Reproductive BioMedicine Online (2010) **20**, 453–469

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REVIEW

Embryo culture: can we perform better than nature?

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Abstract Culture of preimplantation-stage embryos has always been a key element of laboratory embryology and has contributed substantially to the success of many assisted reproduction procedures. During the past decade, its importance has increased as extended in-vitro embryo culture and single blastocyst transfer have become indispensable parts of the approach to decreasing the chance of multiple pregnancy while preserving the overall efficiency of the treatment. However, in spite of the scientific and commercial challenge stimulating research worldwide to optimize embryo culture conditions, a consensus is missing even in the basic principles, including composition and exchange of media, the required physical and biological environment and even the temperature of incubation. This review attempts to summarize the controversies, demonstrate the fragility of some widely accepted dogmas and generate an open-minded debate towards rapid and efficient optimization. New approaches expanding the traditional frames of mammalian embryo culture are also discussed. Although some researchers suppose that the efficiency of the presently applied in-vitro culture systems have already approached the biological limits, authors are confident that substantial improvement may be achieved that may expand considerably the possibilities of future assisted reproduction in humans.
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KEYWORDS: blastocyst, human, incubation, *in vitro*, *in vivo*, stress

Introduction

The first successful culture of mammalian embryos *in vitro* was published in the middle of the last century (Whitten, 1956, 1957). It is probably worth mentioning that the overall level of physical and natural sciences was surprisingly advanced in that period, at least compared with mammalian embryology. Achievements in that particular decade included harnessing nuclear fission for power generation as well as an established military use in atomic and hydrogen bombs, the first space flights, development of synchrotrons, nuclear powered submarines and the cracking of the DNA

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doi:10.1016/j.rbmo.2009.12.018

code, whilst in biological science the production of a single blastocyst in a Petri dish was still an unattainable dream for most embryologists.

According to Bavister (2002), the major reason for this regrettable delay was that scientists did not realize the extreme temperature sensitivity of oocytes and embryos in most mammalian species, particularly at those stages of meiosis involving delicate spindle formation. However, even that eventual discovery (Shettles, 1953; Smith, 1951) did not accelerate progress either. In spite of the impressive achievements in human IVF with millions of babies born worldwide, culture of mammalian embryos remains a demanding task and is still performed using rather primitive techniques and the advancement is slow.

To avoid fiascos, in practice most laboratories simply use their earliest albeit modestly successful routines, painstakingly introducing any innovative procedures which require many years, sometimes a decade or even more to develop. Controversially, even the few new procedures that gain worldwide acceptance are typically implemented and applied without scientifically sound evidence. Consensus among scientists is often lacking. Even the most widely accepted parameter, the application of the core temperature, has been questioned recently and for good reasons (Leese et al., 2008b). Due to these hurdles and other, not purely scientific factors, mammalian embryology may still be regarded as a handicapped branch of science especially when compared with other fields, e.g. information technology, nanotechnology and molecular biology.

Nothing proves that the future cannot be brighter. It is a self-evident truism to refer to the in-vitro environment as 'inappropriate', 'stressful' and 'evidently inferior' compared with the in-vivo counterpart. Ambitions are usually restricted to narrowing the gap between the two systems, although a more daring goal may also be outlined. Indeed there are many examples even in reproductive biology that the natural way is not necessarily the optimal or the most efficient one. In cattle, the overall efficiency of in-vitro embryo production is about 100-fold higher than the in-vivo process if the developmental rate is measured from the antral follicle stage or from the spermatozoon to the blastocyst. The latter difference can be even higher in humans when intracytoplasmic sperm injection is applied.

This review argues that mammalian embryo culture *in vitro* should not be regarded as an imperfect copy of the in-vivo procedures, but an artificial process with its own frames, limitations and possibilities. Acknowledging the current limitations must not restrict the search for future perspectives. By using in-vitro systems, endless choices are offered — even within, but especially behind, the traditional 'medium, gas, temperature, oil' frames — to establish conditions and involve steps that fully exploit all resources of mammalian embryo development, including those not used by the natural process, or to defend embryos from a potentially or definitely harmful maternal effect.

The aim of this work is to provide a focused overview about the state-of-the-art of mammalian, predominantly human, embryo culture. Instead of detailed discussion of all published achievements, approaches and theories, this review attempts to outline major trends, confront contradicting opinions and call attention to some promising new perspectives.

Principles to reconsider

The first group of problems originates from the somatic cell analogue. All standard embryo culture procedures use somatic cell cultures as templates and the existing differences can only be regarded as slight modifications. The frames, rules, tools and equipment of mammalian somatic cell cultures were established decades before the first successes in embryology and served as models for embryo culture. The subsequent innovations on the somatic cell field (including microbeads, smart substrates, microbioreactors, etc.; Mendes, 2008; Miller et al., 1989; Rahman et al., 2009) had little or no effect on embryo culture *in vitro*. This unfortunate approach by embryologists completely disregards that the situation, morphology, function and regulation of somatic cells *in vivo* are basically different from those of preimplantation mammalian embryos.

These differences and their consequences are summarized in the following points:

(i) Preimplantation embryos are not part of the maternal body: they exist physically and biologically as largely independent living beings in an open tube. The physical separation from the maternal body is reinforced by the zona pellucida and becomes complete with the loss of cumulus cell—oocyte gap junctional communication.

(ii) While somatic cells of mammals are surrounded by the extracellular fluid that is rich in nutrients, proteins and other factors, preimplantation embryos are surrounded by an as yet undefined (but most probably very low) amount of oviductal and uterine fluid that is more dilute, contains less resources and its physico-chemical characteristics show spatial and temporal changes (Hunter, 2005; Leese et al., 2001).

(iii) In contrast to somatic cells that are unable to proliferate without external trophic ligands, mammalian zygotes are intrinsically programmed to develop to the hatched blastocyst stage without any external stimuli. However, *in vivo* these embryos are exposed and certainly respond to a number of interrelated endocrine, paracrine and autocrine factors. The complex regulatory mechanism provided by these ligands seems to be supportive for healthy development (O'Neill, 2008).

(iv) The oviductal and uterine fluid is not just the product of the epithelial cells. It contains factors derived from the maternal body, reflects its actual status and may have profound consequences on the future of the fetus and offspring (Leese et al., 2008a). The role of oviductal and uterine epithelial cells includes a passive filtration or active transport of these factors as well; this role cannot be reconstructed by a simple monolayer of a co-culture system.

(v) The oviductal—uterine environment is not necessarily supportive to the embryo development. Depending on the status of the reproductive tract or the whole maternal body, it may also be harmful and may cause serious damage with long-term negative effects on the development of the fetus or offspring (Kwong et al., 2000; Leese, 2002; Leese et al., 2008b; Oliver et al., 2007).

(vi) The establishment and maintenance of the microenvironment plays a crucial role in embryo development both *in vivo* and *in vitro* (Hunter et al., 2003; 2005; O'Neill, 2008). Autocrine factors seem to be decisive constituents of this microenvironment. With the current methods, tools and dishes (established originally for somatic cells), this microenvironment cannot be properly maintained *in vitro*.

