Symposium: Innovative techniques in human embryo viability assessment

Assessing embryo viability by measurement of amino acid turnover

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Abstract

This review assesses the ability of non-invasive 'amino acid profiling' to predict early embryo viability. The history of amino acid supplementation of embryo culture media and the role of amino acids in early embryo development are first considered and these are followed by a review of methods to quantify amino acid depletion and production by single embryos. Data on amino acid profiling of embryos from a number of species are then discussed. It is concluded that this technology has excellent potential to improve the selection of single embryos for transfer in clinical IVF.

Keywords: *animal, culture media, human, IVF, profiling, selection*

Introduction

There can be little debate over the need to select single human embryos with the highest chance of yielding a successful pregnancy in clinical IVF, thus reducing the chances of twins or higher-order births (Martin and Welch, 1998). Currently, embryo selection is based on morphological criteria: most commonly blastomere symmetry and degree of fragmentation, and on the appropriate timing of development. However, this approach is subjective, and points to the need for a more robust method for embryo selection. Such a test should be: (i) non- (or minimally) invasive, i.e. causing no damage to the embryo; (ii) accurate and reproducible; (iii) rapid – since multiple embryos per patient will require testing; (iv) simple – a test must be user-friendly and applicable to the IVF laboratory; (v) cost-effective, i.e. produce a benefit at an appropriate cost; (vi) independent of other predictors, especially embryo morphology; and (vii) ideally, hypothesis based, i.e. the basis by which embryos are selected should be understood.

There has been great interest in the way in which the early embryo modifies its culture environment *in vitro*. Much of the knowledge on this topic has been derived by non-invasive measurement of the depletion and/or appearance of culture medium components, including glucose, lactate, pyruvate and amino acids (Leese and Barton, 1984; Rieger *et al*., 1992; Donnay *et al*., 1999; Gott *et al*., 1999). In this article the potential for measuring amino acid turnover as a marker of early embryo viability is described.

Amino acid classification and characteristics

This review will focus primarily on the ~20 amino acids that are constituents of proteins. Non-protein amino acids will be referred to when appropriate. The standard 20 'protein amino acids' can be classified in various ways: in terms of structure, chemical properties or metabolic features (see **Table 1**). The classic subdivision of amino acids, particularly with respect to embryo culture, is into 'essential' and 'non-essential'. This designation is rather unfortunate.

Table 1. Classification of the standard 20 'protein amino acids'.

Amino acid (abbreviation)	*Essential/non-essential*	*Metabolic*	*Structural*	*Charge*
Alanine (Ala, A)	Non-essential	Glucogenic	Aliphatic	Non-polar
Cysteine (Cys, C)	Non-essential	Glucogenic	Sulphur-containing	Polar neutral
Aspartate (Asp, D)	Non-essential	Glucogenic	Acidic	Polar charged
Glutamate (Glu, E)	Non-essential	Glucogenic	Acidic	Polar charged
Phenylalanine (Phe, F)	Conditionally essential[a]	Ketogenic and glucogenic	Aromatic	Non-polar
Glycine (Gly, G)	Non-essential	Glucogenic	Aliphatic	Non-polar
Histidine (His, H)	Essential	Glucogenic	Basic	Polar charged
Isoleucine (Ile, I)	Essential	Ketogenic and glucogenic	Aliphatic	Non-polar
Lysine (Lys, K)	Essential	Ketogenic	Basic	Polar charged
Leucine (Leu, L)	Essential	Ketogenic	Aliphatic	Non-polar
Methionine (Met, M)	Conditionally essential†	Glucogenic	Sulphur-containing	Non-polar
Asparagine (Asn, N)	Non-essential	Glucogenic	Acidic	Polar neutral
Proline (Pro, P)	Non-essential	Glucogenic	Imino	Non polar
Glutamine (Gln, Q)	Non-essential	Glucogenic	Acidic	Polar neutral
Arginine (Arg, R)	Conditionally essential‡	Glucogenic	Basic	Polar charged
Serine (Ser, S)	Non-essential	Glucogenic	Hydroxyl	Polar neutral
Threonine (Thr, T)	Essential	Ketogenic and glucogenic	Hydroxyl	Polar neutral
Valine (Val, V)	Essential	Glucogenic	Aliphatic	Non-polar
Tryptophan (Trp, W)	Essential	Ketogenic and glucogenic	Aromatic	Non-polar
Tyrosine (Tyr, Y)	Non-essential	Ketogenic and glucogenic	Aromatic	Polar neutral

[a]Phenylalanine is required in significant amounts to allow the formation of tyrosine if tyrosine is lacking from the diet. †Methionine is required to produce cysteine when lacking from the diet. ‡Arginine is often synthesized in inadequate amounts.

All amino acids are essential for protein synthesis and play important roles in cellular function. The subdivision 'essential' versus 'non-essential' distinguishes between those amino acids that can be synthesized by the entire organism (usually based on whole body physiological measurements) and those which must be supplied exogenously in the diet. In the case of the early embryo, there is good reason to assume that all amino acids are required, if not absolutely essential, and should thus be supplied in the culture medium (Summers and Biggers, 2003). Moreover all 20 amino acids are present in the reproductive tract of all species studied, including the rabbit (Gregoire *et al*., 1961; Hood *et al*., 1968), pig (Hood *et al*., 1968; Iritani *et al*., 1974), sheep (Ménézo and Wintenberger-Torres, 1976), cow (Stanke *et al*., 1974; Hayashi and Matsukawa, 1979; Elhassan *et al*., 2001), horse (Engle *et al*., 1984) and, as indirectly determined, the human (Tay *et al*., 1997). It is therefore desirable to provide all amino acids in the culture medium at concentrations that attempt to mimic the physiological environment (Leese, 1991; Leese *et al*., 2001).

Early embryos also express transporters for many amino acids (Dizio and Tasca, 1977; Miller and Schultz 1985) and thus are likely to have pools of endogenous amino acids. It is not within the scope of this article to describe the transport of amino acids by embryos; the reader is referred to the excellent review by van Winkle *et al*. (2006) for further details on this subject.

Amino acid supplementation of embryo culture medium

The importance of amino acids in embryo culture medium was recognized by Brinster (1965) in the mid-1960s who reported that supplementing mouse embryo culture medium with amino acids as a fixed nitrogen source could support preimplantation development in the absence of serum. Much of the early work involved the addition of individual or groups of amino acids to the culture medium. Key experiments on the role of amino acids on embryo development were later carried out by Bavister. Thus, in efforts to culture golden hamster embryos through the 2-cell block, and following earlier work by Gwatkin and Haidri (1973), Juetten and Bavister (1983) demonstrated that the addition of four amino acids (glutamic acid, isoleucine, methionine and phenylalanine) increased cleavage rates of in-vitro–produced hamster zygotes. In a separate study, the same four amino acids were shown to enhance the proportion of eight-cell in-vivo-derived hamster embryos that yielded a blastocyst following in-vitro culture (Bavister *et al*., 1983). Using such empirical trial-and-error approaches, the presence of certain amino acids in embryo culture media has been shown to improve development in the mouse (Chatot *et al*., 1989; Ho *et al*., 1995; Nakazawa *et al*., 1997; Lane and Gardner, 1998), rat (Miyoshi *et al*., 1995), sheep (Gardner *et al*., 1994), cow (Rosenkrans and First, 1994; Lee and Fukui, 1996), pig (Petters *et al*., 1990; Yoshioka, 2002) and human (Devreker *et al*., 2001). However, the precise combination of amino acids that

