Non-invasive amino acid turnover predicts human embryo developmental capacity

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BACKGROUND: IVF is limited by low success rates and a confounding high multiple birth rate contributing to prematurity, increased neonatal mortality and child handicap. These problems could be overcome if single embryos of known developmental competence could be selected for transfer on day 2/3 of development, but current methods, which rely on morphological appearance, are poor predictors of viability. METHODS: We have measured non-invasively the depletion/appearance (i.e. turnover) of a physiological mixture of 18 amino acids by single human embryos during in-vitro culture using high performance liquid chromatography. RESULTS: From the time of transfer (day 2/3), embryos with future competence to develop to the blastocyst stage (day 5/6) exhibit amino acid flux patterns distinct from those of embryos with similar morphological appearance which arrest. Significantly, the profiles of Ala, Arg, Gln, Met and Asn flux predict blastocyst potentiality at \( >95\% \). The amino acid most consistently depleted throughout development by those embryos which form blastocysts was leucine. Of the amino acids which were produced, the most striking was alanine, which appeared in increasing amounts throughout development. CONCLUSIONS: Non-invasive amino acid profiling has the potential to select developmentally competent single embryos for transfer, thereby increasing the success rate and eliminating multiple births in IVF.

Key words: amino acids/developmental potential/human preimplantation embryo culture/IVF

Introduction

Human zygotes produced by IVF can develop to the blastocyst stage in simple salt solutions containing carbon sources such as pyruvate and glucose, a macromolecule such as albumin (Conaghan et al., 1998), and in some cases, maternal serum (Bolton et al., 1989). These systems result in blastocyst yields of 17–23% (Bolton et al., 1989; Conaghan et al., 1998). In order to improve rates of blastocyst formation, mixtures of non-essential and essential amino acids are widely added to human embryo culture media (Devreker et al., 2001). The regime most favoured is supplementation with non-essential amino acids during early cleavage, up to the 8-cell stage, followed by non-essential as well as essential amino acids for the 8-cell to morula and morula to blastocyst transitions (Gardner and Lane, 1997); a sequential system which increases blastocyst yields to ~50% (Schoolcraft et al., 1999). The mechanisms by which amino acids promote human preimplantation development are largely unknown. In early embryos of other species, notably the mouse, amino acids have been shown to serve a variety of functions: in the synthesis of proteins and nucleotides (Epstein and Smith, 1973; Alexiou and Leese, 1992), as sources of energy (Lane and Gardner, 1997, 1998), as osmolites (Van Winkle and Campione, 1996; Dumoulin et al., 1997; Dawson et al., 1998), as antioxidants (Nasr-Esfahani et al., 1992), pH regulators (Bavister and McKiernan, 1993; Edwards et al., 1998), chelators (Van Winkle et al., 1990) and as precursors of signalling molecules such as nitric oxide (Wu et al., 2000).

Amino acid transport mechanisms in early mouse embryos have been studied extensively, with up to 15 different systems identified (Van Winkle, 2001). Many of the uptake processes involve co-transport with \( \text{Na}^+ \), a dependency which increases throughout the preimplantation period (Tasca, 2001). There are relatively few reports on the role of specific amino acids during early human embryo development: glutamine addition increases the proportion of embryos that reach the blastocyst stage (Devreker et al., 1998); taurine acts as an osmolyte (Dumoulin et al., 1997), but provides no additional benefit over glutamine in development to the blastocyst stage (Devreker et al., 1999); glycine has also been shown to be involved in osmoregulation (Hammer et al., 2000).

In these studies, amino acids have been administered singly, whereas in the female reproductive tract, the preimplantation embryo will be exposed to a complete mixture (Leese et al., 1979; Miller and Schultz, 1987). To take account of this, Lamb and Leese measured non-invasively the depletion or appearance of 18 out of a mixture of 20 amino acids by small numbers of mouse blastocysts (Lamb and Leese, 1994). The same
approach was applied to bovine embryos from the zygote to blastocyst stage (Partridge and Leese, 1996; Jung et al., 1998). The data showed that amino acid depletion/appearance differed between the two species and, in the case of the bovine, with stage of development. A notable feature of bovine amino acid metabolism was the appearance of alanine in increasing amounts throughout development (Partridge and Leese, 1996). Subsequently, data from Donnay et al. suggested that alanine was derived from ammonia via transamination with pyruvate (Donnay et al., 1999).