The other group of problems is related to the autocratic approach of dictating to embryos what they should prefer, either by interpreting wrongly the in-vivo situation, or by applying its features mechanically to the in-vitro culture

Examples for this mentality are listed in the following points:

(i) The existence of some commonly cited oviductal factors or phenomena has not been confirmed *in vivo* in pregnant mammals, especially not in humans.
(ii) Several parameters measured as physiological constituents of the oviductal fluid *in vivo* may be detrimental *in vitro* (for example potassium level; Biggers, 2001), whilst others, not present in the oviduct, may not have the widely supposed harmful effect *in vitro*.
(iii) Some factors around and even inside the embryos (e.g. hyaluronic acids in media, or intracytoplasmic lipid droplets in pigs) may not be required in a given in-vitro system.
(iv) Any intervention based on in-vivo analogues and performed with the best intentions may have adverse effects that counterbalance, even reverse the benefits.
(v) The selection of the mouse as the animal model is a convenient solution for the scientist, but may be a suboptimal choice and culprit for erroneous practices where human embryos are concerned.
(vi) For almost all embryologists, the elimination of any kind of stress is the unquestionable principle of the optimal embryo culture system. However, a small but increasing number of data show that a precisely timed and appropriately, provided shock may induce metabolic processes that eventually result in improved embryo quality and developmental competence.

In the following sections, an attempt will be made to justify the above statements and to provide several practical suggestions for future improvements

Chemical components

Media for human embryo culture should contain the following components: pure water, common salts of four metals plus sodium bicarbonate as buffer, sodium salt of EDTA or other chelator, pyruvate and lactate, amino acids, macromolecules and antibiotics. These principles have been established and widely accepted by the final decade of the 20th century and contributed substantially to the dramatic increase in efficiency of human assisted reproduction all over the world around the turn of the millennium (Quinn, 2004).

Unfortunately, a simple list such as the first one in the previous paragraph can summarize everything when a broad consensus exists. Considering the invested energy and the responsibility related to the procedure, the list is disappointingly short and unspecific. Regarding composition and application of media, leading scientists of the field seem to disagree in all details. Not a single component is used in exactly the same concentration in all commonly applied human embryo culture media. There are marked differences in concentration of the simplest elements, such as potassium chloride and magnesium sulphate (Gardner and Lane, 2004). The simple media that have been established and called analogues of oviduct fluid only marginally mimic the natural oviduct environments and differ from each other in almost all parameters (Biggers, 2001). Even the optimal osmolality for development of human embryos in culture has not been determined yet (Gardner, 2008). Moreover, almost all media require supplementation with chemically undefined or partially defined factors as albumin or serum.

Components of media were described in detail in reviews of Bavister (1995), Biggers (2001), Biggers and Summers (2008), Gardner and Lane (2004), Gardner (2008), Leese (2003), Mortimer (2001) and Yovich and Grudzinskas (1990). The summary presented here will focus only on the main tendencies and disputable details.

The first successes of embryo culture were achieved in the mouse with a simple Krebs–Ringer bicarbonate medium (Tyrode's medium) supplemented with lactate (McLaren and Biggers, 1958; Whitten, 1957). For initial human application, complex media (e.g. Earle's balanced salt solution or Ham's F-10) were used; however, supplementation with maternal serum was indispensable (Edwards, 1981).

During the subsequent decades, two major approaches were applied to develop media purposefully for human embryos, both of them using simple media as starting points. The first approach was the 'empirical optimization' of components by bioassays — also known as 'let the embryos choose' principle — established by Biggers (KSOM-Global family; (Biggers, 2001). The other way was the 'back to nature' principle, i.e. to make media with composition similar to the oviductal fluid, outlined and baptized by Leese, 1998, but applied previously to develop solutions including the HTF-Sage and STF-Cook family of Quinn and Mortimer, respectively (Mortimer, 1986; Quinn, 1995; 2000; Quinn et al., 1985). However, none of these strategies was successful enough to be applied purely and exclusively for development of media.

The 'empirical optimization' approach, even with computer modelling, would require astronomic numbers of experiments without acknowledging some principles based on data obtained from in-vivo measurements. Accordingly, some compromises in the interpretation of the mathematical models are required and many of the variables — including amino acid concentrations — need a kind of bulk handling (Biggers, 2001; Summers and Biggers, 2003).

The major problem of the 'back to nature' principle (as also acknowledged by Leese et al., 2008a,b) is the extremely low amount of the fluid in the oviduct and uterus and the technical and ethical problems related to its collection and measurement. So far, investigations have been performed *in vivo* (by micropuncture, chronic or acute in-situ canulation) or *in vitro* (by vascular and luminar perfusion

or excision and rapid sampling; Leese et al., 2008a,b). However, many of these methods cannot be applied to humans and virtually none of them can be used to collect samples during the critical period of early human pregnancy; therefore, all conclusions are based on analogues.

A third approach, to use nutrient consumption data of the embryo, was also listed in the review of Leese (2003). Retrospectively, this latter approach was at least partially used by all researchers who achieved successes in this field and was the basic element of the work of Gardner and Lane (2004) that led to the introduction of the G-Vitrolife family of media.

The final result of these efforts could be a colourful palette of possibilities, with a good chance for the optimal solution to evolve almost spontaneously, by a kind of natural selection. However, there are two serious problems that hamper this advancement. The first can be attributed to the status of the human embryo *in vitro*, with various moral and legal aspects drastically restricting the possibility of comparative studies.

The second is related to the aspect of commercialization. More than 80% of major producers fail to supply information about the exact composition of their media; some do not even provide a list of components. As stated by Biggers (2000) this approach may reduce presentations and papers to uncritical advertisement. Comparisons may only happen between brand names and code numbers, the real research work becomes the privilege of the inner circle of a company making a worldwide unified effort impossible.

Sword of Damocles

There are alerting new data underlining the importance of such worldwide efforts. The first week of mammalian embryo development consists of crucial events including the first cleavage, activation of the maternal genome, compaction of morulae and differentiation with blastocyst development (Lonergan et al., 2006). All these steps are the result of precisely programmed and orchestrated events. Adaptation to an inappropriate environment may cause changes in epigenetics, transcription, metabolism and cell allocation. Components of media can produce serious alterations in the gene expression pattern of preimplantation mammalian embryos (Duranthon et al., 2008; Lonergan et al. 2006; Thompson et al., 2007; Wrenzycki et al. 2005) with potential long-term consequences.

In sheep, serum supplementation may cause up to three- or four-fold increases in birthweight, higher incidence of abortions, neonatal deaths and post-natal malformations. Similar phenomena were also described in less drastic form in cattle (Thompson et al., 2007), whilst in the mouse, decreased fetal weight, increased post-natal organ weight and exencephalopathy have been reported (Fernandez-Gonzalez et al., 2004; Khosla et al., 2001a,b; Lane and Gardner, 1992; Lawrence and Moley, 2008; Zander et al., 2006). In-vitro embryo culture for mouse has also caused behavioural problems (Ecker et al., 2004). Mechanisms involved are most probably related to epigenetic alterations involving imprinted genes, with placental abnormalities and aberrant causal pathways as consequences (Thompson, 2007). Some of the effects may cause morphologically detectable changes even before hatching, but most of them remain hidden from stereomicroscopic investigations.