should be added to the culture medium for an embryo of a given species is unclear. For example, Carney and Bavister (1987) reported that the developmental potential (in terms of blastocyst rate) of hamster 8-cell embryos was significantly reduced when cultured in media with all 20 amino acids. A similar finding was reported for cattle embryos derived *in vitro* (Pinyopummintr and Bavister, 1996), where supplementation with 'essential' amino acids, alone or in combination with 'non-essential' amino acids, led to a fall in blastocyst rates. In the work of Pinyopummintr and Bavister (1996), amino acids were added at concentrations present in tissue culture medium (TCM)-199 medium; these concentrations are significantly higher than those found in the reproductive tract. Despite this, there have been many studies investigating the role of amino acids on embryo development that have considered 'essential' versus 'non-essential' supplementation, often using 'off-the-shelf' preparations of amino acids, usually based on the composition of blood, which have different concentrations of amino acids than oviduct or uterine fluids. For example, arginine is present in TCM-199 at 0.33 mmol/l and in minimum essential medium (MEM)-AA at 0.6 mmol/l (Pinyopummintr and Bavister, 1996), whereas in reproductive tract fluid this value is probably closer to 0.1–0.2 mmol/l (Stanke *et al.*, 1974; Tay *et al.*, 1997). It is therefore unsurprising that many early studies reported inhibitory effects of certain amino acids on preimplantation development. These inhibitory effects may reflect differences in the embryo's ability to transport the various amino acids; something that may be regulated *in vivo* by the provision of amino acids at varying concentrations. Indeed, Liu and Foote (1997) showed that a 50% reduction of the 'essential amino acid' component added to a chemically defined embryo culture medium (potassium simplex optimized medium) allowed development of bovine embryos at a level equal to that obtained in a bovine serum albumin (BSA)-containing medium. However, in this study, amino acids were not added until the embryos had undergone two cell divisions; thus the effects of amino acids on the early cleavage-stage bovine embryo could not be assessed. In similar work, Nakazawa *et al.* (1997) obtained improved blastocyst rates by reducing the amino acid content in mouse embryo culture medium to concentrations similar to those measured in follicular fluid: about 50–60% lower than that of conventional 'off-the-shelf' medium.

Although these data highlight some confusion about the supplementation of embryo culture medium with amino acids, there are good data displaying beneficial effects of adding all 20 amino acids. Biggers *et al.* (2000) reported a positive correlation between the concentrations of 20 amino acids and the ability of mouse embryos to hatch, although there was little difference in overall blastocyst rate. In one particularly elegant study, Lane and Gardner (1998) demonstrated that the presence of all 20 amino acids might reduce the effects of stress *in vitro*. In this study, the metabolic activity of in-vivo-derived mouse embryos in terms of glycolysis was shown to be elevated by a period of in-vitro culture – a potential indicator of suboptimal embryo viability (Leese, 2002; Baumann *et al.*, 2007). However, the presence of all 20 amino acids in the culture medium reduced this glycolytic activity to levels approaching that of the embryo preculture; an effect further augmented by the presence of vitamins. This effect may also be due in part to amino acids entering the tricarboxylic acid cycle, reducing the reliance on glycolysis. Moreover, the post-transfer birth weight of embryos grown in amino acid- and vitamin-containing medium was significantly higher than that of embryos cultured in the absence of amino acids.

For an in-depth discussion of the relevance of adding amino acids to the medium the reader is referred to the excellent and contrasting reviews by Gardner and Lane (1997) and Summers and Biggers (2003). The former discusses in detail one of the possible drawbacks of amino acid supplementation of embryo culture medium: that of ammonium build-up, which is potentially toxic to preimplantation embryos. In the latter review, there is a detailed examination of the literature on embryo culture generally, and multiple factors are discussed comprehensively, including the (mis)-interpretation of data and the effects of different strains of animals. In the Summers and Biggers (2003) review, two approaches for the optimization of culture medium components are proposed: (i) 'let the embryos choose'; and (ii) 'back to nature'. While the 'back to nature' approach is aptly named since it aims to mimic the physiological environment of the embryo, the expression 'let the embryos choose' may be misinterpreted (Leese, 2003). It is meant to represent the experimental approach whereby the concentration of one or more culture medium constituents is varied while the concentrations of the other components are kept constant. In such a situation embryos will choose what to consume whatever medium they are given, be it a 'back to nature' medium or one devised in some other way, since they have the capacity to adapt to their environment (Leese *et al.*, 2008). This returns us to the experimental approach of 'empirical optimization', i.e. literally: 'resting on trial or experiment' and 'to make as efficient as possible'. One way in which this principle has been applied is to use data on the consumption of nutrients to make educated guesses on what an embryo requires (Leese, 2003). For example, it has long been known that embryos consume oxidative substrates such as pyruvate during early preimplantation development, but increase their consumption of glucose as the blastocyst stage is reached; at least *in vitro*, much, if not most of this glucose is converted to lactate, with species variations. This strongly suggests that embryos have a 'need' for glucose during blastocyst formation, but it does not indicate how much is required.

However, this principle may not apply to the consumption/production of amino acids, where Houghton *et al.* (2002) showed that only a limited number were taken up from a full mixture by spare cleavage-stage human embryos that developed to blastocysts (leucine, serine, arginine, methionine and valine), and that only one amino acid, leucine, was consumed at all stages. If the approach 'give the embryo what it needs', were to be adopted, the only amino acids added to the culture medium would be leucine, serine, arginine, methionine and valine, but this would be a misinterpretation of the data on amino acid consumption. First, the term 'consumption' is the *net* flux of amino acids from the medium, and comprises amino acid entry across the plasma membrane, possible exit and re-entry, and metabolic interconversion to other amino acids. Second, the pattern is only derived by providing the embryos with a complete mixture of 20 amino acids; if the amino acids were presented singly or as a small group, embryos would most likely consume whatever amino acid(s) was provided, since there are complex interactions between different amino acid combinations in culture media (Summers and Biggers, 2003).

It therefore seems clear that in order to 'let the embryos decide' it is also necessary to adopt a 'back-to-nature' approach in that

embryo culture media should mimic the in-vivo environment as closely as is technically/practically feasible. In doing so, media should provide a full complement of compounds, including all 20 of the primary amino acids present in reproductive fluids. This will inevitably represent a compromise, as it is not currently feasible to analyse the entire composition of such fluids, and so many as yet undefined compounds will be omitted. Nevertheless, it is the authors' opinion that, while imperfect, data on the composition of reproductive fluids represent a good starting point and an adequate compromise for supplementation of embryo culture media with different concentrations of amino acids.

