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Embryo culture medium: which is the best?

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With the growing move in in-vitro fertilization (IVF) clinics to transfer fewer embryos to women, there is an increasing reliance on the IVF laboratory to maximize embryo viability. Subsequently, there is justified scrutiny on the culture system and the media used to sustain the human embryo in vitro. The transfer of fewer embryos to patients also creates an increased dependence on the ability to cryopreserve embryos successfully. Therefore, in addition to the ability of a culture system to produce a single top-quality embryo for transfer, it is also necessary to enhance the cryotolerance of sibling embryos so that they can survive freezing or vitrification. Therefore, when examining which culture media is the best, it is prudent to not only examine the ability of a culture system to produce a pregnancy with the one or two highest-grade embryos, but also to determine how many embryos from the entire cohort (both fresh and frozen embryos) are capable of producing a live birth. Additionally, research on animal models has demonstrated that stress, and the resultant adaptation to conditions during pre-implantation stages, can affect pregnancy loss and fetal growth. It is therefore important to understand the role of each medium component and to identify possible sources of cellular stress to the embryo that will ultimately affect the function and viability of the conceptus.

Key words: blastocyst; viability; IVF; culture media.

Since the establishment of in-vitro fertilization (IVF) technologies worldwide, a large number of different media formulations and culture systems have been employed for the development of the human embryo. These media have highly varied

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formulations, ranging from simple salt solutions to complex tissue culture media. In the last decade, there has been a substantial increase in knowledge regarding the physiology, biochemistry, genetics and epigenetic control of the mammalian embryo, including several studies performed directly on the human embryo. This advance in our understanding of the pre-implantation embryo has led to significant changes in the philosophy of how best to culture the human embryo, and as a result, there has been significant evolution in the culture media used in clinical IVF.[1–4]

A major challenge facing IVF clinics around the world is to address the epidemic of multiple pregnancies that result following IVF.[5] This has resulted in an increased focus in the last decade on how best to culture the human embryo and which culture media to choose as clinics move towards transferring fewer embryos whilst striving to maintaining acceptable pregnancy rates. In fact, it is now possible to transfer a single embryo to many patient groups while maintaining acceptable pregnancy rates and eliminating multiple pregnancy and its associated complications (such as the increase in premature birth and extremely low birth weight, and the six- to seven-fold increase in cerebral palsy).[6] This review will identify the different types of media systems that are available for growing human embryos in culture, and also focus on important aspects that need to be considered in determining which media system to use. However, it is essential to realize that the culture medium is only one part of the culture system, which is only one component of the laboratory system and clinical management protocol. The developmental competence of embryos can be influenced significantly by other aspects of the culture system, such as gas phase, both carbon dioxide and oxygen levels, and physical factors such as the number of incubator chambers. Several of these aspects have been reviewed extensively elsewhere.[2,7,8]

AT WHAT STAGE SHOULD THE EMBRYO BE TRANSFERRED?

At what stage should embryos be cultured in the laboratory? This is an important consideration as different media systems are designed to support embryo development at different stages. Historically, human embryos have been cultured for 2 or 3 days before transfer to the mother. This is still the preferred procedure for a large number of clinics internationally. However, it is now clear that blastocyst culture and transfer is an important treatment component in an increasing number of clinics.[4,5,9–13] In the authors' clinics, a mixture of cleavage-stage and blastocyst transfers are performed, after the clinicians have identified which patients would best benefit from a cleavage-stage transfer and which from a blastocyst transfer. Therefore, the systems used in the authors' laboratory must be able to support viable cleavage-stage embryos and blastocysts. The use of sequential culture systems gives one the flexibility to use systems that can support cleavage-stage development and optimal subsequent blastocyst development.

DOES THE COMPOSITION OF THE MEDIUM REALLY MATTER?

There has been a long-held view that the choice of culture media for the cleavage-stage embryo is not important due to the fact that many different media have been used with dramatically different formulations. However, it is now apparent that resultant implantation and pregnancy rates are different. Interestingly, there is often a focus on the ability of a given culture system to generate large numbers of blastocysts without assessment of the ability of a culture system to maintain cellular

physiology, gene expression and normal developmental regulation. Significantly, there is an increasing volume of information being generated that challenges the dogma that media formulations do not matter which has revealed that the cost of adaptation in the early stages of development is impaired pregnancy potential. Furthermore, prior to compaction, the embryo is at increased risk for cellular damage, and many of these changes in physiology and gene expression are not evident until later in development. Studies in animal models show that exposure of embryos to suboptimal culture conditions, such as media lacking amino acids or a gas phase comprising 20% oxygen, during the precompaction stages alone has significant effects on postimplantation development and fetal growth following blastocyst transfer.[14–16] Similarly, it has been demonstrated that methylation patterns in the embryo can be altered as early as the two-cell stage, and that gene expression in the blastocyst is affected by the conditions to which the early embryo is exposed.[14,16,17] This is a significant finding as there are now reports of an increase in the incidence of disorders associated with imprinted genes after IVF. The majority of these cases have occurred following transfer of cleavage-stage embryos[18–20] and not solely at blastocyst stage. Therefore, while the early embryo appears to have a great deal of plasticity, it is increasingly recognized that imposing stress on the embryo results in disruption of the co-ordination of developmentally important functions, which results in a loss in viability; this is particularly evident in the precompaction-stage embryo (Figure 1). As such, it is essential that the conditions in which the embryo is grown minimize stress on an embryo and take into account the physiology of the embryo, thus preventing the need for the embryo to adapt, which could affect subsequent embryo health and developmental potential. To reiterate, even if an IVF unit either completely or predominantly performs cleavage-stage embryo transfers, it is essential that there is knowledge of the components and an understanding of the philosophy of how the culture system was developed.

DEVELOPMENT OF MEDIA SYSTEMS

Historically, human embryos were cultured in either simple salt solutions or in complex tissue culture media. The 'simple' media were those such as Earle's, T6 and HTF medium consisting of balanced salt solutions with added carbohydrates glucose, pyruvate and lactate, and commonly supplemented with the patient's serum.[21–23] These media lacked many components that are now known to be important for the maintenance of embryo physiology and health, such as amino acids (see below). Alternatively, embryos used to be grown in tissue culture media. Such media

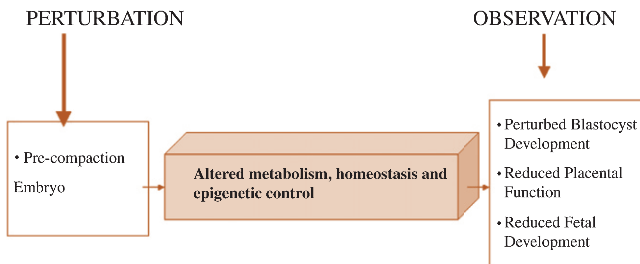

Figure 1. Exposure of pre-compaction stage embryos in a suboptimal environment results in adaptations in cellular physiology that are manifest at later stages of development and adversely affect blastocyst health and pregnancy outcome.

were designed to support immortal cell lines in culture and were far more complex, being supplemented with carbohydrates, amino acids, vitamins, nucleotides and metal ions. Examples of such media are Ham's F-10, MEM and TCM-199.[24,25] Importantly, none of these media were designed to support embryo development, and they actually contain many components that have subsequently been shown to be detrimental to embryo development in vitro, such as high levels of glucose, divalent metal ions, nucleotides and certain hormones.