Considering that developmental anomalies occur in the majority of the handful of species studied in detail so far, in-vitro culture of human embryos could be an extremely risky and morally unacceptable challenge. However, the everyday practice does not support this conclusion. There is only a very slight, if any, increase in the proportion of perinatal complications and developmental abnormalities compared with natural reproduction, and none of these pathologies can be definitely attributed to the in-vitro culture (Chang et al., 2005; Khosla et al., 2001b; Paoloni-Giacobino, 2007).

Various explanations have been proposed attempting to explain these differences between species, especially the privileged position of humans. The most concise summary is that there is no clear idea of the reasons. Retrospectively, it may be interpreted as a pure chance that humans belong to the lucky minority. However, this generous gift of nature can only be accepted while keeping in mind that there are limits of such tolerance. These limits may crossed at anytime, but the consequences may only be realized retrospectively some years or even decades later.

Physical constituents

Media for human embryo culture are usually placed into polystyrene dishes, in 10—80 μl drops covered by oil, then placed in humid dark areas into a mixture of oxygen, carbon dioxide and nitrogen with a consistent 37°C temperature. This system can be called anything but imaginative. Simplicity, practicality and repeatability are its advantages, but these features are to the benefit of embryologists and not the embryos.

Most common dishes applied for human embryo culture are 30- or 60-mm diameter Petri dishes, less frequently four- or five-well dishes. None of them has been developed for embryological purposes. Special surface coatings may be applied in some of these dishes, without proven benefit for mammalian embryo development. Very recently, dedicated dishes for mammalian embryo handling and culture have been produced (Rieger et al., 2007), but conclusive evidence regarding practicality, cost-efficiency and especially the improvement in pre- and post-implantation development is still lacking.

Oil has the bad reputation of being the least stable, most sensitive element of the physical environment, which may become detrimental to embryos at anytime (Gardner and Lane, 2004; Otsuki et al., 2007, 2009). Unfortunately, its application in the current microdrop culture systems is indispensable both to physically separate drops from each other and to prevent rapid evaporation, as well as changes of pH and temperature. Oils may be silicon- or carbon- (paraffin, mineral) based. They are usually supplied in sterile form and can be used without any treatment, although some laboratories prefer sterile filtration and/or washing and equilibration with media.

Light sensitivity of embryos is one of the most disputed and least measured parameters (Oh et al., 2007). The tolerance may differ between species and developmental phases. Mild exposure with a yellowish (Wolfram bulbs)

low-intensity light from the ceiling or the microscope for a limited period does not seem to have a detrimental effect. However, exposure should be restricted to the minimum required level.

A general principle for the physical environment is to keep it free from any uncontrolled insults. Possibilities include a broadly diverse scale from continuous mechanical vibrations to toxic fumes including highly volatile organic compounds. Authors fully agree with the opinion of Thouas (EmbryoMail, 28 August 2008), that the situation '... in a typical working clinical IVF lab with follicular fluid, blood and aerosols floating around all the time from multiple patients, with teams of energetic embryologists, doctors, theatre staff and the patients themselves moving around regularly' is less than favourable to ensure the required calm consistency. Paradoxically, a dull country laboratory in an old separate small building may provide a much better environment, than a famous university laboratory in the middle of a luxuriously equipped skyscraper and surrounded by cutting-edge research, educational status and medical facilities.

Questions to answer

Only the most important questions are listed here, where markedly different opinions between leading scientists exist, and where laboratories have to make their own decisions.

Communal or individual culture?

Microdrop culture offers an easy way to follow the development of each embryo individually. The ultimate benefits of the system are, however, questionable both for the embryos and the embryologists. Most embryologists determine the fate of embryos (developing from normally fertilized 2PN zygotes) at the last evaluation, just before transfer or cryopreservation, regardless of the quality at the previous checkpoint. Therefore, individual identification is rather important for administrative reasons or academic interests. For the embryos, in most mammalian species they seem to dislike solitude. The 'group' or 'communal' effect has been confirmed by numerous studies in mice, cattle and felids; both in polytocous and monotocous species (summarized by O'Neill, 2008; Vajta et al., 2000). In a routine bovine in-vitro culture system, up to 50 embryos can be incubated together in 400 μl medium covered with 400 μl oil, in a well of a four-well dish, grouping embryos close to each other in the middle of the dish. The consistently achievable 50% rate of blastocysts per collected immature oocytes — compared with the 34—39% in individual drop culture — prove the value of this system and resolve concerns regarding the supposed negative effects of dead and dying embyos (Hagemann et al., 1998; Holm et al., 1999; Vajta et al., 2000). Gopichandran and Leese (2006) have even determined the optimal distance between bovine embryos: 165 μm, i.e. close to the commonly achieved average distance when embryos are swirled together.

Although the group effect can be increased by decreasing the volume of the medium, current human culture systems do not exploit this possibility. According to Gardner et al. (1993), 20 μl of synthetic oviduct fluid (SOF) medium contains the minimum energy supply for one embryo over a 6-day in-vitro-culture period. This volume is more than the 10 μl medium suggested by Carolan et al. (1996) for individual bovine embryos. Later, Gardner and Lane (2004) regarded 50 μl drops for four human or domestic animal embryos the minimum requirement, but with medium renewal every 48 h. Similar volume/embryo ratios are applied now worldwide in human IVF laboratories, although these circumstances are far from supportive of communal effects. Even the newly introduced commercially available dishes with both individual identification and communal culture possibility (Rieger et al., 2007) seem to contain too much medium to benefit from the latter arrangement.

Curiously, sporadic empirical evidence indicates that these commonly acknowledged volume/embryo rate limits are far from the real values. According to Ali (personal communication) continuous ultra-microdrop culture, i.e. 1.5—2 μl drops for culture of three to nine human embryos together for 2—3 days. Both in-vitro and in-vivo developmental rates were found significantly increased in the continuous ultra-microdrop system compared with the control. Murine and bovine embryos were also successfully cultured in closed systems containing 0.5—1 μl medium per embryo without refreshment during the whole culture period (Roh et al., 2008; Thouas et al., 2003; Vajta et al., 2001; see also below).

Based on these results, a critical reconsideration of the traditional opinion regarding the volume/embryo requirements is suggested. Modification of the culture system may eventually allow exploitation of the resources of the group effect or may create a physical environment where a similar effect for single embryos is also manifested.

Are co-cultures really needed?

Co-culture is becoming increasingly popular in human embryology (Desai et al., 2008; Mercader et al., 2006; Parikh et al., 2006; Dominguez et al., 2008). A recent meta-analysis of 17 prospective randomized trials has demonstrated that co-cultures increase blastomere numbers and implantation, clinical and ongoing pregnancy rates (Kattal et al., 2008).