Roles of amino acids in early embryo development

Amino acids play a number of roles in early embryo development. The most obvious is to provide precursors for biosynthetic processes, with protein synthesis being the most significant quantitatively. Embryos can develop in the absence of exogenous amino acids, and are likely to rely on intracellular supplies, resulting from protein turnover; there is no net increase in total protein until the blastocyst stage (Thompson *et al.*, 1998). However, it is likely that the entire repertoire of proteins present in the early embryo is turned over within 72 h (Baumann *et al.*, 2007), and so culture in the absence of amino acids may lead to an over-reliance on recycling pathways and endogenous pools for protein synthesis. In addition, it has long been known that exogenous amino acids are incorporated into proteins during early development (Epstein and Smith, 1973). The basic requirements for embryo culture include a mixture of physiological salts, an energy source, and a fixed nitrogen source usually provided as a macromolecule, often from undefined sources such as BSA, human serum albumin or serum (Leese *et al.*, 1993). Defined culture media containing compounds such as polyvinyl alcohol can support development, particularly when supplemented with amino acids in the cow (Kim *et al.*, 1993; Liu and Foote, 1995; Pinyopummintr and Bavister, 1996; Lee *et al.*, 2004), hamster (Kane *et al.*, 1986), sheep (Gardner *et al.*, 1994), pig (Hashem *et al.*, 2006) and mouse (Gardner and Lane, 1993; Bagis and Odaman, 2004). In the absence of an alternate protein source, one must assume that any protein synthesis during the early stages of development in embryo culture medium is making use of the amino acids present in the medium. Direct evidence for protein incorporation of amino acids supplied in the medium comes from work by Epstein and Smith (1973), Van Winkle (1977) and Morris *et al.* (2000) who measured the incorporation of radiolabelled amino acids into protein in mouse and cattle embryos.

Amino acids are also important in metabolic processes. Glutamine, probably the amino acid most commonly added to embryo culture media, can be metabolised via conversion to glutamate and then α-ketoglutarate, which can be oxidised through the tricarboxylic acid cycle to ultimately generate ATP (Wu *et al.*, 2000). Carney and Bavister (1987) and Chatot *et al.* (1989) were among the first to show that the addition of glutamine to hamster and mouse embryo culture media exerted beneficial effects on blastocyst yield. As glutamine improved embryo development when added as the sole amino acid, it was surmised that it was acting through ways other than providing protein precursors (Carney and Bavister, 1987). The oxidative metabolism of glutamine by embryos has been reported in mouse (Chatot *et al.*, 1989) and bovine embryos (Rieger and Guay, 1988; Rieger and Betteridge, 1992) and postulated for porcine embryos (Humpherson *et al.*, 2005), where the provision of glutamine significantly improves blastocyst formation (Petters *et al.*, 1990). In addition, Devreker *et al.* (1998) showed that the presence of glutamine improves human embryo development. However, glutamine has other roles: the provision of carbon and nitrogen for de-novo purine and pyrimidine synthesis as well as GTP and NAD^+ (Leese *et al.*, 1993), protection against oxidative stress (Suzuki *et al.*, 2007), acting as an organic osmolyte (Lawitts and Biggers, 1992), and as a putative regulator in glucose metabolism (Zielke *et al.*, 1976). Similarly, aspartate might be important in regulating metabolic activity via the malate–aspartate shuttle, which Lane and Gardner (2005) showed to play a role in lactate metabolism in mouse embryos. Methionine is also likely to play an important role in metabolic regulation and nucleotide synthesis, particularly with regard to the methylation cycle, involving folate and vitamin B_{12}, and leading to subsequent DNA methylation (Lightfoot *et al.*, 2005; Gilbody *et al.*, 2007).

Amino acids may also act as external paracrine signalling agents. For example, histidine, an amino acid produced by porcine (Booth *et al.*, 2005; Humpherson *et al.*, 2005) and human (Eckert *et al.*, 2007) blastocysts, might play a role in signalling to the uterus since it can be decarboxylated to the signalling agent histamine by the enzyme histidine decarboxylase, which is expressed in the uterus (Wood *et al.*, 2000). It is possible that the production of histidine by the expanded blastocyst acts as a local signal whereby its conversion to histamine helps prepare the uterus for implantation (Johnson and Dey, 1980; Zhao *et al.*, 2000). However, this putative role is hypothetical, and might be species specific, since histidine production has not been detected in mouse and cattle embryos (Lamb and Leese, 1994; Gopichandran and Leese, 2003; Orsi and Leese, 2004a). Other proposed roles for amino acids include the chelation of heavy metals (glycine) and free radical scavenging. For example, the non-protein amino acids taurine and hypotaurine have been shown to improve development of hamster (Barnett and Bavister, 1992) and porcine embryos (Petters and Reed, 1991). Recently, Suzuki *et al.* (2007) reported that taurine in combination with glutamine enhanced pig embryo development, presumably by improving intracellular oxidative status, since there was a reduction in the amount of hydrogen peroxide present.

Arginine is also likely to be important in embryo signalling processes through its metabolism to nitric oxide (NO) by NO synthase. Manser *et al.* (2004) and Kim *et al.* (2004) demonstrated that successful mouse embryo development has an absolute requirement for the production of modest amounts of NO, while Sengupta *et al.* (2005) proposed a possible role for NO in the establishment of pregnancy. Moreover, and highlighting the complex interactions between amino acids alluded to above, glutamine may be important in regulating NO synthesis via a mitochondrial phosphate-dependent glutaminase (Meininger and Wu, 1997). Arginine production has been shown in mouse (Lamb and Leese, 1994), cattle (Partridge and Leese, 1996), pig (Booth *et al.,* 2005; Humpherson *et al.*, 2005), and human embryos (Houghton *et al.*, 2002). Further benefits of alanine supplementation (Moore and Bondioli, 1993) might be due to its action as an intracellular osmolyte (Van Winkle *et al.*, 1990) or in pH regulation (McKiernan *et al.*, 1995). In their

potential role as organic osmolytes, amino acids are essentially benign when present in the cell and in this sense act differently to ionic osmolytes. Glutamine and glycine are the key organic osmolytes for early stage embryos *in vitro*, and probably *in vivo*, since both are present in significant amounts in the oviduct and uterine lumen of mouse (Harris *et al.*, 2005) and bovine (Hugentobler *et al.*, 2007) reproductive tracts. Key work showing the effectiveness of amino acids as organic osmolytes was carried out by Dawson and Baltz (1997) who showed that mouse embryo development can be rescued in high osmolarity medium by the presence of glutamine, glycine, taurine, hypotaurine, proline or β-alanine; all are substrates of the GLY or β transporter systems (Baltz, 2001). Glycine exerted protective effects against high osmolarity at much lower concentrations than the other amino acids. Moreover, a synergistic effect was seen between combinations of amino acids. It was later shown that the GLYT1 transporter is expressed and functional in early embryos (Steeves *et al.*, 2003; Steeves and Baltz, 2005) and is responsible for transporting glycine into the mouse embryo during periods of osmotic stress.

These functions of amino acids are summarized in **Figure 1**, which shows some of the known and proposed roles of amino acids during early embryo development.

While there is still debate on whether all, or a selection, of amino acids should be added during embryo culture, there is little evidence for an intrinsically negative effect, particularly when they are added at low or close-to-physiological concentrations. In the remainder of this commentary, there is a brief discussion of the methods for measuring amino acids within culture media, and the article concludes by reviewing data that suggest that the way in which embryos modify the concentrations of amino acids in culture medium may provide an effective marker of embryo viability.

Determination of amino acid consumption and production by early embryos

The first descriptions of amino acid consumption by early embryos were experiments showing that exogenous amino acids are incorporated into protein (Epstein and Smith, 1973). This largely qualitative determination of amino acid consumption was intended to show that early embryos utilise amino acids for a particular purpose: protein synthesis. However, probably the first quantification of amino acid uptake by embryos was reported by Gardner *et al.* (1989) who used a modification of the non-invasive ultramicrofluorometric assays pioneered by Leese (Leese and Barton, 1984; Gardner and Leese, 1986). The data showed that glutamine consumption by mouse embryos initially fell during early cleavage, but rose significantly by the blastocyst stage. Rieger *et al.* (Rieger and Guay, 1988; Rieger *et al.*, 1992), using radiolabelled substrate, also described the metabolism of glutamine, this time by bovine embryos. In this work, glutamine was shown to be of particular importance during the 2- and 4-cell stages. However, these types of assays only measure the uptake and appearance of individual amino acids. In order to determine usage of each of the 20 amino acids using such methods, one would either need to perform 20 individual ultramicrofluorometric assays, a technically demanding and time-consuming procedure, or use multiple radiolabelled amino acids. However, reverse-phase high performance liquid chromatography represents a good way of studying multiple amino acids present within culture media with a high degree of accuracy, sensitivity and repeatability, in a semi-automated manner.

