevens J, Escudero T, Oter M, Schoolcraft W, Simpson JL *et al*. Preimplantation genetic diagnosis reduces pregnancy loss in women 35 and older with a history of recurrent miscarriages. *Fertil Steril* 2005;**84**:331–335.

Munné S, Fischer J, Warner A, Chen S, Zouves C, Cohen J. And referring centers PGD group. Preimplantation genetic diagnosis significantly reduces pregnancy loss in infertile couples: a multi-center study. *Fertil Steril* 2006;**85**:326–332.

Odom LN, Segars J. Imprinting disorders and assisted reproductive technology. *Curr Opin Endocrinol Diabetes Obes* 2010;**17**:517–522.

Palermo GP. Pregnancies after intracytoplamic injection of single spermatozoon into an oocyte. *Lancet* 1992;**340**:17–18.

Palma GA, Olivier NS, Neumüller Ch, Sinowatz F. Effects of sex-sorted spermatozoa on the efficiency of *in vitro* fertilization and ultrastructure of *in vitro* produced bovine blastocysts. *Anat Histol Embryol* 2008; **37**:67–73.

Park TS, Galic Z, Conway AE, Lindgren A, Van Handel BJ, Magnusson M, Richter L, Teitell MA, Mikkola HKA, Lowry WE. Derivation of primordial germ cells from human embryonic and induced pluripotent stem cells is significantly improved by coculture with human fetal gonadal cells. *Stem Cells* 2009;**27**:783–795.

Partridge RJ, Leese HJ. Consumption of amino acids by bovine preimplantation embryos. *Reprod Fertil Dev* 1996;**8**:945–950.

Porcu E, Fabbri R, Damiano G, Giunchi S, Fatto R, Ciotti PM, Venturoli S, Flamigni C. Clinical experience and applications of oocyte cryopreservation. *Mol Cell Endocrinol* 2000;**169**:33–37.

Rall WF, Fahey GM. Vitrification of embryos. *Nature* 1985;**313**:573–575.

Richter KS. The importance of growth factors for preimplantation embryo development and in-vitro culture. *Curr Opin Obstet Gynecol* 2008; **20**:292–304.

Rienzi L, Romano S, Albricci L, Maggiulli R, Capalbo A, Baroni E, Colamaria S, Sapienza F, Ubaldi F. Embryo development of fresh 'versus' vitrified metaphase II oocytes after ICSI: a prospective randomized sibling-oocyte study. *Hum Reprod* 2010;**25**:66–73.

Sakkas D, Alvarez JG. Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril* 2010; **93**:1027–1036.

Schoolcraft W, Katz-Jaffe MG, Stevens J, Rawlins M, Munne S. Preimplantation aneuploidy testing for infertile patients of advanced maternal age: a randomized prospective trial. *Fertil Stertil* 2009; **92**:157–162.

Seli E, Vergouw CG, Morita H, Botros L, Roos P, Lambalk CB, Yamashita N, Kato O, Sakkas D. Noninvasive metabolomic profiling as an adjunct to morphology for noninvasive embryo assessment in women undergoing single embryo transfer. *Fertil Steril* 2010; **94**:535–542 Epub 2009 July 8.

Shenfield F, de Mouzon J, Pennings G, Ferraretti AP, Nyboe Andersen A, de Wert G, Goossens V. The ESHRE Taskforce on Cross Border Reproductive Care. Cross border reproductive care in six European countries. *Hum Reprod* 2010;**25**:1361–1368.

Sinclair KD, Young LE, Wilmut I, McEvoy TG. *In-utero* overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. *Hum Reprod* 2000;**5**:68–86.

Sjoblom C, Roberts CT, Wikland M, Robertson SA. Granulocyte-macrophage colony-stimulating factor alleviates adverse consequences of embryo culture on fetal growth trajectory and placental morphogenesis. *Endocrinology* 2005;**146**:2142–2153.

Sotovsky P, Hewitson L, Simerly CR, Tengowsky MW, Navara CS, Haavisto A, Schatten G. Intracytoplamic sperm injection for Rhesus monkey fertilization results in unusual chromatin, cytoskeletal, and membrane events, but leads to pronuclear development and sperm aster assembly. *Hum Reprod* 1996;**11**:1703–1712.

Staessen C, Platteau P, Van Assche E, Michiels A, Tournaye H, Camus M, Devroey P, Liebaers I, Van Steirteghem A. Comparison of blastocyst transfer with or without preimplantation genetic diagnosis for aneuploidy screening in couples with advanced maternal age: a prospective randomized controlled trial. *Hum Reprod* 2004; **19**:2849–2858.

Staessen C, Verpoest W, Donoso P, Haentjens P, Van der Elst J, Liebaers I, Devroey P. Preimplantation genetic screening does not improve delivery

rate in women under the age of 36 following single-embryo transfer. *Hum Reprod* 2008;**23**:2818–2825.

Steptoe P, Edwards R. Birth after the re-implantation of a human embryo. *Lancet* 1978;**12**:366.

Stevens J, Wale P, Surrey ES, Schoolcraft WB. Is aneuploidy screening for patients aged 35 or over beneficial? A prospective randomized trial. *Fertil Steril* 2004;**82**(Suppl. 2):249.

Sturmey RG, Hawkhead JA, Barker EA, Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. *Hum Reprod* 2009; **24**:81–91.

Surani A. Stem cells: how to make eggs and sperm. *Nature* 2004;**427**: 106–107.

Thadani VM. Injection of sperm heads into immature rat oocytes. *J Exp Zool* 1979;**210**:161–168.

Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 1983; **305**:707–709.

Ubaldi F, Anniballo R, Romano S, Baroni E, Albricci K, Colamaria S, Capalbo A, Sapienza F, Vajta G, Rienzi L. Cumulative ongoing pregnancy rate achieved with oocyte vitrification and cleavage stage transfer without embryo selection in a standard infertility program. *Hum Reprod* 2010;**25**:1199–1205.

Vail A, Gardener E. Common statistical errors in the design and analysis of subfertility trials. *Hum Reprod* 2003;**18**:1000–1004.

Van Steirteghem A. What next for assisted reproductive technology? A plea for an evidence-based approach. *Hum Reprod* 2008;**12**:2615–2616.

Vergouw CG, Botros LL, Roos P, Lens JW, Schats R, Hompes PG, Burns DH, Lambalk CB. Metabolomic profiling by near-infrared spectroscopy as a tool to assess embryo viability: a novel, non-invasive method for embryo selection. *Hum Reprod* 2008; **23**:1499–1504 Epub 2008 April 18.

Verlinsky Y, Cieslak J, Freidine M, Ivakhnenko V, Wolf G, Kovalinskaya L, White M, Lifchez A, Kaplan B, Moise J *et al.* Pregnancies following pre-conception diagnosis of common aneuploidies by fluorescent in-situ hybridization. *Hum Reprod* 1995;**10**:1923–1927.

Watkins AJ, Fleming TP. Blastocyst environment and its influence on offspring cardiovascular health: the heart of the matter. *J Anat* 2009; **215**:52–59.

Wells DN. Animal cloning: problems and prospects. *Rev Sci Tech* 2005; **1**:251–264.