The fact that monolayers from various sources (e.g. primary hepatocytes or even cell lines) can completely replace oviduct and uterine cells for co-culture purposes indicates that the factors — if really present and contributing in the improvement — are not reproductive tract-specific. One explanation for the beneficial effect is the decrease of the oxygen concentration in the medium or the elimination of other toxic factors (reviewed by Bavister, 1992; 1995; Donnay et al., 1997). For the former, a decrease of atmospheric oxygen concentration from 20% to 5% eliminated the need for co-cultures in cattle (Nagao et al., 1994; Trounson et al., 1994; Voelkel and Hu, 1992). For neutralization of other toxic factors, it may also play a limited role, as simplification of media by excluding unnecessary, potentially toxic chemicals has definitely contributed to the tendency to abandon co-cultures in domestic animals (Thompson et al., 2007).

Accordingly, co-cultures seem to be beneficial when the basic embryo culture is handicapped including the lack of

communal effect. Unfortunately, a high price has to be paid for the benefits. Co-cultures have serious disadvantages, including extra work and technical difficulties with standard establishment and handling, the risk of contamination, the possible involvement of animal or human serum in phases of isolation and culture; and the lack of appropriate control over factors involved. Moreover, just like serum, co-cultures were found to cause developmental abnormalities in cattle (Khosla et al., 2001b; Van Wagtendonk-de Leeuw et al., 1998). In the future, less complicated and more reliable techniques are expected to replace communal effect and to overcome problems for which co-culture seems to be the best solution today.

Oxygen concentration: 21% or 5%?

In domestic animals, considerable confusion was created in the early 1990s by the identical observation of three independent research groups (Nagao et al., 1994; Trounson et al., 1994; Voelkel and Hu, 1992) that co-cultures of embryos with somatic cells require atmospheric oxygen concentrations, while embryos without somatic cell support develop better in decreased oxygen. Various explanations of this phenomenon may exist, none of them was fully proven, and with the introduction of simple media and omission of co-cultures the whole problem has lost its significance. All successful research groups in cattle, pig, sheep and goat embryology use simple media with supplementation and 5% or 6% oxygen concentration, in complete accordance with the in-vivo observations. Oxygen concentrations in oviduct and uterus of all investigated species are considerably lower than the atmospheric oxygen concentrations (1.5—6% versus 21%, respectively). Unless convincing strong evidence occurs, the low oxygen concentration should be a principle for all mammalian embryo culture systems including humans (Meintjes et al., 2009b). This is one of the few questions were a definite answer is available and a worldwide consensus is being formed right now.

Further decrease of the oxygen concentration *in vitro* may have negative consequences. Oxygen at 2%, although leading to increased blastocyst rates, may cause developmental abnormalities in ruminants (Thompson and Peterson, 2000). On the other hand, there is no evidence that embryos need a continuous gas supply. A gas mixture volume of 50 ml in a closed system generously covers the requirements of 200 bovine embryos for 1 week, from the zygote to the blastocyst stage (Vajta et al., 1997).

Supplementation with macromolecules

Over decades, reports in both domestic animal and human fields were repeatedly published about completely defined culture systems that approach or even reach the overall efficiency of those supplemented by proteins.

The most frequently used chemically defined macromolecule supplement is polyvinyl-alcohol (PVA), as it provides surfactant activity similar to albumin (Thompson, 2000) and prevents gametes attaching to each other or any surface they contact (dish, pipette, tubes, etc.) during in-vitro manipulation. Another common feature is the maintenance of the colloidal osmotic pressure (Gardner, 2008). However, the physical characteristics are only one, and maybe not the most important, feature of albumin. It may support embryo development in many different ways, e.g. by modifying oxidation of pyruvate (Eckert et al., 1999; Lee et al., 1998). Albumin also acts as a carrier for vitamins, hormones, bioactive lipids and autocrine ligands (O'Neill, 2008). Additionally, albumin may neutralize some toxins occurring in the culture media (Gardner, 2008). Enrichment of media with more complex protein supplementation may result in further improvement in blastocyst and implantation rates (Meintjes et al., 2009a).

Albumin is the most common protein of the female genital tract fluids. This fact is often cited when compared with serum. On the other hand, the argument that serum is not physiological because embryos do not meet serum during preimplantation development is not really convincing: embryologists have welcomed many substances that help embryos to develop including EDTA and would welcome PVA if its supportive effect would be comparable to serum or albumin, although neither EDTA nor PVA are natural constituents of oviductal or uterine fluid.

In the early decades of embryo culture, serum was a common constituent of media. Its presence was especially required for co-cultures, as somatic cells need serum supplementation for healthy growth. However, since the late 1990s, serum has become the 'pathological fluid', a culprit that embryos should never meet during culture. Even short-term application was declared unwelcome.

There are serious reasons to support this opinion but the baby should not be thrown with the bath water, even if the analogy is rather frivolous in this context. As mentioned above, in sheep (and to a lesser extent in cattle) presence of serum was found to cause morphological alterations of preimplantation embryos and serious fetal, neonatal and post-natal abnormalities, summarized as large offspring syndrome. One of the most cited studies also involved application of 20% human serum in sheep (Thompson et al., 1995). However, Lawrence and Moley has stated recently that 'because of the change in media involved several variables, it was not possible to definitely prove that human serum was responsible for the large offspring syndrome of the offspring' (Lawrence and Moley, 2008). Additionally, large offspring syndrome occurs in sheep after in-vitro culture without serum, in the presence of PVA or bovine serum albumin as well (Rooke et al., 2006; Sinclair, 2008). Although Khosla et al. (2001b) have found an increase in developmental abnormalities in mice after culture in 20% serum, they also state that only few studies have dealt with this phenomenon, which is surprising compared with the extensive use of the mouse as the animal model for humans. A thorough search of the available literature proves that most direct evidence has been obtained from sheep (which is most probably predisposed to large offspring syndrome) or from a very limited number of studies performed in other species. Moreover, reviews often talk about general effects of serum without specifying the species of embryos, the origin of serum or the applied concentration.

The latter seems to be very important. All cited studies are based on very high (15—20%) concentrations of serum, disregarding other possibilities. However, the observations of O'Neill (1997) that nanograms of albumin are more efficient to support embryo development than the usual

mg/ml formulae may also be valid for serum, at least to some extent. Supplementation of modified synthetic oviduct fluid (SOFaaci) medium with 5% cattle serum allows blastocyst production from 50% of collected immature cattle oocytes after in-vitro maturation and fertilization. These blastocysts are morphologically similar to those developing *in vivo* and are without the pathological features (accumulation of lipid droplets, mitochondrial degeneration) observed after 15–20% supplementation. The cryotolerance of embryos cultured in 5% cattle serum was comparable with their in-vivo counterpart (Vajta et al., 1998). In pilot studies, no increase of large offspring after transfer of these embryos was observed (G Vajta et al., unpublished data; Lewis and Callesen, unpublished data). According to Thompson et al. (2007), patient serum was widely used in the early days of human IVF with no apparent dire consequences.

Application of serum for several embryological procedures including cryopreservation has been found beneficial and difficult to replace with additives of known composition. Unknown factors present in serum (e.g. endocrine ligands) may also have a supportive effect for embryos.