Although these different media support the development of the human embryo through the first three cleavage divisions, they are not able to support acceptable levels of blastocyst development. Consequently, it was necessary to transfer the embryos to the female reproductive tract at the cleavage stages on day 2 or day 3 of development. However, in recent years, there has been the change in the way that media are formulated, and several media have been designed specifically to support development to blastocyst stage, such as DM1/2/3, G1/G2 media, KSOM and Quinns Advantage.[26–28]

Until relatively recently, it was common practice for clinics to prepare their own culture media in house, either by making them from individual chemicals in the case of simple media or by reconstituting preformulated powder in the case of tissue culture media. However, a significant change has occurred over the last decade with a major shift away from 'in-house' media preparations to commercially produced media manufactured specifically for use in clinical IVF applications. The ability to purchase aseptically produced media, formulated in a specialized factory under good manufacturing procedures and which has undergone regulatory and quality control inspection, is an attractive alternative for all IVF clinics. Only a few specialized clinics have the capacity to match the level of quality control that commercial media undergoes. Interestingly, this move to commercially produced media has also resulted in a convergence of the formulations used for clinical IVF worldwide. Currently, there are essentially two types of media that are widely used for clinical IVF: sequential culture systems or monoculture systems. Some of the scientific basis for these two different styles of media systems are discussed below, and knowledge of media components and ability to support normal embryo development is reviewed.

DYNAMIC PHYSIOLOGY OF THE PRE-IMPLANTATION EMBRYO

The pre-implantation embryo is a highly dynamic period during which the pronuclear stage embryo develops from the relatively quiescent oocyte under the genetic control of maternally derived transcripts, to a metabolically and biosynthetically active group of cells predominantly under their own genetic control by the blastocyst stage. Furthermore, the oocyte, and pronuclear and cleavage-stage embryos appear to resemble unicellular organisms in that they possess relatively primitive mechanisms for the regulation of cellular homeostasis.[29,30] A series of highly co-ordinated developmental events then occur in a period of 4–5 days. Firstly, there is completion of the sperm-derived calcium waves initiates oocyte activation[31–34], followed by the first cleavage division some 21 h later.[35] Subsequently, cleavage divisions occur every 12–15 h, while there is gradual activation of the embryonic genome.[36] On day 3 of development, compaction occurs[37] and finally the blastocoel is formed[38] with the differentiation of the inner cell mass (ICM) and the trophectoderm. As a result of such changes, there are different demands and requirements as the embryo develops and

Table 1. Differences in the physiology of the mammalian embryo for development from the zygote to the blastocyst stage.

Precompaction stage	Postcompaction stage
Low biosynthetic activity	High biosynthetic activity
Low QO_2 (Metabolic Quotient)	High QO_2 (Metabolic Quotient)
Pyruvate-based metabolism	Glucose-based metabolism
Maternal genome	Embryonic genome
Single cell	Transporting epithelium
Low ability to maintain cellular homeostasis	Complex systems for maintenance of cellular homeostasis
Totipotent	Differentiation into inner cell mass and trophectoderm

differentiates, which require the precise regulation of many cell functions such as cellular homeostasis, metabolism and gene expression (Table 1).

Dynamics of pre-implantation embryo metabolism

Studies on mammalian embryos, including the human embryo, have determined that the morphological changes that occur during development from the zygote to the blastocyst stage occur concomitantly with dynamic changes in the metabolism of the embryo. At the zygote stage, the embryo is initially quiescent with a low metabolic and biosynthetic activity. At these early stages, the embryo predominantly uses the carboxylic acids pyruvate and lactate as its preferred energy substrates[39–41], together with specific amino acids, such as aspartate.[42] Therefore, at these early stages of development, the embryo is completely reliant on mitochondrial-based metabolism for ATP generation. However, the embryo does take up low levels of glucose[39,40], which are thought to be metabolised by the pentose phosphate pathway for the maintenance of reducing power in the cell, particularly in the production of reduced glutathione for protection against oxidative stress, in nucleic acid and lipid biosynthesis. Furthermore, there is a more recent understanding that the metabolism of glucose by the hexosamine biosynthesis pathway in the early embryo may be important in establishing O-linked glycolyslation for later stage development.[43] Therefore, it may be that this metabolism of glucose, although at a low level during the early stages of pre-implantation development, is essential for setting the subsequent metabolic programme.

With the onset of embryonic genome activation and the concomitant increase in biosynthesis, there is a switch in nutrient preference to a glucose-based metabolism, so that by the blastocyst stage, glucose has become the preferred nutrient.[39,40] However, unlike somatic cells that metabolise virtually all of the glucose taken up via the tricarboxylic acid (TCA) cycle, glucose taken up by the blastocyst is metabolised by both the TCA cycle and by aerobic glycolysis (approximately 30–50% of all of the glucose being converted to lactate).[44,45] This unusual conversion of glucose to lactate in the presence of significant levels of oxygen is thought to result from the high biosynthetic demand[46], or as a result of an inability to maintain redox control in the cell, as reported for other rapidly dividing cells. Interestingly, there appears to be a difference in the metabolism of the ICM cells of the blastocyst compared with the cells of

the surrounding trophectoderm. ICM cells have been shown to be completely reliant on glycolysis for their energy, and appear to have limited functional oxidative metabolism. In contrast, trophectoderm cells metabolise the majority of glucose through oxidative-based metabolism.[47,48]

Significantly, the nutrients available within the human female reproductive tract mirror the changing nutrient preference of the developing embryo. At the precompaction stages when the embryo resides in the oviduct, the fluid within is characterized by relatively high concentrations of pyruvate and lactate, and a relatively low concentration of glucose (Table 2). In contrast, uterine fluid is characterized by relatively low levels of pyruvate and lactate, and a higher concentration of glucose.[49,50] Therefore, at the time that the embryo is in the reproductive tract, there is a declining gradient of pyruvate and lactate from the oviduct to the uterus, while the reverse is true for glucose. These changing levels of carbohydrates reflect the switch from a carboxylic-acid-based metabolism to a glucose-based metabolism in the embryo.

