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ORIGINAL RESEARCH

Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development *in vitro*

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ABSTRACT: This study investigated the relationship between human preimplantation embryo metabolism and aneuploidy rates during development *in vitro*. One hundred and eighty-eight fresh and cryopreserved embryos from 59 patients (33.9 ± 0.6 years) were cultured for 2–5 days. The turnover of 18 amino acids was measured in spent media by high-performance liquid chromatography. Embryos were either fixed for interphase fluorescent *in situ* hybridization analysis of chromosomes 13, 18, 19, 21, X or Y, or were assayed for mitochondrial activity. Amino acid turnover was different ($P < 0.05$) between stage-matched fresh and cryopreserved embryos due to blastomere loss following warming. The proportion of embryos with aneuploid cells increased as cell division progressed from pronucleate- (23%) to late cleavage stages (50–70%). Asparagine, glycine and valine turnover was significantly different between uniformly genetically normal and uniformly abnormal embryos on Days 2–3 of culture. By Days 3–4, the profiles of serine, leucine and lysine differed between uniformly euploid versus aneuploid embryos. Gender significantly ($P < 0.05$) affected the metabolism of tryptophan, leucine and asparagine by cleavage-stage embryos. Pronucleate zygotes had a significantly higher proportion of active:inactive mitochondria compared with cleavage-stage embryos. Furthermore, mitochondrial activity was correlated ($P < 0.05$) with altered aspartate and glutamine turnover. These results demonstrate the association between the metabolism, cytogenetic composition and health of human embryos *in vitro*.

Key words: aneuploidy / mitochondria / metabolomics / embryo development / FISH

Introduction

Assisted reproduction technologies (ART) are hampered by two major problems, namely low success rates and the high incidence of multiple pregnancies (The ESHRE Capri Workshop Group, 2000; Adamson and Baker, 2004). A major priority in human assisted conception clinics is therefore the development of a reliable diagnostic test which can be used to select those embryo(s) with the greatest developmental competence for transfer. Although the number of embryos transferred after ART can be limited to reduce the number of multiple gestations, more sophisticated methods must be employed if the success rates of IVF are to be improved as >40% of ART-derived embryos are known to harbour chromosomal abnormalities (Delhanty *et al.*, 1997; Munné *et al.*, 1998; Gianaroli *et al.*, 1999; Kahraman *et al.*, 2000; Marquez *et al.*, 2000). Errors in both meiotic and mitotic segregation of chromosomes in the oocyte and early embryo, respectively, can lead to different patterns of aneuploidy including polyploidy and chaotic mosaics. Indeed, the latter account for ~30% of aneuploidies in which multiple chromosomes are affected in each cell (Munné and Wells, 2003). However, despite the lethality of grossly aberrant chromosome complements, it is rarely possible to distinguish morphologically between healthy embryos and those with abnormal chromosomal complements. Consequently, many of these embryos are graded as suitable for transfer (Rienzi *et al.*, 2005; Nagy, 2008; Sturmey *et al.*, 2008). Overall, the evidence suggests that subjective scoring systems based on embryo

morphology are an ineffective means of selecting developmentally competent embryos (Munné et al., 2009). There is therefore a clear need to develop alternative objective approaches which can be used to select the best embryos for transfer and cyropreservation.

A considerable amount of research has been conducted to develop techniques which will reliably identify which *in vitro*-derived embryos have the potential to produce a successful pregnancy. For many years, embryo selection has been based on the subjective evaluation of embryo cell number, blastomere size, symmetry and fragmentation, and cleavage rate (Puissant et al., 1987; Steer et al., 1992; Van Royen et al., 1999; Hardarson et al., 2001; Racowsky et al., 2003; Rienzi et al., 2005). Recently, these methods have been extended to include pronucleate scoring of zygotes (Tesarik and Greco, 1999; Scott, 2003), cumulative scoring systems (Scott and Smith, 1998; Lan et al., 2003; Ciray et al., 2005; Chen and Kattera, 2006) and embryo respirometry (Scott et al., 2008a). The development of new generation, sequential embryo culture media, which promoted blastocyst development *in vitro* in conjunction with blastocyst grading (Gardner et al., 2000; Balaban et al., 2006) and elective single blastocyst transfer, has also been proposed as a means of improving the success of IVF while at the same time reducing multiple births (Gardner et al., 2004; Cutting et al., 2008). However, despite the advantages of many of these approaches as research tools, they have frequently proved difficult to incorporate into routine clinical practice. For example, blastocyst generation *in vitro* has the advantage that it is a non-invasive approach; however, it is costly in time and resources and there are concerns about the safety of prolonged culture to the blastocyst stage and the risk of potential aberrant epigenetic programming events during extended *in vitro* development (for review, see Huntriss and Picton, 2008). In contrast, as chromosomal aneuploidy is known to affect all stages of preimplantation embryo development (Marquez et al., 2000; Bielanska et al., 2002), recent attention has focused on the application of aneuploidy screening of oocytes and embryos as a means of improving embryo developmental potential and pregnancy outcome.