In some species (e.g. cattle, as discussed above), low concentration of serum may be a better choice than albumin while, in pig, addition of bovine serum albumin seems to be superior to serum (G Vajta, unpublished data). In the widely used human embryo culture systems, supplementation with human serum albumin seems to be appropriate. However, further investigations are required including critical overview of available data to find the optimal macromolecule supplementation. Eventually, serum or some of its constituents may become useful elements of many techniques in human embryology, and after thorough clarification of safety issues, the future application of these constituents as substitutes in embryo culture media cannot be excluded, either.

Sequential or single medium?

This is probably the most exciting question in human embryo culture today. Papers supporting either sequential or single media have been published repeatedly (Gardner and Lane, 1997; 2003; Gardner, 2008; Gardner and Lane 2007; Lane and Gardner, 2007; Pool, 2002; 2005; versus Biggers, 2001; Summers and Biggers, 2003; Biggers et al., 2005; Biggers and Summers, 2008; Sepúlveda et al. , 2008, see also direct arguments: Gardner and Lane, 2006 versus Biggers et al., 2006). While most human IVF laboratories use some kind of sequential media today, there is an increasing group of scientists preferring the latest versions of single media. This review refrains from making any general judgement. The discussion of the problem will be restricted to listing the published pros and cons (**Table 1**), to adding some new or usually disregarded points and to adding personal opinion where it seems to be relevant.

At present, the overwhelming majority of data used for development of commercially available human media are derived from experiments performed on murine embryos. On the other hand, successful embryo culture media have been developed in cattle and pig including SOFaaci (Holm et al., 1999) and the porcine zygote medium (PZM) family (Yoshioka et al., 2002), respectively. These media with serum or albumin supplementation are capable of supporting 60–90% blastocyst development from parthenogenetically activated in-vitro-matured oocytes. The common special features of these media include: (i) they are single media from zygote to blastocyst stage; (ii) none of them contains glucose or EDTA; and (iii) the potassium content is relatively high. As suggested by Bavister (2002) more intensive dialogue between human and domestic animal embryologists may eventually lead to useful changes in the composition of human media.

Data obtained from domestic animals may also help to establish a more balanced opinion regarding the need of medium change during preimplantation embryo development *in vitro*, especially in light of the fact that most human-research scientists completely disregard that neither SOF nor PZM requires medium renewal during the whole culture period (see below).

Renew medium or not?

In their extensive review listing many arguments for sequential versus single media, Biggers and Summers (2008) also mention the non-renewal of media as a possibility with potential benefits. However, in spite to their earlier good results in the mouse (Biggers et al., 2005), they seem to avoid dealing with the non-renewal in detail. The possibility has been listed in one of their tables, describing many positive features of this approach: (i) the embryos are left undisturbed; (ii) accumulated endogenous growth factors are left in place; (iii) the relative environmental stress is 'low' (iv) labour intensity is lower; (v) cost is lower; and (vi) less quality control is required. Two possible disadvantages are also mentioned: essential nutrients are not replaced and toxins may accumulate.

The former disadvantage should not be a major problem. If single media are accepted as appropriate for the full in-vitro period, the supply with nutrients is only the question of volume/embryo ratio. As mentioned earlier, according to data of Roh et al. (2008), Thouas et al. (2003), Vajta et al. (2001), murine and bovine embryos may achieve high blastocyst rates in medium volumes that are with an order of magnitude lower than those routinely applied in humans (0.5 μl and 1 μl per embryo during the whole culture period, for mice and cattle, respectively).

For the possible accumulation of toxic substances, the same publications may be referred to. Theoretically, materials with potential negative effect may originate from different sources, including dead embryos in communal culture, ageing of the medium, metabolism of healthy embryos and diffusion of toxic substances from the oil, atmosphere or plastic dish. Again, theoretically, medium exchange may keep accumulation of these toxins under control. However, the common laboratory practice does not support the idea. There are no data or signs that dying or dead embryos hamper the development of their neighbours. In an appropriate embryo culture system, the concentration of toxic substances derived from the environment should be basically low and their control should not require medium change. Regarding the metabolism of normal embryos and the spontaneous degradation of medium components, the

Table 1 Arguments for sequential media versus a single medium.

Sequential media	*Single medium*
Evidence	
In-vivo data obtained from humans show different concentrations of pyruvate, lactate and glucose in the oviduct and the uterus, changing also during the menstrual cycle (Gardner et al., 1996)	There are no direct data obtained from pregnant women (Biggers, 2001) The metabolism of the embryo is not strictly determined by the environment. *In vitro*, embryos may adapt to constant environment with their changing metabolism as long as the constituents fall between tolerable ranges (Biggers and Summers, 2008)
Carbohydrate metabolism	
In-vitro cleavage-stage mouse and human embryos use pyruvate as primary energy source, while glucose after the 8-cell stage (Leese and Barton, 1984; Leese et al., 1993). Pyruvate values in the oviduct are higher than in the uterus (Gardner and Lane, 1996) Glucose may have an inhibitory effect on development of cleavage-stage mammalian embryos including humans (Schini and Bavister, 1988; Conaghan et al., 1993). Accordingly, in the first interval, glucose should be reduced to 0.5 mmol/l or omitted completely (Pool, 2002; 2005)	Pyruvate in 2 mmol/l concentration seems to be sufficient to support embryo development *in vitro* (Biggers, 2001). There are no data about harmful effects of this concentration of pyruvate to further embryo development Glucose is present in a considerable concentration at any phase of the cycle in human oviduct and uterus (0.5 mmol/l and 3.15 mmol/l, respectively (Gardner et al., 1996). Complete omission of glucose is unlikely to benefit the embryo as glucose plays an important role in ribose synthesis, which essential in early development as well (Thompson, 2000). Media with up to 4.7 mmol/l glucose concentration for the entire culture period may support mammalian embryo development including humans (Summers and Biggers, 2003)
EDTA	
Ethylenediamine tetraacetic acid (EDTA) supports early embryo development to overcome the 2-cell block in mouse (Abramczuk et al., 1977) The generally acknowledged efficient concentration of EDTA (0.01 mmol/l) may compromise glycolytic activity in the inner cell mass; therefore it is potentially harmful for further embryo development (Lane and Gardner, 2001; Gardner and Lane, 2006)	The harmful effect of EDTA in 0.01 mmol/l concentration was demonstrated only in cell-free extracts. Direct evidence shows that 0.01 mmol/l EDTA during the whole culture period does not impair embryo development in mouse (Biggers et al., 2005; 2006)
Glutamine metabolism	
Ammonium accumulation in the medium, as the result predominantly of glutamine deamination, has been shown to induce exencephaly in mice (Lane and Gardner, 1994a,b) Addition of exogenous ammonium impairs embryo development and induces exencephaly (Lane and Gardner, 2003) When high concentration of ammonium was measured in the culture medium, human embryos had impaired development (Virant-Klun et al., 2006)	Occurrence of exencelopathy was never confirmed in the same system or in other animal models with similar glutamine concentrations (Biggers and Summers (2008)) Exogenous addition of ammonium may not follow the kinetics of glutamine breakdown (Biggers and Summers, 2008). Ammonium may be more harmful for early-stage embryos, while at the end of culture period when ammonium accumulation may occur, embryos are relatively insensitive (Zander et al., 2006) Glutamine can be replaced by stable dipeptides of glutamine which do not give rise to ammonium (Biggers et al., 2004) The volume of culture medium was unusually high (0.5 ml for small groups or single embryos) and was changed every day. It is not clear whether increased ammonium was the cause or the consequence of compromised embryo quality. The system does not seem to provide a strong argument for sequential media (G Vajta, unpublished data)