Changes in cellular homeostasis

In addition to the metabolic changes that occur in the embryo, there are significant changes in the ability of the embryo to regulate ionic homeostasis as development proceeds. The oocyte and early embryo in the first few hours following egg activation have no active transport systems for the regulation of intracellular pH.[30,51,52] Transport systems for regulating pH are not detected until 6–8 h after fertilization and are not completely functional until around 10 h after fertilization. In some mammalian species, the transport systems for regulating an acid load function are either reported to be absent or are unable to restore pH and the embryo cannot recover completely from a pH stress.[30,51–53] Additionally, embryos that are collected from the reproductive tract in the first few hours after egg activation also appear to have a reduced ability to regulate intracellular calcium levels compared with embryos collected 9 h following egg activation.

An increasing ability of the embryo to regulate ionic and metabolic control as development proceeds can be observed by the increased developmental capacity in vitro when embryos are collected at later stages from the reproductive tract. It is clear that the earlier an embryo is collected from the tract, the more susceptible it is to its environment. As a result, the culture media for the early embryo must be able to support the embryo in its ability to maintain homeostatic functions (Figure 2).

Table 2. Differences between oviduct and uterus in mammalian embryos.

Component	Oviduct	Uterus
Glucose concentration	0.5 mM	3.15 mM
Pyruvate concentration	0.32 mM	0.10 mM
Lactate concentration	10.5 mM	5.2 mM
Oxygen concentration	8%	1.5%
Carbon dioxide concentration	12%	10%
pH	7.5	7.1
Glycine concentration	2.77	19.33
Alanine concentration	0.5	1.24
Serine concentration	0.32	0.80

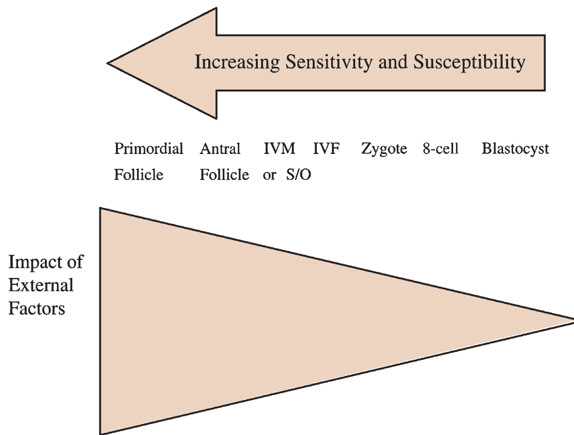

Figure 2. The early stages of development, when the oocyte and early embryo have primitive mechanisms for maintaining metabolic homeostasis, are more susceptible to stress. Therefore, there is a greater reliance on external factors to maintain homeostasis and developmental competence at the early stages of embryo development. When these precompaction-stage embryos are placed in culture, there is an increased reliance on the components of the culture to provide this protection. S/O=superovulation.

Therefore, regulators of homeostatic functions such as amino acids that can protect against ionic and pH stress (see below) are essential components of culture medium for the precompaction-stage embryo.

SIGNIFICANCE OF COMPACTION IN REGULATING THE PHYSIOLOGY OF THE PRE-IMPLANTATION EMBRYO

It is clear that one of the most significant events to occur during the development of the early embryo is the process of compaction and the formation of a transporting epithelium. At this time, the embryo changes from a group of individual cells to a structure containing a functioning epithelium that enables the embryo to control its physiology and to create a specific environment around the ICM. An example of this is the increased ability of the postcompaction-stage embryo to regulate its internal environment against ionic stress. Precompaction-stage embryos take up significant levels of the amino acid glycine in response to an increase in salt concentration in the medium.[29,54,55] However, after embryos have compacted, their reliance on external osmolytes to regulate ionic stress is reduced significantly.[29] Similarly, the postcompaction-stage embryo has an enhanced ability to regulate intracellular pH. The pre-compaction stage embryo has a low capacity to regulate intracellular pH when challenged with an acid load. However, after compaction, the embryo is able to maintain normal levels of intracellular pH when challenged with an acid stress. Significantly, when embryos were decompacted experimentally by exposure to medium lacking calcium and magnesium, the ability to regulate intracellular pH was lost.[56] To reiterate, the process of compaction, with the formulation of the tight junctions, facilitates the development of a functioning transporting epithelium which helps to regulate ionic homeostasis, thereby making the embryo less sensitive to its environment and hence less susceptible to trauma.

DEVELOPMENT OF CULTURE MEDIA/RELIANCE ON ANIMAL MODELS

One of the limitations in the development of culture media for the human embryo is the inability to perform empirical studies on the human embryo or to perform large-scale controlled studies on individual medium components. As a result, the majority of what is known about the mammalian pre-implantation embryo comes from research conducted on animal models. The most commonly used models for the human embryo have been the mouse and the cow. Subsequently, strong opinions have been penned regarding which model might be the most appropriate.[57] However, as with all animal research, models have both benefits and limitations, and therefore it is proposed that no single species may provide an appropriate basis for the backbone of a clinical media system. Rather, a mixture of models have been used that can determine common traits pertaining to the physiology of the mammalian embryo. One example of this would be the observation that amino acid requirements change as the embryo develops from the cleavage stage to the blastocyst stage; this was demonstrated in several animal models, specifically the mouse[58], the cow[59] and the sheep.[60] As a result of these commonalities, a biphasic exposure to amino acids has been factored into many media system formulations for the human embryo. It was subsequently demonstrated that amino acid requirements of the human embryo are similar to those of other species.[61]

As the majority of basic research is performed on animal models, it is important to critically assess the endpoint used as the criteria to assess the effects of a medium component. It is now acknowledged that blastocyst development, especially at a single timepoint, is a relatively poor indicator of embryo quality. Therefore, it is important that the endpoints used in basic research reflect subsequent viability of the embryo, such as the determination of the ability of resultant embryos to implant and develop into a normal fetus after embryo transfer. For example, many embryos may be able to form blastocysts under specific conditions, but they could have fewer cells and perturbed cell signalling and nuclear function that is incompatible with subsequent development. Several components of a culture medium, that do not necessarily affect blastocyst development, can have significant effects on the viability and postimplantation development of the fetus.[14,62] Some culture systems have been formulated solely on the basis of the ability of a culture medium to support delayed development to the blastocyst stage, and were never assessed in terms of their ability to produce blastocysts of normal physiology or on pregnancy outcomes, e.g. the medium KSOM. In contrast, we have used numerous markers of cellular health and physiology, such as metabolic and homeostatic control, differentiation and gene expression, and assessment of viability in multiple species, on each individual component as well as the final medium formulations in the development of our sequential culture media G1/G2.