Of the 20 protein amino acids, 18 (plus taurine and hypotaurine), will derivatize with *o*-phthaldialdehyde and β-mercaptoethanol, forming highly fluorescent isoindole compounds (Hill *et al.*, 1979), which can then be separated on the basis of different retention times by chromatography, usually on octadecyl (C-18)-type silica columns. When excited by light at 330 nm, each derivatized amino acid gives a fluorescent signal at 450 nm that can be measured using a fluorescence detector. The quantified signal can then be correlated directly with the amount of compound, using standards of known concentration. The accuracy and robustness of such methods are greatly improved by the addition of an internal standard to the medium. Such a standard should be inert, non-toxic, and unmodifiable by the embryo. One such compound is D-α-amino-butyric acid. The presence of this compound allows a reference point against which the concentrations of all other amino acids can be normalised, thus correcting for any 'non-specific' changes in amino acid concentration, not attributable to embryo activity.

Amino acid profiling of early embryos

The term 'amino acid profile' or AAP is used describe the depletion and/or appearance of amino acids in embryo culture media. The first description came in 1994 by Lamb and Leese (1994) who reported the uptake of amino acids by groups of up to 46 day-4 mouse blastocysts over a 4-h period. The volume of the incubation medium was 1 µl. The presence of differing concentrations of amino acids resulted in a variation in the pattern and amount of amino acids depleted and appearing in the culture medium; these profiles also depended on the stage of blastocyst development. For example, when day-4 embryos were cultured in medium with amino acids present at 'physiological concentrations' (based on that of rabbit oviduct fluid), glycine, alanine, isoleucine, leucine, lysine, aspartate and arginine were significantly depleted from the medium. However, when the concentration of amino acids was 0.1 mmol/l (i.e. lower than in the previous experiment) only five amino acids were significantly depleted (aspartate, glutamate, arginine, isoleucine and leucine). These data highlight the way in which a culture medium supplementation strategy can significantly influence the findings on metabolite usage. The day-5 mouse blastocysts depleted more amino acids and in higher amounts than on day 4, regardless of supplement concentration. This work illustrates the complexity of amino acid metabolism, and the strong influence of amino acid concentration in the culture medium; further evidence in support of the aforementioned contention that supplementing at physiological concentration is a good starting point.

This initial work was followed up by Partridge and Leese (1996) who measured the depletion and appearance of amino acids by groups of in-vitro- and in-vivo-derived cattle embryos at different stages of development, using similar methodology to that of Lamb and Leese (1994). Embryos were cultured in

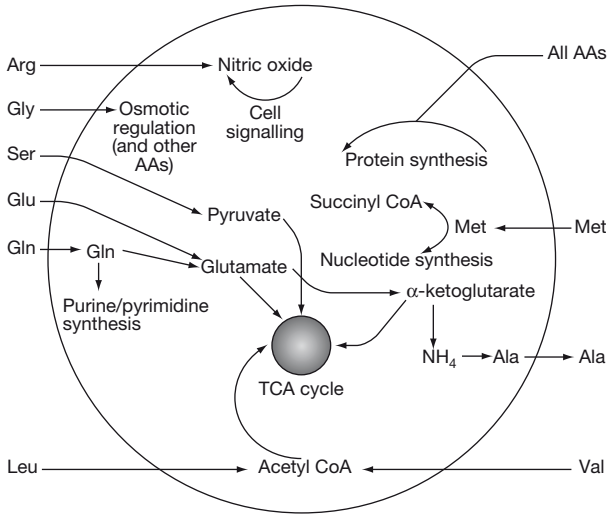

Figure 1. Summary of proposed roles and interactions between amino acids (AA) during early embryo development. TCA = tricarboxylic acid.

synthetic oviductal fluid (Tervit *et al.*, 1972) containing amino acids (Gardner *et al.*, 1994). The principal finding of this work was that the pattern of amino acid depletion and appearance (AAP) varied with the stage of development. For example, zygotes depleted only glutamine in significant amounts, whereas the 4-cell bovine embryo depleted 13 amino acids in significant quantities, and the blastocyst only four amino acids, while absolute amounts of each amino acid consumed increased throughout development. One observation consistent at all stages of development was the appearance of alanine in the culture medium, suggestive of an important role for this amino acid as a means of exporting ammonium ions (Donnay *et al.*, 1999), since the embryo does not posses a functional urea cycle (Orsi and Leese, 2004a). Another finding was that the profile of in-vivo-derived blastocysts was significantly different to that given by in-vitro-produced blastocysts. Of note was the export of glycine by in-vivo-derived embryos, possibly as a means of adjusting intracellular osmolarity (Baltz, 2001) in response to the reduced osmolarity of the culture medium compared with the reproductive tract (290 versus 360 Osmol/l; Baltz, 2001).

In a separate study, Jung *et al.* (1998) measured the production and depletion of amino acids by bovine blastocysts produced by IVF or parthenogenetic activation, and also compared profiles between cryopreserved and non-cryopreserved blastocysts. Significant differences were observed between IVF and parthenotes, and between fresh and frozen embryos. Again, certain patterns seemed consistent, notably the significant depletion of arginine and the significant appearance of alanine. This pattern was apparent qualitatively irrespective of treatment, while quantitatively there was variation between fresh and frozen and IVF and parthenotes. The same pattern for arginine consumption/alanine production by bovine embryos has also been reported by Gopichandran and Leese (2003) and Orsi and Leese (2004a), the latter reporting quantitative differences in amino acid profiles between bovine blastocysts grown in a BSA-containing medium compared with a polyvinyl-alcohol-containing medium. In addition, Orsi and Leese (2004b) showed that the absence of pyruvate altered the amino acid profile significantly, particularly with respect to alanine production, supporting the contention that alanine production is a means of disposing of ammonium ions.

These differences between the AAP of bovine embryos *in vitro* and *in vivo*, and those generated or exposed to different conditions, offered the first hint that the way in which an embryo modifies the amino acid content of a droplet of medium might relate to its developmental characteristics. It has long been known that in-vivo-derived embryos have a higher developmental potential than their in-vitro counterparts, and the data on amino acid metabolism reported by Partridge and Leese (1996) reflected this difference. Qualitatively, in-vivo-derived cattle embryos tended to deplete fewer amino acids, and in lower amounts than those produced *in vitro*. Similarly, Jung *et al.* (1998) found different amino acid profiles between frozen–thawed embryos, a stressful intervention likely to reduce viability, when compared with fresh embryos, and Gopichandran and Leese (2003) reported that the amino acid metabolism of the bovine inner cell mass (ICM) differed from that of the trophectoderm; generally the ICM had a higher requirement for amino acids. However, as this work was carried out on isolated ICM, it is possible that the observed metabolic activity was a response to the stress of disruption, although the authors did take care to minimize this. Additionally, it appears that certain amino acids accumulate in blastocoel fluid: namely aspartate, glutamate, glycine, threonine and alanine (Gopichandran and Leese, 2003).