Table 1 (*continued*)

Sequential media	*Single medium*
Amino acids	
Non-essential amino acids stimulate cleavage between the zygote and 8-cell stage and consequently facilitate blastocyst formation and hatching (Gardner and Lane, 1993; Lane and Gardner, 1994) Essential amino acids that are in low concentration in oviduct appear to reduce cell numbers of blastocysts, by inhibition during the first 4-cell cycle (Gardner and Lane, 1993; Steeves and Gardner, 1999)	Human embryos make no distinction between essential and non-essential amino acids (Leese, 2003) It is prudent to add a full mixture of 20 amino acids, according to the composition of the tubal fluid (Leese, 1998; Tay et al., 1997)

only material that was supposed to have detrimental effect is ammonium: by replacing glutamine with stable dipeptides (Biggers et al., 2004), this danger can be safely eliminated.

In conclusion, a single medium without renewal may be a realistic alternative of the present culture systems. It may offer many practical benefits for embryologists and it may also help embryos to build up and maintain their microenvironment.

Alternative possibilities for embryo culture

In sharp contrast to the enormous efforts invested to improve culture media, surprisingly few attempts have been made to establish the appropriate tool to hold embryos during in-vitro development. One consequence of the adoption of monolayer culture systems for embryo culture was the acceptance of the two-dimensional approach, confirmed also by the stereo- and inverted microscopes. Although this approach has simplified all manipulations, it provided a suboptimal environment for globes that are completely surrounded *in vivo* by the microvilli of the oviductal epithelium. Until recently, attempts to exploit the potential benefits of a three-dimensional embryo culture system were sparse and none of the recently introduced alternative methods has obtained wide acceptance.

Tubes

The simplest three-dimensional culture system was applied first by Trounson and Conti (1982) and Bavister et al. (personal communication) by placing embryos in common test tubes for the whole culture period. Although the system was practical and easy to use, the lack of the possibility to visually follow-up embryo development was a major disadvantage for human embryologists. Very recently, a similar system with 250 μl PCR microtubes, 10 μl volume and 20 mouse embryos per tube and without oil overlay was published by Roh et al. (2008) and the obtained blastocyst rates were higher than in the traditional drop culture system.

Thompson (1996) have established a more sophisticated system with the definite purpose to improve efficiency. By using a modification of an early idea of continuous perifusion (Deter; 1977) a semi-automated perfusion culture system was created by culturing embryos in tubes. The system provided embryos the optimal culture medium at all phases of preimplantation development according to their changing metabolism and supposedly changing requirements. Unfortunately no significant increase in the embryo developmental rates was obtained. Subsequent versions of the system (summarized by Thompson, 2007) also failed to demonstrate benefits over the traditional culture methods and did not get wide acceptance for practical use.

Microchannel microfluidic system

Microfluidics is a multidisciplinary approach developed initially for inkjet printers in the 1980s but soon applied for various purposes in physics, chemistry, microtechnology and biotechnology, including the lab-on-a-chip for biotechnology. The principle is that no turbulence occurs in small channels (from 100 nm to several hundred m), i.e. the flow of solutions remains laminar and the components mingle by diffusion only. This special characteristic helps to control various manipulations. With the use of computer-controlled pneumatic valves and micropumps, a rather complicated system can be designed, resembling an electric integrated circuit, and the microchannels may be made suitable for various manipulations. Macroscopically, the device usually consists of a glass microscope slide base and a plastic (e.g. polydimethylsiloxane) layer with the channels and valves connected with outlets to mechanical or automatic pumps.

Microfluidics offer an excellent possibility to establish and maintain optimal microenvironment for embryos. However, further potential applications include almost all steps of human IVF, e.g. oocyte maturation, sperm selection, cumulus or zona removal, in-vitro fertilization, embryo culture with or without medium change and zona thinning. Furthermore, microfluidics are also capable for complicated tasks including fluorescence activated cell sorting (reviewed by Beebe et al., 2002; Smith and Takayama, 2007; Thompson, 2007; Vajta et al., 2004). As integration of the manipulations into a production line is also possible, there is still a theoretical but quite realistic chance of making complex procedures completely automated, including the whole human IVF procedure and even somatic cell nuclear transfer (Smith and Takayama, 2007; Vajta et al., 2004).

Although the first commercially produced versions of microfluidics are already available, the widespread application is hampered by simple technical problems (Thompson, 2007). Additionally, in the field of embryo culture, almost all published applications have exploited one of the most fascinating intrinsic potentials of the microfluidic technique,

the continuous or stepwise medium exchange. However, it may not be the optimal approach, as proven by the success of the glass oviduct and Well of the Well systems.

Glass oviduct system

The glass oviduct (GO) system (Thouas et al., 2003) can be regarded as an extremely simplified microfluidic device that provides a completely static environment. It is based on an open-ended 2 μl sterile capillary with 200 μm inner diameter. Loading of the embryos is performed manually by immersing one end of the capillary in a Petri dish containing the embryos in a standard microdrop system. While passing through the oil layer, a small oil column enters into the glass tube as the result of the capillary effect, followed by the medium and one or two embryos, for mice or cattle, respectively. Upon retraction of the capillary, a small oil column enters again; therefore, the approximately 1 μl of medium inside the capillary is separated from the atmospheric environment at both ends by oil. The capillary is inverted subsequently and cultured vertically for the entire culture period undisturbed in a carbon dioxide incubator. Comparing the system with a standard microdrop technique, similar blastocyst rates were achieved in mice, but the cell numbers and hatching rates were higher in the GO system. The efficiency of the technique was also confirmed in cattle, both for zona-intact and zona-free embryos cultured individually (Vajta et al., 2001; G Vajta, unpublished data).

Well of the Well system

An alternative solution to use the benefits of the three-dimensional arrangement is the Well of the Well (WOW) system (Vajta et al. 2000). It consists of a small microwell of conical shape, 200–300 μm diameter and depth, produced in a well of a four-well dish or in a Petri dish.

The system was originally established for in-vitro culture of zona-free cloned embryos to keep the blastomeres together before compaction. It was based on the darning needle hole system used previously for aggregation chimeras (Wood et al., 1993). However, the requirements of a short aggregation and a long embryo culture are different. The latter needs larger and deeper wells to avoid floating out of embryos and smooth walls to permit visual evaluation of their development. The possible repeated movements of the dish also require larger wells. These modifications were achieved first by melting the plastic (Vajta et al., 2000), later with a modified mechanical method (Booth et al., 2001; Vajta et al., 2005). With all these modifications, the first goal, i.e. culture of zona-free embryos from the zygote to the blastocyst stage) was fully accomplished and the system has contributed significantly in the success of the handmade cloning technique (reviewed by Lagutina et al., 2007; Vajta et al., 2005; Vajta et al., 2007).