KEY COMPONENTS OF CULTURE MEDIA

Several extensive reviews have focused on the individual components present in the majority of embryo culture media.[21,63,64] This review will highlight important components that differ between media systems. Understanding their effects on embryo physiology may alter the choice of which culture system to use in a clinic.

It is also important to consider that sequential media were developed specifically to work together, and that mixing different pairs of media from different sequential systems is of limited value and could conceivably induce some homeostatic stress that may influence pregnancy establishment and health.

Glucose

Over several years, a number of publications reported that the addition of glucose to the culture medium for the mammalian embryo resulted in impaired development to the blastocyst stage. Studies on the mouse[65], hamster[66], sheep[67], cattle[68] and human[69] have all demonstrated that a high level of glucose in culture is responsible for retardation or developmental arrest of cleavage-stage embryos. However, such observations appear to contradict the situation in vivo where glucose is present in the reproductive tract, and the oocyte and the embryo have transporters for glucose at all stages of development.[70] Significantly, this apparent toxicity of glucose appears to be manifest in medium containing phosphate and typically lacking amino acids. Embryos cultured in the presence of high levels of glucose and phosphate had reduced respiratory capacity, and mitochondrial function appeared to be reduced.[71] This reduction in metabolic control culminated in a loss in ATP production and therefore developmental arrest. Subsequently, several culture media have been designed to support the cleavage-stage embryo, in which either glucose alone or both glucose and phosphate were removed from the formulation.

In the following decade, several studies determined that this inhibitory effect of glucose in the presence of phosphate could be alleviated by the presence of amino acids, EDTA or vitamins.[26,45] The addition of amino acids, vitamins or EDTA to the culture medium containing glucose and phosphate helped to prevent a loss in respiration and metabolic control. Embryos were therefore able to maintain a more normal metabolism and adequate ATP production for development to proceed. Interestingly, and perhaps more significantly, it was determined that the lack of glucose in the media for the postcompaction-stage embryo resulted in impaired development to the blastocyst stage and a loss of viability.[26,72] Therefore, the observed inhibitory effect of glucose appears to be an in-vitro-induced artifact that is manifest as a result of lack of other important regulators in the medium.

Amino acids and ammonium

Among the most important components of all handling and culture media for the mammalian oocyte and pre-implantation embryo is amino acids. Studies on the embryos from all mammalian species to date, i.e. mouse[26,62,73–76], hamster[77,78], sheep[60,79], cow[59] and human[61], have all demonstrated that the inclusion of amino acids in the culture medium enhances embryo development to the blastocyst stage and increases subsequent viability. Even a transient exposure (less than 5 min) of mouse zygotes to medium lacking amino acids impairs subsequent developmental potential.[26] This has implications for all media for the handling and collection of oocytes and embryos.

Oviduct and uterine fluids contain significant levels of free amino acids[80], while both oocytes and embryos possess specific transport systems for amino acids[81] to maintain an endogenous pool.[82] As amino acids are readily taken up and metabolised by the embryo, the above information supports the notion that amino acids have a physiological role in the pre- and peri-implantation periods of mammalian embryo development.

Significantly, amino acids have now been shown to have many important roles in culture media, including chelators, osmolytes, pHi buffers, antioxidants, regulators of energy metabolism, biosynthetic precursors and energy substrates[4], confirming the above hypothesis.

The utilization of amino acids by the early embryo has been demonstrated to change as the embryo develops from the precompaction stage to the postcompaction stage. Up to the eight-cell stage, those amino acids present in relatively high levels in oviduct fluid (which are also present in the group defined as non-essential, together with glutamine), increase cleavage rates[58,59] and viability. However, after compaction, non-essential amino acids and glutamine increase blastocoel formation and hatching, while those amino acids classified as essential amino acids are required for ICM development and viability.[62] In the mouse, equivalent implantation rates to in-vivo-developed blastocysts were achieved when pronucleate embryos were cultured with non-essential amino acids to the eight-cell stage followed by culture with all 20 amino acids from the eight-cell stage to the blastocyst.[58]

Furthermore, as well as a reduction in blastocyst development and viability, it has also been demonstrated that the absence of amino acids in the culture medium significantly alters blastocyst gene expression[83], and significantly disrupts the normal imprinted expression of H19 with high proportions of blastocysts demonstrating bi-allelic expression.[84] The observation that a change in the culture system can significantly alter methylation status and chromatin silencing is significant, especially if examined in the context of the reported increase in imprinted gene disorders after IVF.[19,85,86] Significantly, the majority of reported imprinting disorders following human assisted reproductive technology (ART) are the result of day 2 or day 3 transfers, although cases have been reported following blastocyst transfer.[85] However, cases of abnormal human imprinting in resultant offspring have also resulted following ovulation induction alone, i.e. without any laboratory treatments. Therefore, the importance of understanding how ovarian stimulation and culture systems affect the physiology of the oocyte and resultant embryo are increasingly important.

Ammonium

One of the consequences of having amino acids, particularly glutamine, in the culture medium is that they breakdown spontaneously at 37 °C to produce ammonium.[74,87] The levels of ammonium that are produced by medium containing glutamine (1 mM) incubated at 37 °C in just 24 h (around 150 μM) are significantly higher than those that have been shown to be inhibitory to embryo development.[74,87] Significantly in media containing amino acids, this level of ammonium does not always alter blastocyst development; however, the cellular health of the blastomeres is affected significantly.[14,74] More importantly, the effects of ammonium on embryos from animal models resulted in a significant reduction in the ability of embryos to implant, and also resulted in a significant fetal loss after pregnancy was established, with a significant number of fetuses resorbing and not developing to term (Figure 3). Furthermore, at moderate levels of ammonium (300 μM), fetal growth rates were affected such that resultant fetuses were smaller than control embryos.[14,62,87] Recently, a study found that the levels of ammonium that are produced after incubation of medium containing glutamine inhibits development of the human blastocyst in vitro.[88]

Although glutamine is quite labile in culture, it can be substituted with a stable dipeptide of glutamine such as alanyl glutamine or glycyl glutamine, which have the