The key breakthrough in the development of AAP for determining embryo developmental potential was made by Houghton *et al.* (2002) who showed that the manner in which human embryos on day 2 of development modified the amino acid content of a droplet of culture medium predicted development to the blastocyst stage. Thus, spare human embryos, donated for research with patients' consent were cultured singly in small droplets of culture medium containing a close to physiological mixture of amino acids (Tay *et al.*, 1997), and their development to blastocyst was monitored. Development was related retrospectively to the amino acid content of the drop in which each embryo had been cultured

from day 2–3 of development. Amino acid profiles were also presented for 8-cell/morula and blastocysts. Again, a striking qualitative pattern common to all stages was the consumption of arginine and the production of alanine. In addition, human embryos tended to consume glutamine. For example, day 2 embryos that developed to blastocysts consumed significantly *less* glutamine and arginine, and produced alanine and threonine in significantly lower quantities than those that arrested. Additionally, when the total amino acid 'turnover' (i.e. the sum of production and consumption of all 18 amino acids measured) was compared, developmentally competent day 2 embryos had a lower amino acid turnover than those that arrested. This work was the first to demonstrate a link between the viability of an embryo and its consumption/production of amino acids, determined non-invasively. This relationship was independent of other predictors of embryo viability such as morphological grade. This is a crucial requirement for practical application of a non-invasive assay, since if AAP 'merely' identified morphologically healthy embryos this would be of great interest scientifically, but it would not add much to the power of embryo selection in the clinic.

Brison *et al.* (2004) followed up these findings in a clinical study in which the day-2 amino acid profiles of human embryos were related to the chance of an ongoing pregnancy and live birth after transfer to the uterus in a clinical IVF programme. Embryos from 52 IVF cycles in patients of diverse reproductive history were cultured individually in 4-µl drops of medium for 24 h from early day 1 post-fertilization to early day 2, in a medium containing a mixture of 18 amino acids. After this, one or two embryos were selected for transfer back into the uterus according to conventional grading. The data on AAP, which were not available at the time of transfer, were then retrospectively analysed with respect to the outcome of the cycle. Cycles were included in which a single transferred embryo implanted, both of two embryos implanted, and only one of two transferred implanted. Obviously in the latter case it could not be known which of the two embryos implanted; however, this study design was deliberately chosen over one in which only single embryos were transferred, or data analysed only from '100% implantations', in order to capture as wide a range of clinical scenarios as possible. It was shown that across all cycles, concentrations of asparagine, glycine and leucine remaining in the medium after the 24 h culture period were significantly associated with the chance of a live birth. The odds ratios for this were strikingly high, between 8:1 and 10:1 for the three amino acids, compared for example with 2.3 for cell number and 6.8 for embryo grade. In addition to these three key amino acids, principal component analysis of the overall variation in the dataset showed that the pattern of consumption/depletion of all 18 amino acids was also significantly related to the chance of a live birth. **Figure 2** shows the relative concentrations of amino acids present in spent culture droplets of embryos that gave a pregnancy compared with those that did not. It is particularly encouraging that even given the 'noise' in the outcome data generated (i.e. it was not known which embryo had implanted in some cases), there was a high statistical significance between amino acid profile and cycle outcome. If anything, the data analysis is therefore likely to be an underestimate of the power of AAP in a clinical setting. Crucially, AAP parameters were not associated with embryo grade or cell number, suggesting strongly that AAP measures some other aspect of embryo viability. This leads to the suggestion that an algorithm combining embryo cell number, grade and AAP would have an additive and very high predictive power, an essential requirement for a clinical method of embryo selection.

When compared with the data of Houghton *et al.* (2002), there is a striking similarity between the overall profiles that predicted blastocyst formation in that study, and those that predict clinical pregnancy. While the particular amino acids shown to have predictive power differed between the two studies, this may be attributable to the fact that Brison *et al.* (2004) measured profiles a day earlier than Houghton *et al.* (2002). Alternatively, the differences between the two studies may suggest that the blastocysts that formed in the study by Houghton *et al.* (2002) were not necessarily viable, or in other words that AAP may be a more powerful predictor of long-term embryonic and fetal viability than the ability to form a blastocoel in culture.

More recently, Eckert *et al.* (2007) have demonstrated links between the expression of tight junction complexes occludin and ZO-1α^{+} in the trophectoderm and the amino acid metabolism of surplus human embryos donated for research. In particular, there was a significant relationship between amino acid turnover (that is, summed depletion and appearance of all measured amino acids) and membrane assembly of these tight junctional complexes. Intriguingly, particular patterns of metabolism of individual amino acids varied between stages, suggestive of very specific requirements for amino acids at different stages of blastocyst formation. Moreover, the authors reported an inverse relationship between embryo morphology and amino acid metabolism, consistent with the Quiet Embryo Hypothesis (Leese, 2002; Baumann *et al.*, 2007; Leese *et al.*, 2007).

The ability to predict developmental and pregnancy outcome by non-invasive AAP would be of great value in the move towards single embryo transfer. However, no test is ever likely to be 100% reliable, and non-embryo-related factors such as uterine receptivity have to be considered. Thus, supernumerary embryos will continue to be cryopreserved for the foreseeable future. In this context, Stokes *et al.* (2007) measured the amino acid profiles of frozen–thawed human embryos and found that the ability to predict embryos that developed to the blastocyst stage post-thaw survived the cryopreservation process irrespective of grade. Strikingly, the AAP of grade 1 embryos (i.e. 'the best' according to conventional morphological grading) that subsequently gave rise to a blastocyst differed from that obtained of grade 1 embryos that failed to develop.

The most convincing evidence that AAP can offer some selection power over and above embryo grade comes from the recent work of Booth *et al.* (2007) who, in a substantial paper, showed that by combining AAP with a variety of morphological and kinetic parameters (i.e. time to cleave, evenness of blastomeres, day-1 and day-2 cell numbers) it was possible to predict with greater than 80% accuracy which porcine zygotes would generate a blastocyst.

Conclusion

Based on animal and human embryo studies over many years, this review leads to the suggestion that using amino acid turnover (AAP) to select embryos for transfer in clinical