Another application possibility of the WOW system is to use it for zona-enclosed embryos. Although it is an individual culture system, it is suitable to replace the communal effect. In cattle, culture of single embryos in WOW resulted in a considerable increase of blastocyst rates compared with drop culture (from 32% to 60%), and the results were similar to those achieved in communal culture (Vajta et al., 2000). Later studies have shown a dramatic increase in developmental rates of parthenogenetically activated pig embryos and increased speed of development of in-vivo-produced mouse zygotes (Vajta et al., 2008). A very recent study has demonstrated that gene expression patterns of bovine embryos cultured in WOW show closer resemblance to in-vivo-derived embryos than those cultured on flat surfaces (Ghanem et al., 2009). Similar microwell systems produced in agar gels have also been reported to be successful in various animal models (Peura and Vajta, 2003; Thompson, 2007).

The first human application has resulted in higher blastocyst rates when sibling embryos were cultured in WOW versus traditional culture (56% versus 37%, respectively). Pregnancy and birth rates achieved with embryos cultured in the WOW system are still preliminary but very promising (Vajta et al., 2008).

The mechanism explaining the successes of the GO and WOW systems still requires further clarification. The systems' common features are the small amount of medium surrounding the embryo and the static or semi-static arrangement. Both factors allow the establishment of a stable microenvironment. In the GO system, the embryos sink to the oil–medium border and are closely surrounded by 0.1–0.2 μl medium; the thin column in the capillary allows considerable concentration gradients for factors selected or eliminated by the embryos. The volume with WOW is approximately 0.05 μl. Although the system is open upwards, it may serve as a nest for the embryos, to establish and maintain the optimal microenvironment and to minimize the need of constant adaptation. It is also worth considering that the size and shape of the WOW may have considerable effect on the outcome: the deeper and narrower the WOW, the better embryo development can be achieved (Feltrin et al., 2006).

Various factors were supposed to contribute to the beneficial effect of an appropriate microenvironment (reviewed by; Gandolfi; Thompson, 2000; Vajta et al., 2000; Richter, 2008). An excellent detailed analysis of both communal culture and microclimate has been published by O'Neill (2008). He concludes that the communal effect may create both deleterious and beneficial effects, with a net beneficial outcome, and that much of the beneficial effect is accounted for by the action of autocrine trophic ligands in defined culture media. The effect can be induced by increasing the number of the embryos in a given medium volume or by decreasing the volume that surrounds the embryo(s). The review, based on his previous work, provides convincing evidence, and also a detailed analysis of the possible molecular mechanism of this autocrine ligand effect, showing that it may have a critical role – together with paracrine and endocrine factors – in embryo development *in vivo* as well.

The lack of a breakthrough with sophisticated perfusion systems, the successes achieved with simple static culture methods (GO, PCR tubes, WOW), the commonly acknowledged communal effect and its molecular explanation are all strong indicators of the importance of microenvironment. It cannot be further neglected in practical work. When designing embryo culture systems, a priority should be given to the maintenance of the milieu that embryos create themselves.

Table 2 Differences and similarities between embryos of some mammalian species.

Characteristic	*Mice*	*Cattle and sheep*	*Pigs*	*Humans*
Volume of the zygote, metabolic reserves[a]	5—8 times smaller than human	Similar to human	Similar to human	—
Cytoplasmic lipid content	Low	High	Extremely high	Moderate
Development to blastocyst	On day 4—5	On day 6—8	On day 5—6	On day 5—6
Embryo genome activation[b]	2-cell stage	8—16-cell stage	4—8-cell stage	4—8-cell stage
Amino acid metabolism[c]	Different from humans	Different from humans	Similar to humans	—
Pyruvate/lactate versus glucose[d]	Switches to glucose at 48 h	No absolute need for glucose before hatching	No absolute need for glucose before hatching	No absolute need for glucose before hatching
Overall sensitivity *in vitro*	Low	High	Extremely high	High
Genome sequencing	Completed	Proceeding	Almost completed	Complete
Genome structure[e]	Far from humans	Unknown	Close to humans	—
Demethylation and methylation during early embryo development[b]	Extensive	Considerable (cattle), moderate (sheep)	Moderate	Probably moderate
Time and location of embryo transfer[a]	Exact match required	Exact match required	Flexible	Flexible
Developmental anomalies after in-vitro culture	May occur	Frequent and serious	Very rare	Very rare

[a] Leese, 2003.
[b] Duranthon et al., 2008.
[c] Booth et al., 2005.
[d] Bavister, 1995; Vajta et al., 2004
[e] Wernersson et al., 2005.

Alternative animal model

Many practical characteristics predispose the mouse to become the animal model for almost anything in mammalian biology. Accordingly, selection for early embryological research was evident and has led to fundamental discoveries. Quinn and Horstman (1998) have acknowledged its use as model animal for human embryology, justifying the practice of the previous decades. However, the fact that almost everything that is going on today in human embryo culture is based on murine experiments may be an exaggeration of the relevance and applicability of this species (Betteridge and Rieger, 1993; Ménézo and Hérubel, 2002). Recent research has proven the existence of fundamental differences in culture requirements between mammalian species. Although appropriate embryo culture is available only for a handful of these species, a comparative analysis of relevant factors may demonstrate that some of them may be more suitable for special purposes of embryology research. The main evaluation points are summarized in **Table 2**.

According to these data, pig should be considered as an alternative animal model for human embryo culture. This suggestion meets the overall tendency in medical research (Vodicka et al., 2005). Moreover, during the past 5 years, the efficiency of porcine embryo culture has improved exponentially, mainly due to changes that have also increased efficiency in humans, but without sequential culture and without medium change. Today, blastocyst rates with parthenogenetically activated in-vitro-matured oocytes may reach 60—90% (Vajta et al., 2005). Finally, some common features between mice and pigs (relatively short pregnancy

compared with the size of the latter species, large litter, relatively low costs and experience in breeding) and the easy access to oocytes by using slaughterhouse-derived ovaries also support the feasibility of changing the model system, or at least involving pigs in tests when improvements of human embryo culture are attempted.

Quietness versus stress

In 2002, in a much cited paper, Leese launched the following hypothesis: '... preimplantation embryo survival is best served by a relatively low level of metabolism; a situation achieved by reducing the concentrations of nutrients in culture media, and encouraging the use of endogenous resources'. According to the author (and supported by many previous observations), the consequence of in-vitro embryo culture is increased metabolic activity and compromised developmental competence, even if the final consequence is manifested only in the fetal or post-natal phase. Qualitative assessments including higher glycogen content, reactive oxygen species formation and glucose consumption *in vitro* support the hypothesis. On the other hand, reduction of metabolism by specific agents including low atmospheric oxygen and chemicals blocking oxidative metabolism improve development (Gardner, 2008). It has also been observed that human embryos with reduced amino acid turnover have higher potential to develop to blastocysts (Houghton et al., 2002).