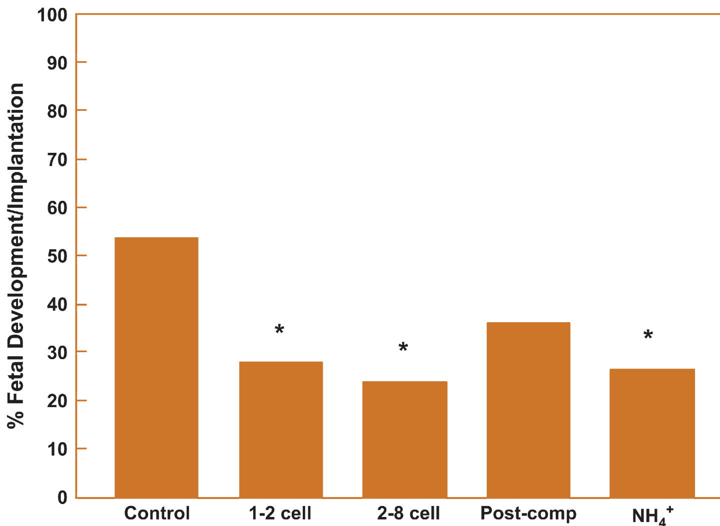

Figure 3. Fetal development of mouse blastocysts cultured with 300 μM ammonium for various stages of development: control (no ammonium); 1–2 cell (from the zygote to the two-cell stage); 2–8 cell (from the two-cell stage to the eight-cell stage); Post-comp (from the eight-cell compacted stage to the blastocyst stage); and NH_4^+ (from the zygote to the blastocyst stage). Fetal development is expressed as a percentage of the blastocysts that implanted. *Significantly different from control ($P < 0.05$). Exposure of the embryo at the precompaction stage significantly reduced fetal development rates and therefore increased fetal loss after implantation. This was despite all blastocysts developing in vitro at similar rates and appearing morphologically similar.[14]

beneficial effects of glutamine without the problematic build-up of the toxin ammonium. Therefore, one of the significant choices that must be made is whether to use a medium that contains glutamine in a clinical setting. The use of a medium containing glutamine results in significant levels of ammonium build-up, even when the medium is changed every 24 h, and therefore requires high levels of vigilance in laboratory protocols to constantly monitor the length of time that media is placed in the incubator with or without embryos. However, with the increasing body of evidence highlighting the inhibitory and perturbing nature of the presence of ammonium in the medium on embryo health and viability, it is strongly recommended that the media chosen for human ART does not contain glutamine but rather a stable dipeptide form.

Chelators: EDTA

The addition of EDTA to culture media has been shown to be important in the development of the early-stage embryo, with the first beneficial effect reported some 30 years ago.[89] Subsequently, many studies have demonstrated the beneficial effect of EDTA for development of the embryo from the zygote stage.[65,90–92]

Significantly, the beneficial effects of EDTA are confined to the cleavage-stage embryo.[26,91] When cultured with 100 μM EDTA, postcompaction-stage embryos have reduced ICM development and fetal development after transfer.[26,91] A possible explanation for this biphasic effect of EDTA could be due to its effects in inhibiting

glycolysis by inhibiting cytosolic kinases such as 3-phosphoglycerate kinase at concentrations as low as 10 μM.[93] As the ICM of the embryo uses glycolysis as its main energy-producing pathway[47], the presence of EDTA would inhibit its development and therefore fetal development. Therefore, media designed specifically for development of the postcompaction-stage embryo omit EDTA from their formulations such as G2[4,27], DM3[26] and Ham's F-10 (blastocyst medium).[94]

Macromolecules

In the early years of IVF, embryo culture media was most commonly supplemented with patient's serum at a concentration of 5–20%.[21] However, evidence is mounting that serum can be detrimental to the developing mammalian pre-implantation embryo in culture, and may be involved in alterations in metabolism, ultrastructure, methylation of imprinted genes and fetal overgrowth.[95] This has resulted in a major change to culture systems which are now routinely supplemented with serum albumin. Importantly, recombinant human serum albumin has recently become available, which can potentially eliminate the problems inherent with using blood-derived products, and lead to the standardization of medium formulations.[96]

The fluid of the reproductive tract is rich with macromolecules. One macromolecule that is present at increasing levels in the tract when the embryo is present is hyaluronan. Subsequently, it has been demonstrated that hyaluronan has an important role in embryo culture media. Hyaluronan can substitute for albumin in culture media but what is perhaps more significant for human IVF applications is that the addition of hyaluronan to a culture medium containing albumin significantly increased development but also improved the cryosurvival of embryos (Figure 4).[97,98] This is a highly significant outcome for clinical IVF applications, as one of the consequences of transferring fewer embryos for the fresh transfer is that more embryos are available for cryopreservation. Therefore, a component that can improve the ability of embryos to survival cryopreservation is highly significant and increases the efficacy of the culture system.

In addition to its role in culture medium, an elevated concentration of hyaluronan in the medium for embryo transfers in the mouse has also been shown to increase pregnancy and implantation rates. A recent randomized controlled trial in clinical IVF confirmed that elevating the levels of hyaluronan increased pregnancy and implantation rates, which was particularly evident in selected patient groups.[99,100]

MEDIA SYSTEM TYPES: MONOCULTURE SYSTEMS

Monoculture systems are defined here as a single medium formulation that is used to support zygote development to the blastocyst stage. A monoculture system does not reflect any changes in the physiology of the embryo. Direct comparisons of monoculture systems with sequential media in animal models of 'on-time' development to different stages resulted in slower rates of development in monoculture.[8,101] Interestingly, when monoculture systems were included in a meta-analysis for blastocyst transfer, there was no increase in the implantation rate of blastocysts over cleavage-stage embryos, while there was a significant increase in implantation rates of blastocysts cultured in a sequential media system.[102] This meta-analysis supports the animal data which indicate that adaptation by embryos in culture, which must occur in a monoculture system, may not affect