We have now examined the depletion or appearance of amino acids brought about by single, spare, human embryos conceived by IVF as a first approach to understanding the mechanisms by which mixtures of amino acids promote human preimplantation development. It should be emphasised that this approach is not intended to be a strict, kinetic analysis of amino acid uptake; rather, it represents the way in which embryos modify the composition of a mixture of amino acids present at concentrations believed to be close to physiological. Amino acid profiles have been obtained for human embryos from day 2–3 post-insemination and during the compacting 8-cell to morula and morula to blastocyst transitions.

Our data indicate that amino acid depletion/appearance differ with embryo stage and with embryo quality assessed in terms of developmental capacity. Specifically, we have identified groups of amino acids whose depletion or appearance, measured on day 2 post-fertilization, predict the ability of human embryos to develop to the blastocyst.

Materials and methods
Spare human embryos donated to research were obtained with informed consent from patients undergoing IVF at the Assisted Conception Unit, Leeds General Infirmary. Ethical approval for the work was granted by the Human Fertilisation and Embryology Authority and the Ethics Committees of the collaborating institutions. Ovarian stimulation and oocyte collection were performed as previously described (Balen, 2001). In general, a long pituitary desensitization protocol was used, with intranasal nafarelin followed by gonadotrophin stimulation with either HMG (Menogon; Ferring Pharmaceuticals Ltd, Langley, Berks, UK) or recombinant FSH (Puregon; Organon Laboratories Ltd, Cambridge, UK). Briefly, oocytes were collected by follicular aspiration 36 h after HCG administration and cultured at 37°C in 5% CO₂ in Medi-Cult IVF medium under oil. The oocyte–cumulus complexes were inseminated with a final concentration of 70 000 motile sperm per ml at ~40 h post-HCG and incubated overnight until fertilization was confirmed by the presence of two pronuclei (day 1 post-insemination). Prior to being used for research, zygotes were cultured in 70 µl drops of Medi-Cult IVF medium under oil, as above. A maximum of three embryos was transferred on day 2 post-insemination and any remaining surplus embryos, from day 2 to day 5 post-fertilization, were transported to York in the same medium at 37°C.

Developmental grade (Table I) and stage were recorded and the embryos cultured individually in 4 µl drops of EBSS at 37°C in 5% CO₂ in air for 24 h. The embryos were then transferred every 24 h to fresh 4 µl drops until the blastocyst stage. The EBSS was supplemented with 0.5% HSA, 1 mmol/l glucose, 5 mmol/l lactate, 0.47 mmol/l pyruvate and a physiological mixture of amino acids (Tay et al., 1997). The spent medium was stored at −80°C. Following thawing, an aliquot (2 µl) was removed and diluted 1:12.5 in high performance liquid chromatography (HPLC) grade water. Embryo-free control drops were incubated alongside the embryo-containing drops to allow for any non-specific amino acid degradation/appearance.

The amino acids were analysed by reverse-phase HPLC as previously described (Lamb and Leese, 1994), but using a Kontron 500 series automated HPLC system fitted with a Jasco F920 fluorescence detector and a 4.5×250 mm Hypersil ODS-16 (column (Jones Chromatography, Hengoed, Mid Glamorgan, UK). Derivatization was achieved by the automated reaction of a 25 µl sample with an equal volume of reagent [10 µl 2-mercaptoethanol and 5 ml o-phthalaldehyde (OPA) reagent]. The elution gradient operated at a flow rate of 1.3 ml/min. Solvent A consisted of 18 ml tetrahydrofuran (Fisher Scientific, Loughborough, Leics, UK), 200 ml methanol and 800 ml sodium acetate (83 mmol/l, pH 5.9). Solvent B consisted of 800 ml methanol and 200 ml sodium acetate (83 mmol/l, pH 5.9). Using this method, it was not possible to detect proline and cysteine.