Another observation also supports the 'quiet embryo' hypothesis. Ovaries and Fallopian tubes seem to be considerably cooler than the surrounding tissues, and the difference is greater around ovulation and around mating (Hunter and Nichol, 1986; Hunter et al., 2000; Hunter et al., 2006; Leese et al., 2008b). The mechanism of this strange cooling mechanism inside a warm body requires further research, but the consequence of the lower temperature is most probably a decreased rate of embryo metabolism, and possibly a better embryo quality.

For the practical work in human embryology, this observation may indicate that a radical change is needed in the temperature of all incubators, workstations, heated stages, dishes, tube heaters etc. These instruments are now scrupulously adjusted to 37°C, the routine calibration and control is one of the most important quality assurance activities in a human assisted reproduction laboratory. A preliminary study compared blastocyst development in cattle at 37°C with the conventional 39°C and resulted in the same blastocyst rate but lower amino-acid turnover, a quieter state that may be interpreted as a more in-vivo-like status (Sturmey et al., unpublished; cited by Leese et al., 2008b).

The 'quiet embryo' theory has, however, been challenged by a series of recent observations. Based on the experiments of Wemekamp-Kamphuis et al. (2002) in bacteria, gametes and early embryos were exposed to high hydrostatic pressure (HHP) to induce a sublethal stress. The level of pressure varied between species and developmental phases, but was extremely high (between 20 and 80 MPa) compared with the pressures these cells meet under physiological circumstances. After exposure for 1 or 2 h and a

Table 3 Achievements with sublethal stress to induce stress tolerance in gametes and embryos.

Sample	*Stress*	*Consequence*	*Reference*
Mouse in-vivo-derived blastocysts	HHP60 MPa, 30 min	Improved development after slow-rate freezing	Pribenszky et al., 2005a
Bovine in-vitro-produced blastocysts	HHP 80 MPa, 45 min	Improved development after slow-rate freezing	Pribenszky et al., 2005b
Bovine in-vitro-produced blastocysts	HHP 60 MPa, 60 min	Improved development after vitrification	Pribenszky et al., 2007a
Porcine ejaculated semen	HHP 30 MPa, 90 min	Increased post-thaw motility and integrity	Pribenszky et al., 2005c
Porcine ejaculated semen	HHP 30 MPa, 90 min	Increased litter number after AI	Pribenszky et al., 2008a
Porcine ejaculated semen	HHP 30 MPa, 90 min	Increased litter number after cryopreservation and AI	Kuo et al., (2007)
Bovine ejaculated semen	HHP 30 MPa, 90 min	Increased post-thaw motility and integrity	Pribenszky et al., 2007b
Porcine in-vitro-matured oocytes	HHP 20 MPa, 60 min	Increased development after vitrification and PA	Pribenszky et al., 2008b; Du et al., 2008a
Porcine in-vitro-matured oocytes	HHP 20 MPa, 60 min	Increased development after vitrification and SCNT cloned offspring born	Du et al., 2008b
Porcine in-vitro-matured oocytes	1% (v/w) extra NaCl added, 60 min	Increased development after PA or SCNT	Lin et al., 2009a
Porcine in-vitro-matured oocytes	Osmotic stress with sucrose and trehalose, 60 min	Increased development after PA or SCNT	Lin et al., 2009b

AI = artificial insemination; HHP = high hydrostatic pressure; PA = parthenogenetic activation; SCNT = somatic cell nuclear transfer.

recovery period, gametes and embryos presented an improved performance (increased fertilizing ability, developmental competence after activation, IVF or somatic cell nuclear transfer, increased cryotolerance, etc.). Later, similar results were achieved by applying osmotic stress instead of HHP. The detailed discussion of these observations exceeds the scope of this review; the published achievements are summarized in **Table 3**. According to the preliminary investigations performed in cattle, mice and pigs, the treatment had no long-term consequence on sex ratios, malformations, bodyweights and other detectable characteristics of offspring.

Very recently, independent research has led to a similar conclusion: elevated temperature applied for hours after either in-vitro fertilization, parthenogenetic activation or somatic cell nuclear transfer has dramatically increased the in-vitro developmental potential of porcine embryos (Isom et al., 2009). The earlier observation of Leach et al. (1993) regarding the supportive effect of short ethanol treatment to the development of mouse embryos *in vitro* may also be related to the events observed after HHP, osmotic stress or elevated temperature.

The phenomenon that a sublethal stress induces a response with temporary increase of a general, aspecific resistance to various further stresses has been observed in almost all levels of life, from bacteria to multicellular organisms including humans (general adaptation syndrome; Selye, 1998). On the cellular level, the reaction incorporates sensing, assessing and then counteracting stress-induced damage, consequently increasing temporary tolerance of such damage (Kültz, 2005). Factors that are involved in the key functions of stress response are conserved in all cells and participate in cellular functions including protein, DNA and chromatin stabilization and repair, cell cycle control, redox regulation, energy metabolism, fatty acid/lipid metabolism and elimination of damaged proteins (Kültz, 2003). Available data are still insufficient to give a detailed explanation of which factors are involved in the stress response of gametes and embryos. Nevertheless, it is rather surprising that, in spite of the relative silence in transcription and translation in gametes and early embryos, they can react so rapidly and efficiently to stress.

In summary, controversial observations have created a new question: to stress or not to stress? The right answer might be that in general, stress, especially in uncontrolled and continuous form, may cause serious harm and should be avoided. However, there are some situations where a well-defined and properly applied stress may help gametes and embryos to increase their tolerance towards other stresses, including those created during laboratory procedures. Thorough investigation of the molecular basis and safety of the procedures are still required, but eventually HHP, osmotic stress or other treatments with the same principle may improve the overall efficiency of assisted reproductive techniques.

Conclusion

Based on the impressive achievements during the past decade, there is an increasing, although rarely declared, doubt about whether the present efficiency of in-vitro embryo culture can be considerably expanded in the future. The basis of this opinion is that there are natural limits of advancement and that, essentially, the laws of nature cannot be crossed.

As demonstrated by the present review, the authors respectfully disagree with this opinion. Present embryo culture systems miss the consensus and solid scientific evidence in basic elements, accordingly open debates challenging the currently applied principles may eventually result in considerable improvements. On the other hand, as mentioned above, there are proven possibilities outside the present frameworks that may result in radical changes in both quantitative and qualitative efficiency. To refer the relevant words of one of the most creative experimental embryologists, Steen Malte Willadsen: 'The role of the scientist is to break the laws of nature, rather than to establish, let alone accept them.' (Silver, 2007).

Acknowledgements

The authors thank the sharp and most relevant critical remarks of Professor Ronald HF Hunter regarding an earlier version of this manuscript. They hope that this work now more or less meets his high standards.

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Declaration: Gabor Vajta is a minority shareholder in the company that produces tools for HHP and WOW studies. Other authors declare no commercial interest.

Received 19 May 2009; refereed 20 July 2009; accepted 9 December 2009.