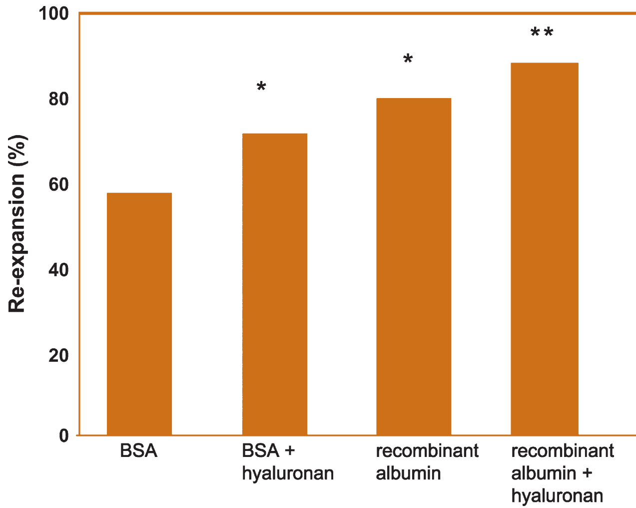

Figure 4. Effect of macromolecules in the culture medium on re-expansion after freezing and thawing of bovine blastocysts. Culture medium was supplemented with either 8 mg/mL bovine serum albumin (BSA), 8 mg/mL BSA and 0.125 mg/mL hyaluronan, recombinant albumin 2.5 mg/mL, or recombinant albumin 2.5 mg/mL and 0.125 mg/mL hyaluronan. *Significantly different from BSA ($P < 0.05$). **Significantly different from BSA ($P < 0.01$). Addition of hyaluronan to the culture medium significantly enhanced cryotolerance of blastocysts. Furthermore, purified recombinant albumin in the medium also increased cryosurvival. Therefore, macromolecular supplementation of the medium can significantly alter cryotolerance, which will affect the overall efficacy of the culture system.[98]

the ability to develop to the blastocyst stage by results in perturbations in cellular function that reduce subsequent viability.

MEDIA SYSTEM TYPES: SEQUENTIAL MEDIA SYSTEMS

The majority of commercial culture systems available for the human embryo are sequential culture systems designed around the known requirements of the developing embryo and the changing composition of the reproductive tract, examples of which have been given above. Sequential media systems are designed initially to provide optimal support for the development of the cleavage-stage embryo, and the culture media is subsequently changed to allow for optimal conditions for extended culture to the blastocyst stage. These media systems are formulated to address the changing requirements of the developing embryo, thereby reducing cellular stress and limiting the need for the embryo to undergo adaptation to grow to the blastocyst stage. In a practical sense, what has been determined is that culture conditions that support optimal blastocyst development and differentiation are actually inhibitory to the development of the early-cleavage-stage embryo.[103] Conversely, those conditions that support good growth of the zygote in culture do not sustain optimal blastocyst development and differentiation.

These sequential culture systems have been shown to produce high pregnancy rates after both cleavage stage and blastocyst culture, and have been shown by a meta-analysis to be essential for blastocyst viability. Therefore, if a clinic intends to use blastocyst

transfer as a treatment option for any patients, the answer to the question of which media is best is a sequential system.

CONCLUSION

While human embryos can grow in vitro in a wide variety of media from simple systems to complex tissue culture media, such media do not seem to be able to maintain normal blastomere physiology. Therefore, although morphological assessments may not differ between culture systems, only sequential media systems designed specifically for the embryo are able to reduce perturbations associated with in-vitro development, and as a result, the embryo is better able to maintain normal development and viability. Culture of embryos in suboptimal or perturbing conditions compels the embryo to undergo adaptations, the consequences of which are lower pregnancy rates and significantly greater fetal loss. However, it is also important to recognize that the culture media is only one part of the culture system, and that optimal performance of the medium is dependent on the quality of other aspects such as contact supplies and oil. The importance of the quality of the entire system increases with extended culture. Therefore, it is essential that high levels of quality control and assurance exist in the laboratory to enable any culture medium to perform to its maximum.[7]

REFERENCES

1. Gardner DK, Lane M & Schoolcraft WB. Physiology and culture of the human blastocyst. *J Reprod Immunol* 2002; **55:** 85–100.
2. Pool TB. An update on embryo culture for human assisted reproductive technology: media, performance, and safety. *Semin Reprod Med* 2005; **23:** 309–318.
3. Behr B & Wang H. Effects of culture conditions on IVF outcome. *Eur J Obstet Gynecol Reprod Biol* 2004; **115**(supplement 1)**:** S72–S76.
4. Gardner DK & Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update* 1997; **3:** 367–382.
5. Gardner DK & Balaban B. Choosing between day 3 and day 5 embryo transfers. *Clin Obstet Gynecol* 2006; **49:** 85–92.
6. Adashi EY, Barri PN, Berkowitz R et al. Infertility therapy-associated multiple pregnancies (births): an ongoing epidemic. *Reprod Biomed Online* 2003; **7:** 515–542.
7. Gardner DK, Reed L, Linck D et al. Quality control in human in vitro fertilization. *Semin Reprod Med* 2005; **23:** 319–324.
8. Gardner DK & Lane M. Towards a single embryo transfer. *Reprod Biomed Online* 2003; **6:** 470–481.
9. Schoolcraft WB, Gardner DK, Lane M et al. Blastocyst culture and transfer: analysis of results and parameters affecting outcome in two in vitro fertilization programs. *Fertil Steril* 1999; **72:** 604–609.
10. Schoolcraft WB & Gardner DK. Blastocyst culture and transfer increases the efficiency of oocyte donation. *Fertil Steril* 2000; **74:** 482–486.
11. Blake D, Proctor M, Johnson N et al. Cleavage stage versus blastocyst stage embryo transfer in assisted conception. *Cochrane Database Syst Rev* 2002; **2:** CD002118.
12. Gardner DK & Lane M. Blastocyst transfer. *Clin Obstet Gynecol* 2003; **46:** 231–238.
13. Papanikolaou EG, Camus M, Kolibianakis EM et al. In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. *N Engl J Med* 2006; **354:** 1139–1146.

*14. Zander DL, Thompson JG & Lane M. Perturbations in mouse embryo development and viability caused by ammonium are more severe after exposure at the cleavage stages. *Biol Reprod* 2006; **74:** 288–294.