Results were expressed as amino acid depletion/appearance in pmol/embryo/h ± SEM. The term ‘turnover’ has been used to describe the sum, in pmol/embryo/h of amino acid depletion from, and appearance in, the culture medium. Embryos received on day 2 varied in developmental stage from 2- to 4-cell. After 24 h incubation they ranged from 4-cell to compacting 8-cell (c8-cell). Moreover, embryos were of variable grade; from 1–4. The data were therefore expressed in terms of amino acid depletion/appearance from day 2–3. Due to variation in the rate of human preimplantation development, amino acid profiles for the later stage embryos were best grouped according to embryo stage; c8-cell to morula and morula to blastocyst, rather than by day post-insemination. Approximately equal numbers of embryos were at the c8-cell stage on day 3 and 4 in both the arresting and developing groups. Developing embryos progressed from the c8-cell stage to the morula stage within the 24 h culture period and subsequently developed from the morula to the blastocyst stage in the next 24 h period. Each amino acid value was tested for significance from zero depletion/appearance using Student’s t-test. Differences between amino acid turnover for arresting and developing embryos were also analysed by Student’s t-test, as were differences between embryo grade.

Results
The average grades of embryos which subsequently developed to the blastocyst stage and those that arrested prior to blastocyst formation are shown in Table II. There were no significant differences in grade between arresting and developing embryos at either stage of development. Metabolic profiling in terms of amino acid depletion/appearance could readily be carried out on single human embryos incubated for 24 h, from as early as day 2 post-insemination. Profiles for the embryos

<table>
<thead>
<tr>
<th>Table I. Human embryo scoring system</th>
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<tbody>
<tr>
<td>Embryo grade</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<tr>
<td>2.5</td>
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<tr>
<td>3</td>
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<td>4</td>
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<td>5</td>
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</table>
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Figure 1. Amino acid depletion (negative values) and appearance (positive values) ± SEM by individual, spare human preimplantation embryos which subsequently formed blastocysts (n = 14), or which arrested in culture prior to blastocyst formation (n = 28), measured non-invasively from day 2–3 of development. *P < 0.05; **P < 0.01; ***P < 0.001 significance from zero. Bars with the same superscript are significantly different: a, P = 0.0004; b, P = 0.023; c, P = 0.026; d, P = 0.033; e, P = 0.044.

Table II. Mean grade ± SEM for day 2 and compacting 8-cell embryos which either developed to the blastocyst or arrested prior to blastocyst formation

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Embryo grade</th>
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<tbody>
<tr>
<td></td>
<td>Developing</td>
</tr>
<tr>
<td>Day 2</td>
<td>2.50 ± 0.18</td>
</tr>
<tr>
<td>Compacting 8-cell</td>
<td>1.80 ± 0.14</td>
</tr>
</tbody>
</table>

incubated from day 2–3, during the c8-cell to morula and morula to blastocyst transitions were as follows:

(i) Amino acid turnover by embryos from day 2–3 post-insemination

Leucine was the only amino acid significantly depleted (P < 0.001) between day 2 and 3 of development by embryos that subsequently developed to the blastocyst stage (Figure 1) while glutamate (P < 0.01) and alanine (P < 0.001) appeared in significant quantities in the culture medium. The other amino acids were not significantly different from zero, i.e. there was no net disappearance from the medium, nor did they appear. Embryos that were morphologically indistinguishable (P = 0.067; Table II) from any other in this cohort, but which subsequently failed to develop to the blastocyst stage displayed a different pattern of amino acid depletion/appearance. Asparagine (P < 0.05), glutamine (P < 0.001), arginine (P < 0.001), methionine (P < 0.01), valine (P < 0.0001), isoleucine (P < 0.001) and leucine (P < 0.001) were all depleted while aspartate (P < 0.05), glutamate (P < 0.001), glycine (P < 0.001), alanine (P < 0.001) and lysine (P < 0.05) appeared. The amino acid profiles of asparagine (P = 0.044), glutamine (P = 0.026), arginine (P = 0.023), alanine (P = 0.0004) and methionine (P = 0.033) of those day 2–3 embryos which went on to form blastocysts differed from those which subsequently arrested (Figure 1). In addition, when the values for the significantly depleted and produced amino acids were summed, there was a 7.2- and 2.1-fold increase in amino acid uptake and production respectively for day 2–3 embryos which subsequently arrested compared with those that continued to develop. In other words, the amino acid turnover (i.e. the sum of depletion and appearance) for embryos which arrested was 3.7-fold greater (P < 0.001) than those which developed to the blastocyst stage (Figure 2).
(ii) Amino acid turnover during the compacting 8-cell to morula transition