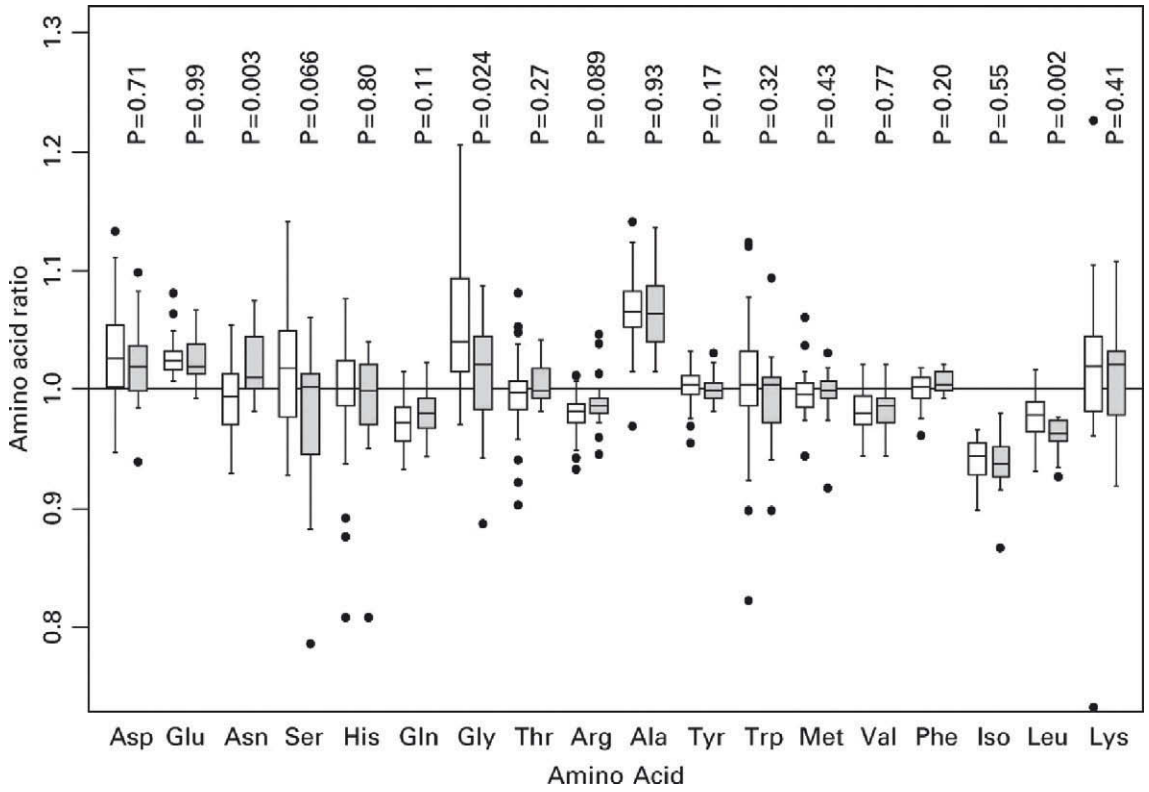

Figure 2. Box plots of the 18 individual amino acids showing the medians (thicker lines), inter-quartile ranges (boxes), ranges (whiskers; excluding outliers) and outlying observations (solid circles) for amino acid appearance in the culture medium. Open boxes are the cycles that did not achieve a pregnancy and shaded boxes are those yielding a pregnancy. *P*-values are for a Mann–Whitney test comparing the two groups for each amino acid. Reproduced from Brison *et al*. (2004), with the permission of Oxford University Press.

IVF or domestic animal production has a sound scientific and physiological basis. AAP is probably unique in this respect, in contrast to alternative methods such as metabolomics or metabolic profiling (Brison *et al*., 2007; Seli *et al*., 2007) that rely on analysis of all metabolites in the medium using methods such as infrared spectroscopy, and show an apparent association with embryo viability. However, these analyses by their nature are not hypothesis based, do not identify the important molecule(s) on which embryo selection is based, and do not have data from animal testing, all of which may need to be addressed before they can be put into clinical practice. Current ongoing studies of AAP in routine clinical IVF settings suggest that this method has the capacity to be sufficiently simple, rapid and cost-effective to improve significantly the ability to select embryos for transfer.

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References

Bagis H, Odaman MH 2004 Effect of chemically defined culture medium supplemented with beta-mercaptoethanol and amino acids on implantation and development of different stage *in vivo*- or *in vitro*-derived mouse embryos. *Molecular Reproduction and Development* **69**, 52–59.

Baltz JM 2001 Osmoregulation and cell volume regulation in the preimplantation embryo. *Current Topics in Developmental Biology* **52**, 55–106.

Barnett DK, Bavister BD 1992 Hypotaurine requirement for in vitro development of golden hamster one-cell embryos into morulae and blastocysts, and production of term offspring from *in vitro*-fertilized ova. *Biology of Reproduction* **47**, 297–304.

Baumann CG, Morris DG, Sreenan JM, Leese HJ 2007 The quiet embryo hypothesis: molecular characteristics favoring viability. *Molecular Reproduction and Development* **74**, 1345–1353.

Bavister BD, Leibfried ML, Lieberman G 1983 Development of preimplantation embryos of the golden hamster in a defined culture medium. *Biology of Reproduction* **28**, 235–247.

Biggers JD, McGinnis LK, Raffin M 2000 Amino acids and preimplantation development of the mouse in protein-free potassium simplex optimized medium. *Biology of Reproduction* **63**, 281–293.

Booth PJ, Watson TJ, Leese HJ 2007 Prediction of porcine blastocyst formation using morphological, kinetic, and amino acid depletion and appearance criteria determined during the early cleavage of in vitro-produced embryos. *Biology of Reproduction* **77**, 765–779.

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

Brinster RL 1965 Studies on the development of mouse embryos *in vitro*: III The effect of fixed-nitrogen source. *Journal of Experimental Zoology* **158**, 69–78.

Brison DR, Hollywood K, Arnesen R, Goodacre R 2007 Predicting human embryo viability: the road to non-invasive analysis of the