*15. Lonergan P, Rizos D, Gutierrez-Adan A et al. Effect of culture environment on embryo quality and gene expression – experience from animal studies. *Reprod Biomed Online* 2003; **7:** 657–663.
16. Lonergan P, Rizos D, Gutierrez-Adan A et al. Oocyte and embryo quality: effect of origin, culture conditions and gene expression patterns. *Reprod Domest Anim* 2003; **38:** 259–267.
17. Shi W & Haaf T. Aberrant methylation patterns at the two-cell stage as an indicator of early developmental failure. *Mol Reprod Dev* 2002; **63:** 329–334.
18. Gosden R, Trasler J, Lucifero D et al. Rare congenital disorders, imprinted genes, and assisted reproductive technology. *Lancet* 2003; **361:** 1975–1977.
19. Halliday J, Oke K, Breheny S et al. Beckwith-Wiedemann syndrome and IVF: a case-control study. *Am J Hum Genet* 2004; **75:** 526–528.
20. Li T, Vu TH, Ulaner GA et al. IVF results in de novo DNA methylation and histone methylation at an Igf2-H19 imprinting epigenetic switch. *Mol Hum Reprod* 2005; **11:** 631–640.
21. Gardner DK & Lane M. Embryo culture systems. In Gardner DK & Trounson A (eds.). *Handbook of In Vitro Fertilization*. New York: CRC Press, 1999, pp. 123–150.
22. Whittingham DG. Culture of mouse ova. *J Reprod Fertil* 1971; **14**(supplement)**:** 7–21.
23. Quinn P, Kerin JF & Warnes GM. Improved pregnancy rate in human in vitro fertilization with the use of a medium based on the composition of human tubal fluid. *Fertil Steril* 1985; **44:** 493–498.
24. Ham RG. An improved nutrient solution for diploid Chinese hamster and human cell lines. *Exp Cell Res* 1963; **29:** 515–526.
25. Eagle H. Amino acid metabolism in mammalian cell cultures. *Science* 1959; **130:** 432–437.
26. Gardner DK & Lane M. Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. *Hum Reprod* 1996; **11:** 2703–2712.
27. Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int* 1994; **18:** 1163–1179.
28. Lawitts JA & Biggers JD. Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture media. *Mol Reprod Dev* 1992; **31:** 189–194.
29. Lane M & Gardner DK. Blastomere homeostasis. In Lane M & Gardner DK (eds.). *Art and the Human Blastocyst*. Boston: Serono Symposia, 2001.
30. Phillips KP & Baltz JM. Intracellular pH regulation by $HCO3^-/Cl^-$ exchange is activated during early mouse zygote development. *Dev Biol* 1999; **208:** 392–405.
31. Whitaker M. Calcium at fertilization and in early development. *Physiol Rev* 2006; **86:** 25–88.
32. Dumollard R, Duchen M & Sardet C. Calcium signals and mitochondria at fertilisation. *Semin Cell Dev Biol* 2006; **17:** 314–323.
33. Jones KT. Mammalian egg activation: from $Ca2^+$ spiking to cell cycle progression. *Reproduction* 2005; **130:** 813–823.
34. Saunders CM, Larman MG, Parrington J et al. PLC zeta: a sperm-specific trigger of $Ca(2^+)$ oscillations in eggs and embryo development. *Development* 2002; **129:** 3533–3544.
35. Schultz RM. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Hum Reprod Update* 2002; **8:** 323–331.
36. Braude P, Bolton V & Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 1988; **332:** 459–461.
37. Fleming TP, McConnell J, Johnson MH et al. Development of tight junctions de novo in the mouse early embryo: control of assembly of the tight junction-specific protein, ZO-1. *J Cell Biol* 1989; **108:** 1407–1418.
38. Biggers JD, Bell JE & Benos DJ. Mammalian blastocyst: transport functions in a developing epithelium. *Am J Physiol* 1988; **255:** C419–C432.
39. Gott AL, Hardy K, Winston RM et al. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod* 1990; **5:** 104–108.
40. Hardy K, Hooper MA, Handyside AH et al. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum Reprod* 1989; **4:** 188–191.
41. Gardner DK, Lane M, Stevens J et al. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril* 2001; **76:** 1175–1180.
42. Lane M & Gardner DK. Mitochondrial malate–aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem* 2005; **280:** 18361–18367.

43. Pantaleon M & Kaye PL. Nutrient sensing by the early mouse embryo: hexosamine biosynthesis and glucose signalling during preimplantation development. *Reprod Fertil Dev* 2005; **17:** 70.
44. Gardner DK & Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil* 1990; **88:** 361–368.
45. Lane M & Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998; **13:** 991–997.
46. Gardner DK. Embryo development and culture techniques. In Clark AE (ed.). *Animal Breeding Technology for the 21st Century.* New York: Harwood Publishers, 1999, pp. 13–47.
47. Hewitson LC & Leese HJ. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. *J Exp Zool* 1993; **267:** 337–343.
48. Hewitson LC, Martin KL & Leese HJ. Effects of metabolic inhibitors on mouse preimplantation embryo development and the energy metabolism of isolated inner cell masses. *Mol Reprod Dev* 1996; **43:** 323–330.
49. Gardner DK, Lane M, Calderon I et al. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996; **65:** 349–353.
50. Leese HJ, Tay JI, Reischl J et al. Formation of fallopian tubal fluid: role of a neglected epithelium. *Reproduction* 2001; **121:** 339–346.
51. Baltz JM, Biggers JD & Lechene C. Apparent absence of Na+/H+ antiport activity in the two-cell mouse embryo. *Dev Biol* 1990; **138:** 421–429.
52. Lane M, Baltz JM & Bavister BD. Na+/H+ antiporter activity in hamster embryos is activated during fertilization. *Dev Biol* 1999; **208:** 244–252.
53. Steeves CL, Lane M, Bavister BD et al. Differences in intracellular pH regulation by Na(+)/H(+) antiporter among two-cell mouse embryos derived from females of different strains. *Biol Reprod* 2001; **65:** 14–22.
54. Hammer MA & Baltz JM. Beta-alanine but not taurine can function as an organic osmolyte in preimplantation mouse embryos cultured from fertilized eggs. *Mol Reprod Dev* 2003; **66:** 153–161.
55. Steeves CL & Baltz JM. Regulation of intracellular glycine as an organic osmolyte in early preimplantation mouse embryos. *J Cell Physiol* 2005; **204:** 273–279.
56. Edwards LJ, Williams DA & Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998; **13:** 3441–3448.
57. Menezo YJ & Herubel F. Mouse and bovine models for human IVF. *Reprod Biomed Online* 2002; **4:** 170–175.
*58. Lane M & Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. *J Reprod Fertil* 1997; **109:** 153–164.
*59. Steeves TE & Gardner DK. Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol Reprod* 1999; **61:** 731–740.
*60. Gardner DK, Lane M, Spitzer A et al. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod* 1994; **50:** 390–400.
*61. Devreker F, Hardy K, Van den Bergh M et al. Amino acids promote human blastocyst development in vitro. *Hum Reprod* 2001; **16:** 749–756.
*62. Lane M & Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil* 1994; **102:** 305–312.
63. Bavister BD. Culture of mammalian embryos: facts and artifacts. *Hum Reprod Update* 1995; **1:** 91–148.
64. Leese HJ. Metabolic control during preimplantation mammalian development. *Hum Reprod Update* 1995; **1:** 63–72.
65. Chatot CL, Ziomek CA, Bavister BD et al. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. *J Reprod Fertil* 1989; **86:** 679–688.
66. Schini SA & Bavister BD. Two-cell block to development of cultured hamster embryos is caused by phosphate and glucose. *Biol Reprod* 1988; **39:** 1183–1192.
67. Thompson JG, Simpson AC, Pugh PA et al. Requirement for glucose during in vitro culture of sheep preimplantation embryos. *Mol Reprod Dev* 1992; **31:** 253–257.