Human embryos incubated from the c8-cell to morula stage of development depleted leucine (P < 0.001), arginine (P < 0.01) and serine (P < 0.01) significantly from the medium (Figure 3), while aspartate (P < 0.01), glutamate (P < 0.05), alanine (P < 0.001) and tryptophan (P < 0.05) were significantly produced. Embryos over this developmental period which failed to develop to the blastocyst stage gave greater amino acid appearance and disappearance values than those which developed normally [significance values were: asparagine (P < 0.05), glutamine (P < 0.01), arginine (P < 0.001), valine (P < 0.05), isoleucine (P < 0.05) and leucine (P < 0.001) in terms of depletion, and aspartate (P < 0.01), glutamate (P < 0.001), glycine (P < 0.01) and alanine (P < 0.001) in terms of appearance]. There was no significant difference in morphology at the c8-cell stage between embryos that developed to the blastocyst stage and those that arrested (Table II). However, the developmental potential of c8-cell embryos was reflected in the amino acid profiles for specific amino acids (Figure 3). Thus, serine (P = 0.019), glycine (P = 0.015) and alanine (P = 0.037) depletion/appearance values differed for developing versus arrested embryos. Compacting 8-cell to morula stage embryos which arrested prior to blastocyst formation displayed a 2.2- and 1.6-fold increase in amino acid uptake and production respectively compared with those that continued to develop. In total, amino acid turnover for c8-cell embryos that arrested during development was 2-fold greater (P < 0.001) than those which developed to the blastocyst stage (Figure 2).

(iii) Amino acid turnover during the morula to blastocyst transition

Serine (P < 0.001), arginine (P < 0.001), methionine (P < 0.001), valine (P < 0.001) and leucine (P < 0.01) were significantly depleted while aspartate (P < 0.001), glutamate (P < 0.001) and alanine (P < 0.001) appeared in the medium (Figure 4).

Amino acid turnover throughout development

Table III summarizes the amino acids whose depletion or appearance was statistically significant from zero for the three developmental transitions: day 2–3; c8-cell to morula; morula to blastocyst. For embryos which developed to the blastocyst stage, there was an increase in uptake; from one amino acid (leucine) from day 2–3, to three amino acids (serine, arginine and leucine) during the compacting 8-cell to morula transition, to five amino acids (serine, arginine, methionine, valine and leucine) during the morula to blastocyst transition. By contrast, embryos which arrested had a greater amino acid uptake; for seven amino acids during the day 2–3 transition, and six amino acids during the compacting 8-cell to morula stage. Total amino acid depletion was also shown to increase in absolute terms, from 4.3 ± 0.4 pmol/embryo/h from day 2–3 to 14.6 ± 1.9 pmol/embryo/h over the morula to blastocyst transition (P = 0.01; Figure 5).

Discussion

Non-invasive assays have been used to measure amino acid depletion/appearance (i.e. turnover) by single human embryos throughout development to the blastocyst stage. Although the data are expressed as rates of depletion or appearance of given amino acids, it should be emphasised that these were not measured under conditions where initial rates of uptake or release could be determined; rather, the data represent net, embryo-mediated fluxes of amino acids, in an inward or outward direction. Because of this, the data cannot be interpreted in kinetic terms, but do provide an indication of the nutritional requirements of early human embryos and the possible relationship with embryo physiology and development. Thus, it is likely that the major quantitative use of amino acids, as in somatic cells, is in protein synthesis, which, in other
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Figure 4. Amino acid depletion/appearance ± SEM by individual human embryos over the morula to blastocyst transition (n = 42). **P < 0.01; ***P < 0.001 significance from zero.

Table III. Amino acid uptake/production by developing and arresting embryos

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Amino acids taken up by the embryo</th>
<th>Amino acids produced by the embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Developing embryos</td>
<td>Arresting embryos</td>
</tr>
<tr>
<td></td>
<td>Developing embryos</td>
<td>Arresting embryos</td>
</tr>
<tr>
<td>Day 2–3</td>
<td>Leu(^E)</td>
<td>Asn(^{NE})</td>
</tr>
<tr>
<td>Compact 8-cell to morula</td>
<td>Ser(^{C})</td>
<td>Asp(^{NE})</td>
</tr>
<tr>
<td>Morula to blastocyst</td>
<td>Ser(^{C})</td>
<td>Asp(^{NE})</td>
</tr>
<tr>
<td></td>
<td>Met(^{E})</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leu(^{E})</td>
<td></td>
</tr>
</tbody>
</table>