secretome using metabolic footprinting. *Reproductive BioMedicine Online* **15**, 296–302.
Brison DR, Houghton FD, Falconer D *et al*. 2004 Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Human Reproduction* **19**, 2319–2324.
Carney EW, Bavister BD 1987 Stimulatory and inhibitory effects of amino acids on the development of hamster eight-cell embryos *in vitro*. *Journal of In Vitro Fertilization and Embryo Transfer* **4**, 162–167.
Chatot CL, Ziomek CA, Bavister BD *et al*. 1989 An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *Journal of Reproduction and Fertility* **86**, 679–688.
Dawson KM, Baltz JM 1997 Organic osmolytes and embryos: substrates of the Gly and beta transport systems protect mouse zygotes against the effects of raised osmolarity. *Biology of Reproduction* **56**, 1550–1558.
Devreker F, Hardy F, Van den Bergh M *et al*. 2001 Amino acids promote human blastocysts development *in vitro*. *Human Reproduction* **16**, 749–756.
Devreker F, Winston RM, Hardy K 1998 Glutamine improves human preimplantation development *in vitro*. *Fertility and Sterility* **69**, 293–299.
DiZio SM, Tasca RJ 1977 Sodium dependent amino acid transport in preimplantation mouse embryos III. Na^+–K^+ ATP-ase linked mechanism in blastocysts. *Developmental Biology* **59**, 198–205.
Donnay I, Partridge R J, Leese HJ 1999 Can embryo metabolism be used for selecting bovine embryos before transfer? *Reproduction, Nutrition, Development* **39**, 523–533.
Eckert JJ, Houghton FD, Hawkhead JA *et al*. 2007 Human embryos developing *in vitro* are susceptible to impaired epithelial junction biogenesis correlating with abnormal metabolic activity. *Human Reproduction* **22**, 2214–2224.
Elhassan Y, Wu G, Leanz A *et al*. 2001 Amino acid concentrations in fluids from the bovine oviduct and uterus and in KSOM-based culture media. *Theriogenology* **55**, 1907–1918.
Engle CC, Foley CW, Plotka ED, Witherspoon DM 1984 Free amino acids and protein concentrations in reproductive tract fluids of the mare. *Theriogenology* **21**, 919–931.
Epstein CJ, Smith SA 1973 Amino acid uptake and protein synthesis in preimplantation mouse embryos. *Developmental Biology* **33**, 171–184.
Gardner DK, Lane M 1997 Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Human Reproduction Update* **3**, 367–382.
Gardner DK, Lane M 1993 Amino acids and ammonium regulate mouse embryo development in culture. *Biology of Reproduction* **48**, 377–385.
Gardner DK, Leese HJ 1986 Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. *Human Reproduction* **1**, 25–27.
Gardner DK, Lane M, Spitzer A, Batt PA 1994 Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage *in vitro* in the absence of serum and somatic cell: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biology of Reproduction* **50**, 390–400.
Gardner DK, Clarke RN, Lechene CP, Biggers JD 1989 Development of a noninvasive ultramicrofluorometric method for measuring net uptake of glutamine by single preimplantation mouse embryos. *Gamete Research* **24**, 427–438.
Gilbody S, Lewis S, Lightfoot T 2007 Methylene tetrahydrofolate reductase (MTHFR) genetic polymorphisms and psychiatric disorders: a HuGE review. *American Journal of Epidemiology* **165**, 1–13.
Gopichandran N, Leese HJ 2003 Metabolic characterization of the bovine blastocyst, inner cell mass, trophectoderm and blastocoel fluid. *Reproduction* **126**, 299–308.
Gott A, Hardy K, Winston R, Leese HJ 1999 Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Human Reproduction* **5**, 104–108.
Gregoire AT, Gongsadki D, Rakoff AE 1961 The free amino acid content of the female rabbit genital tract. *Fertility and Sterility* **12**, 322–327.
Gwatkin RB, Haidri AA 1973 Requirements for the maturation of hamster oocytes *in vitro*. *Experimental Cell Research*. **76**, 1–7.
Harris SE, Gopichandran N, Picton HM *et al*. 2005 Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology* **64**, 992–1006.
Hashem A, Hossein MS, Woo JY *et al*. 2006 Effect of potassium simplex optimization medium and NCSU23 supplemented with beta-mercaptoethanol and amino acids of *in vitro* fertilized porcine embryos. *Journal of Reproduction and Development* **52**, 591–599.
Hayashi T, Matsukawa T 1979 Free amino acid composition of bovine genital tract fluid. *Japanese Journal of Animal Reproduction* **25**, 1–5.
Hill DW, Walters FH, Wilson TD, Stuart JD 1979 High performance liquid chromatographic determination of amino acids in the picomole range. *Analytical Chemistry* **51**, 1338–1341.
Ho Y, Wigglesworth K, Eppig JJ, Schultz RM 1995 Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Molecular Reproduction and Development* **41**, 232–238.
Hood D, Williams DJ, Foley CW, Trout HF 1968 Amino acids in sow and rabbit oviduct fluids. *Journal of Animal Reproduction and Animal Science* **27**, 1786.
Houghton FD, Hawkhead JA, Humpherson PG *et al*. 2002 Non-invasive amino acid turnover predicts human embryo developmental capacity. *Human Reproduction* **17**, 999–1005.
Hugentobler SA, Diskin MG, Leese HJ *et al*. 2007 Amino acids in oviduct and uterine fluid and blood plasma during the estrous cycle in the bovine. *Molecular Reproduction and Development* **74**, 445–454.
Humpherson PG, Leese HJ, Sturmey RG 2005 Amino acid metabolism of the porcine blastocyst. *Theriogenology* **64**, 1852–1866.
Iritani A, Sato E, Nishikawa Y 1974 Secretion rates and chemical composition of oviduct and uterine fluids in sows. *Journal of Animal Science* **39**, 582–589.
Johnson DC, Dey SK 1980 Role of histamine in implantation: dexamethasone inhibits estradiol-induced implantation in the rat. *Biology of Reproduction* **22**, 1136–1141.
Juetten J, Bavister BD 1983 The effects of amino acids, cumulus cells, and bovine serum albumin on in vitro fertilization and first cleavage of hamster eggs. *Journal of Experimental Zoology* **227**, 487–490.
Jung YG, Sakata T, Lee ES, Fukui Y 1998 Amino acid metabolism of bovine blastocysts derived from parthenogenetically activated or *in vitro* fertilized oocytes. *Reproduction, Fertility and Development* **10**, 279–287.
Kane MT, Carney EW, Bavister BD 1986 Vitamins and amino acids stimulate hamster blastocysts to hatch *in vitro*. *Journal of Experimental Zoology* **239**, 429–432.
Kim BH, Kim CH, Jung KY *et al*. 2004 Involvement of nitric oxide during *in vitro* fertilization and early embryonic development in mice. *Archives of Pharmacal Research* **27**, 86–93.
Kim JH, Niwa K, Lim JM, Okuda K 1993 Effects of phosphate, energy substrates, and amino acids on development of in vitro-matured, in vitro-fertilized bovine oocytes in a chemically defined, protein-free culture medium. *Biology of Reproduction* **48**, 1320–1325.
Lamb VK, Leese HJ 1994 Uptake of a mixture of amino acids by mouse blastocysts. *Journal of Reproduction and Fertility* **102**, 169–175.
Lane M, Gardner DK 2005 Mitochondrial malate–aspartate shuttle regulates mouse embryo nutrient consumption. *Journal of Biological Chemistry* **280**, 18361–18367.
Lane M, Gardner DK 1998 Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Human Reproduction* **13**, 991–997.
Lawitts JA, Biggers JD 1992 Joint effects of sodium chloride, glutamine, and glucose in mouse preimplantation embryo culture