68. Takahashi Y & First NL. In vitro development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* 1992; **37:** 963–978.
69. Quinn P. Enhanced results in mouse and human embryo culture using a modified human tubal fluid medium lacking glucose and phosphate. *J Assist Reprod Genet* 1995; **12:** 97–105.
70. Pantaleon M & Kaye PL. Glucose transporters in preimplantation development. *Rev Reprod* 1998; **3:** 77–81.
71. Seshagiri PB & Bavister BD. Glucose and phosphate inhibit respiration and oxidative metabolism in cultured hamster eight-cell embryos: evidence for the "crabtree effect". *Mol Reprod Dev* 1991; **30:** 105–111.
72. Martin KL & Leese HJ. Role of glucose in mouse preimplantation embryo development. *Mol Reprod Dev* 1995; **40:** 436–443.
73. Biggers JD, McGinnis LK & Raffin M. Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. *Biol Reprod* 2000; **63:** 281–293.
*74. Gardner DK & Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 1993; **48:** 377–385.
75. Lane M & Gardner DK. Nonessential amino acids and glutamine decrease the time of the first three cleavage divisions and increase compaction of mouse zygotes in vitro. *J Assist Reprod Genet* 1997; **14:** 398–403.
76. Rinaudo P & Schultz RM. Effects of embryo culture on global pattern of gene expression in preimplantation mouse embryos. *Reproduction* 2004; **128:** 301–311.
77. Bavister BD & Arlotto T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. *Mol Reprod Dev* 1990; **25:** 45–51.
78. Carney EW & Bavister BD. Stimulatory and inhibitory effects of amino acids on the development of hamster eight-cell embryos in vitro. *J In Vitro Fert Embryo Transf* 1987; **4:** 162–167.
79. Thompson JG. In vitro culture and embryo metabolism of cattle and sheep embryos – a decade of achievement. *Anim Reprod Sci* 2000; **60/61:** 263–275.
80. Miller JG & Schultz GA. Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol Reprod* 1987; **36:** 125–129.
81. Van Winkle LJ. Amino acid transport regulation and early embryo development. *Biol Reprod* 2001; **64:** 1–12.
82. Schultz GA, Kaye PL, McKay DJ et al. Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. *J Reprod Fertil* 1981; **61:** 387–393.
83. Ho Y, Wigglesworth K, Eppig JJ et al. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 1995; **41:** 232–238.
*84. Doherty AS, Mann MR, Tremblay KD et al. Differential effects of culture on imprinted H19 expression in the preimplantation mouse embryo. *Biol Reprod* 2000; **62:** 1526–1535.
85. Chang AS, Moley KH, Wangler M et al. Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients. *Fertil Steril* 2005; **83:** 349–354.
86. DeBaun MR, Niemitz EL & Feinberg AP. Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19. *Am J Hum Genet* 2003; **72:** 156–160.
87. Lane M & Gardner DK. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biol Reprod* 2003; **69:** 1109–1117.
88. Virant-Klun I, Tomazevic T, Vrtacnik-Bokal E et al. Increased ammonium in culture medium reduces the development of human embryos to the blastocyst stage. *Fertil Steril* 2006; **85:** 526–528.
89. Abramczuk J, Solter D & Koprowski H. The beneficial effect of EDTA on development of mouse one-cell embryos in chemically defined medium. *Dev Biol* 1977; **61:** 378–383.
90. Erbach GT, Bhatnagar P, Baltz JM et al. Zinc is a possible toxic contaminant of silicone oil in microdrop cultures of preimplantation mouse embryos. *Hum Reprod* 1995; **10:** 3248–3254.
91. Gardner DK, Lane MW & Lane M. EDTA stimulates cleavage stage bovine embryo development in culture but inhibits blastocyst development and differentiation. *Mol Reprod Dev* 2000; **57:** 256–261.
92. Nasr-Esfahani MH, Winston NJ & Johnson MH. Effects of glucose, glutamine, ethylenediaminetetraacetic acid and oxygen tension on the concentration of reactive oxygen species and on development of the mouse preimplantation embryo in vitro. *J Reprod Fertil* 1992; **96:** 219–231.
93. Lane M & Gardner DK. Inhibiting 3-phosphoglycerate kinase by EDTA stimulates the development of the cleavage stage mouse embryo. *Mol Reprod Dev* 2001; **60:** 233–240.

94. Behr B, Pool TB, Milki AA et al. Preliminary clinical experience with human blastocyst development in vitro without co-culture. *Hum Reprod* 1999; **14:** 454–457.
95. Thompson JG, Gardner DK, Pugh PA et al. Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol Reprod* 1995; **53:** 1385–1391.
*96. Bungum M, Bungum L, Humaidan P et al. Day 3 versus day 5 embryo transfer: a prospective randomized study. *Reprod Biomed Online* 2003; **7:** 98–104.
97. Stojkovic M, Kolle S, Peinl S et al. Effects of high concentrations of hyaluronan in culture medium on development and survival rates of fresh and frozen-thawed bovine embryos produced in vitro. *Reproduction* 2002; **124:** 141–153.
98. Lane M, Maybach JM, Hooper K et al. Cryo-survival and development of bovine blastocysts are enhanced by culture with recombinant albumin and hyaluronan. *Mol Reprod Dev* 2003; **64:** 70–78.
99. Valojerdi MR, Karimian L, Yazdi PE et al. Efficacy of a human embryo transfer medium: a prospective, randomized clinical trial study. *J Assist Reprod Genet* 2006; **23:** 207–212.
100. Gardner DK, Rodriegez-Martinez H & Lane M. Fetal development after transfer is increased by replacing protein with the glycosaminoglycan hyaluronan for mouse embryo culture and transfer. *Hum Reprod* 1999; **14:** 2575–2580.
101. Lane M, Gardner DK, Hasler MJ et al. Use of G1.2/G2.2 media for commercial bovine embryo culture: equivalent development and pregnancy rates compared to co-culture. *Theriogenology* 2003; **60:** 407–419.
102. Blake DA, Proctor M & Johnson NP. The merits of blastocyst versus cleavage stage embryo transfer: a Cochrane review. *Hum Reprod* 2004; **19:** 795–807.
103. Gardner DK & Lane M. Culture of viable human blastocysts in defined sequential serum-free media. *Hum Reprod* 1998; **13**(supplement 3)**:** 148–159 [discussion 160].