Superscripts depict amino acid category.
E = essential, NE = non-essential and C = conditional amino acid.

species, increases during the later stages of preimplantation development (Leese, 1991). This was reflected by an increase in amino acid depletion as the embryos developed from day 2 through to the blastocyst stage (Figure 5). Curiously, amino acid depletion by embryos which arrested were 7.2 and 2.2 times greater on day 2–3 than during the c8-cell to morula transition respectively than for those which developed normally. In other words, the data indicate that preimplantation embryos competent to develop into blastocysts have a lower amino acid turnover than those which arrest. These results are intriguing and suggest that arresting embryos are more metabolically active than developmentally competent embryos. The conclusion that viable human embryos are less active metabolically was also reached by Conaghan et al. who measured the consumption of pyruvate by 590 individual in-vitro produced human embryos from the pronucleate stage following fertilization, up to the day of transfer on day 2 or 3 post-insemination (Conaghan et al., 1993). For those embryos which gave rise to a successful pregnancy after transfer, the pyruvate consumption was significantly lower than those which failed to implant. The same pattern was apparent in a related study carried out on human embryos conceived through natural cycle IVF (Turner et al., 1994).

The amino acid most consistently depleted throughout development by those embryos which formed blastocysts was leucine. This was an interesting finding; firstly because leucine is an ‘essential amino acid’ (see below) and also since it has an important role as a signalling molecule which stimulates protein synthesis in a variety of cell types (Kimball et al., 1999; Anthony et al., 2000; Lynch et al., 2000).

An interesting observation in the present study was that glutamine was not significantly depleted from the medium at
Culture media for preimplantation mammalian embryos are widely supplemented with amino acids, which are most commonly divided into ‘essential’ and ‘non-essential’. For human embryos, non-essential amino acids are recommended for early cleavage, with a mixture of non-essential and essential for the 8-cell to blastocyst stages (Lane and Gardner, 1997). This regime has to be questioned on two grounds. Firstly, the traditional division of amino acids into ‘essential’ and ‘non-essential’ has now been replaced, albeit in terms of human nutrition, by the addition of a third category of ‘conditionally essential’ amino acids (Frayn, 1997); those which may be synthesized from other essential amino acids, but may be required in the diet under some circumstances to satisfy nutritional requirements. On this basis, all amino acids showing a net depletion from day 2 through to the blastocyst stage, are either ‘essential’ or ‘conditional’ (Table III); none fall into the non-essential category. This requirement would obviously not be fulfilled by culture media which included only non-essential amino acids during the early preimplantation phase. Moreover, the division of amino acids into even three groups may mask considerable subtleties in their requirements (Jackson, 1999).

Our data lead us to favour including all 20 amino acids in human embryo culture media, at concentrations believed to be physiological, and to let the embryos choose which to consume or release, and in what quantities.

**Metabolic profiling and embryo selection.**

Our data have revealed quite marked differences in amino acid turnover between embryos which develop to the blastocyst stage and those which arrest. Such differences are apparent in embryos incubated from day 2–3 post-insemination. This implies that oocyte quality must play a major role in determining embryo viability since zygotic genome activation is not detected in the human embryo until the 4- to 8-cell stage (Braude et al., 1988). Zygotes incubated for 24 h from day 1 have not been available for this study, since decisions on which embryos to transfer, freeze or release for research purposes were not made until day 2. An alternative method to detect differences in amino acid turnover between viable and non-viable embryos was to group the sum of selected amino acids. From day 2–3 post-development, the sum of lysine, alanine and glycine gave the greatest significant difference between embryos that reached the blastocyst stage and those that arrested prior to cavitation. During the c8-cell to morula transition, the sum of glycine, alanine and lysine gave the same degree of significance between developmentally competent and arrested embryos as the sum of serine, glycine, alanine, leucine and lysine. Thus, there are a number of ways of using amino acid profiling data to predict non-invasively the ability of a human embryo to reach the blastocyst stage. These results have implications for the selection of human embryos for transfer following IVF: if the ability to cavitate can be determined as early as day 2 of development, this would permit the selection of single, developmentally competent embryos and obviate the need for prolonged culture to the blastocyst stage as a means of selection prior to transfer.

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