media. *Molecular Reproduction and Development* **31**, 189–194.
Lee ES, Fukui Y 1996 Synergistic effect of alanine and glycine on bovine embryos cultured in a chemically defined medium and amino acid uptake by *in vitro*-produced bovine morulae and blastocysts. *Biology of Reproduction* **55**, 1383–1389.
Lee ES, Fukui Y, Lee BC *et al*. 2004 Promoting effect of amino acids added to a chemically defined medium on blastocyst formation and blastomere proliferation of bovine embryos cultured *in vitro*. *Animal Reproduction Science* **84**, 257–267.
Leese HJ 2003 What does an embryo need? *Human Fertility (Camb)* **6**, 180–185.
Leese HJ 2002 Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* **24**, 845–849.
Leese HJ 1991 Metabolism of the preimplantation mammalian embryo. *Oxford Reviews of Reproductive Biology* **13**, 35–72.
Leese HJ, Barton AM 1984 Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *Journal of Reproduction and Fertility* **72**, 9–13.
Leese HJ, Hugentobler SA, Gray SM *et al*. 2008 Female reproductive tract fluids: composition, mechanism of formation and potential role in the developmental origins of health and disease. *Reproduction, Fertility and Development* **20**, 1–8.
Leese HJ, Sturmey RG, Baumann CG, McEvoy TG 2007 Embryo viability and metabolism: obeying the quiet rules. *Human Reproduction* **22**, 3047–3050.
Leese HJ, Donnay I, Macmillan D, Houghton F 2001 Metabolism of the early embryo: energy production and utilisation. In: Gardner D, Lane M (eds) *ART and the Human Blastocyst*. Springer, Massachusetts, pp. 61–68.
Leese HJ, Conaghan J, Martin KL, Hardy K 1993 Early human embryo metabolism. *Bioessays* **15**, 259–264.
Lightfoot TJ, Skibola CF, Willett EV *et al*. 2005 Risk of non-Hodgkin lymphoma associated with polymorphisms in folate-metabolizing genes. *Cancer Epidemiology, Biomarkers and Prevention* **14**, 2999–3003.
Liu Z, Foote RH 1997 Effects of amino acids and α-amanitin on bovine embryos development in a simple protein-free medium. *Molecular Reproduction and Development* **46**, 278–285.
Liu Z, Foote RH 1995 Effects of amino acids on the development of *in-vitro* matured/*in-vitro* fertilization bovine embryos in a simple protein-free medium. *Human Reproduction* **10**, 2985–2991.
Manser RC, Leese HJ, Houghton FD 2004 Effect of inhibiting nitric oxide production on mouse preimplantation embryo development and metabolism. *Biology of Reproduction* **71**, 528–533.
Martin PM, Welch HG 1998 Probabilities for singleton and multiple pregnancies after *in vitro* fertilization. *Fertility and Sterility* **70**, 478–481.
McKiernan SH, Clayton MK, Bavister BD 1995 Analysis of stimulatory and inhibitory amino acids for development of hamster one-cell embryos *in vitro*. *Molecular Reproduction and Development* **42**, 188–199.
Meininger CJ, Wu G 1997 L-Glutamine inhibits nitric oxide synthesis in bovine venular endothelial cells. *Journal of Pharmacology and Experimental Therapeutics* **281**, 448–453.
Ménézo Y, Wintenberger-Torres S 1976 Free amino acid content of ewe uterine fluid under various hormonal treatments during early pregnancy. *Annales de Biologie Animale Biochimie Biophysique* **16**, 537–543.
Miller JG, Schultz GA 1985 Amino acid transport in mouse blastocyst compartments. *Journal of Embryology and Experimental Morphology* **89**, 149–158.
Miyoshi K, Abeydeera LR, Okuda K, Niwa K 1995 Effects of osmolarity and amino acids in a chemically defined medium on development of rat one-cell embryos. *Journal of Reproduction and Fertility* **103**, 27–32.
Moore K, Bondioli KR 1993 Glycine and alanine supplementation of culture medium enhances development of *in vitro* matured and fertilized cattle embryos. *Biology of Reproduction* **48**, 833–840.
Morris DG, Diskin MG, Sreenan JM 2000 Protein synthesis and phosphorylation by elongating 13–15-day-old cattle blastocysts. *Reproduction, Fertility and Development* **12**, 39–44.
Nakazawa T, Ohashi K, Yamada M *et al*. 1997 Effect of different concentrations of amino acids in human serum and follicular fluid on the development of one-cell mouse embryos *in vitro*. *Journal of Reproduction and Fertility* **111**, 327–332.
Orsi NM, Leese HJ 2004a Amino acid metabolism of preimplantation bovine embryos cultured with bovine serum albumin or polyvinyl alcohol. *Theriogenology* **61**, 561–572.
Orsi NM, Leese HJ 2004b Ammonium exposure and pyruvate affect the amino acid metabolism of bovine blastocysts *in vitro*. *Reproduction* **127**, 131–140.
Partridge RJ, Leese HJ 1996 Consumption of amino acids by bovine preimplantation embryos. *Reproduction, Fertility and Development* **8**, 945–950.
Petters R, Reed M 1991 Addition of taurine or hypotaurine to culture medium improves development of one and two cell pig embryos *in vitro*. *Theriogenology* **35**, 253.
Petters R, Johnson B, Reed M, Archibong A 1990 Glucose, glutamine, and inorganic phosphate in early development of the pig embryo *in vitro*. *Journal of Reproduction and Fertility* **89**, 269–275.
Pinyopummintr T, Bavister B 1996 Effects of amino acids on development *in vitro* of cleavage-stage bovine embryos into blastocysts. *Reproduction, Fertility and Development* **8**, 835–841.
Rieger D, Betteridge KJ 1992 Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced *in vitro*. *Reproduction Fertility and Development* **4**, 547–557.
Rieger D, Guay P 1988 Measurement of the metabolism of energy substrates in individual bovine blastocysts. *Journal of Reproduction and Fertility* **83**, 585–591.
Rieger D, Loskutoff NM, Betteridge KJ 1992 Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced *in vitro*. *Reproduction, Fertility and Development* **4**, 547–557.
Rosenkrans CF Jr, First NL 1994 Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes *in vitro*. *Journal of Animal Science* **72**, 434–437.
Seli E, Sakkas D, Scott R *et al*. 2007 Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing *in vitro* fertilization. *Fertility and Sterility* **88**, 1350–1357.
Sengupta J, Dhawan L, Lalitkumar PG, Ghosh D 2005 Nitric oxide in blastocyst implantation in the rhesus monkey. *Reproduction* **130**, 321–332.
Stanke DF, Sikes JD, DeYoung DW, Tumbleson ME 1974 Proteins and amino acids in bovine oviductal fluid. *Journal of Reproduction and Fertility* **38**, 493–496.
Steeves CL, Baltz JM 2005 Regulation of intracellular glycine as an organic osmolyte in early preimplantation mouse embryos. *Journal of Cell Physiology* **204**, 273–279.
Steeves CL, Hammer MA, Walker GB *et al*. 2003 The glycine neurotransmitter transporter GLYT1 is an organic osmolyte transporter regulating cell volume in cleavage-stage embryos. *Proceedings of the National Academy of Sciences USA* **100**, 13982–13987.
Stokes PJ, Hawkhead JA, Fawthrop RK *et al*. 2007 Metabolism of human embryos following cryopreservation: implications for the safety and selection of embryos for transfer in clinical IVF. *Human Reproduction* **22**, 829–835.
Summers MC, Biggers JD 2003 Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. *Human Reproduction Update* **9**, 557–582.
Suzuki C, Yoshioka K, Sakatani M, Takahashi M 2007 Glutamine and hypotaurine improves intracellular oxidative status and in vitro development of porcine preimplantation embryos. *Zygote* **15**, 317–324.
Tay JI, Rutherford AJ, Killick SR *et al*. 1997 Human tubal fluid: production, nutrient composition and response to adrenergic agents. *Human Reproduction* **12**, 2451–2456.
Tervit HR, Whittingham DG, Rowson LE 1972 Successful culture *in*

vitro of sheep and cattle ova. *Journal of Reproduction and Fertility* **30**, 493–497.

Thompson JG, Sherman AN, Allen NW *et al*. 1998 Total protein content and protein synthesis within pre-elongation stage bovine embryos. *Molecular Reproduction and Development* **50**, 139–145.

Van Winkle LJ 1977 Low Na^+ concentration: a factor contributing to diminished uptake and incorporation of amino acids by diapausing mouse blastocysts? *Journal of Experimental Zoology* **202**, 275–281.

Van Winkle LJ, Tesch JK, Shah A, Campione AL 2006 System BO^+ amino acid transport regulates the penetration stage of blastocyst implantation with possible long term developmental consequences through adulthood. *Human Reproduction Update* **12**, 145–157.

Van Winkle LJ, Haghighat N, Campione AL 1990 Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *Journal of Experimental Zoology* **253**, 215–219.

Wood GW, Hausmann EH, Choudhuri R, Dileepan KN 2000 Expression and regulation of histidine decarboxylase mRNA expression in the uterus during pregnancy in the mouse. *Cytokine* **12**, 622–629.

Wu F, Orlefors H, Bergstrom M *et al*. 2000 Uptake of ^{14}C- and ^{11}C-labelled glutamate, glutamine and aspartate *in vitro* and *in vivo*. *Anticancer Research* **20**, 251–256.

Yoshioka K, Suzuki C, Tanaka A *et al*. 2002 Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biology of Reproduction* **66**, 112–119.

Zhao X, Ma W, Das SK *et al*. 2000 Blastocyst H(2) receptor is the target for uterine histamine in implantation in the mouse. *Development* **127**, 2643–2651.

Zielke HR, Ozand PT, Tildon JT *et al*. 1976 Growth of human diploid fibroblasts in the absence of glucose utilization. *Proceedings of the National Academy of Sciences USA* **73**, 4110–4114.

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