The rationale for using aneuploidy screening as the basis for embryo selection is that it will ensure that the transferred embryos are chromosomally normal (Marquez et al., 2000). Although some of the observed chromosomal errors such as pre-division of chromosomes in oocytes can be attributed to suboptimal culture environments and oocyte ageing *in vitro* (Clyde et al., 2003), review of the literature on the genetic composition of ART embryos has identified two groups of patients who consistently have a high risk of aneuploid embryos (Gianaroli et al., 1999; Kahraman et al., 2000). These include patients of advanced maternal age who have embryos with the most common aneuploidies, such as trisomy 21 and sex chromosome aneuploidies, which have been shown to increase 7- to 70-fold with age (Hook et al., 1983) and patients with repeated IVF failure (Gianaroli et al., 1999; Kahraman et al., 2000). In the latter group, the percentage of chromosomally abnormal embryos has been found to be directly proportional to the number of IVF failures. These observations are important as an increasing proportion of women undergoing assisted conception are over 35 years old (SART, 1992; Human Fertilisation and Embryology Authority, 2007). Although this evidence supports the use of aneuploidy screening in defined patient groups, the absence of convincing supporting data from prospective randomized clinical trials, together with the invasive nature of the biopsy procedure and the extra time and expense incurred, may not justify the widespread application of this technique as a routine method for embryo selection across all patients groups. Indeed, the use of aneuploidy screening for this purpose has recently been questioned (Platteau et al., 2006; Anderson and Pickering, 2008; Harper et al., 2008; Jansen et al., 2008; Mastenbroek et al., 2008; Yakin and Urman, 2008).

An alternative, non-invasive strategy which has consistently been shown to be of value in predicting the developmental potential of human embryos *in vitro* is the measurement of different aspects of the metabolomic changes in the embryo's culture environment (Brison et al., 2007; Nagy et al., 2008; Sturmey et al., 2008). Non-invasive metabolic profiling methods are frequently based on the analysis of spent embryo culture media. These include the use of near infrared and Raman spectroscopy (Seli et al., 2007; Scott et al., 2008b) and scanning electrochemical microscopy (Utsunomiya et al., 2008). One metabolomic approach which has proved robust across a number of species is the measurement of amino acid turnover in spent embryo culture media by high-performance liquid chromatography (HPLC) (Houghton et al., 2002; Brison et al., 2004). On the basis of the turnover of a small number of key amino acids, it is possible to discriminate accurately between morphologically similar fresh or frozen-thawed (Stokes et al., 2007) cleavage-stage embryos which have the capacity to form blastocysts (Houghton et al., 2002) and achieve pregnancy (Brison et al., 2004). However, although amino acid turnover appears to be predictive of fresh and cryopreserved embryo developmental potential *in vitro* (Houghton et al., 2002), it is unclear whether the regulation of amino acid metabolism is linked to the genetic health of the embryo. In this context, for example, according to the data available through the NCBI gene website, several amino acid transporters are localized on chromosome 19—a chromosome we have previously shown to be susceptible to abnormality in *in vitro* cultured human metaphase II oocytes (Clyde et al., 2003).

This study was therefore conducted to quantify the aneuploidy levels of a panel of six chromosomes that are frequently involved in early pregnancy loss (Munné et al., 1998) in human preimplantation embryos in relation to their amino acid metabolism profile and to investigate the possible role of mitochondrial potential and activity in this process. Spent culture media were collected from embryos cultured from Days 2 to 5 post-insemination for analysis of amino acid turnover. Embryos were then disaggregated and chromosomal health of all blastomeres was quantified using fluorescence *in situ* hybridization (FISH) analysis for chromosomes 13, 18, 19, 21, X and Y.

Materials and Methods

Unless otherwise stated, all chemicals and reagents used were obtained from Sigma Aldrich Chemical Company (Poole, Dorset, UK). Preimplantation human embryos surplus to therapeutic needs were donated to this research programme by patients being treated for infertility at the Reproductive Medicine Unit at Leeds General Infirmary, Leeds, and at the Bourn Hall Clinic, Cambridge. The experimental protocol was approved by the local research ethics committees and licensed by the UK Human Fertilisation and Embryology Authority. Ovarian stimulation was conducted using conventional GnRH agonist and antagonist protocols (Rutherford et al., 1988; Out et al., 2004), and transvaginal ultrasound-guided oocyte collection and fertilization were performed as described previously (Tang et al., 2006). Surplus cleavage-stage embryos not

required for cryopreservation on Day 2 post-insemination were donated in the Leeds clinic, whereas surplus cryopreserved two pronuclear (2PN) stage as well as cleavage-stage embryos were donated from the Bourn Hall Clinic.

Embryo cryopreservation and thawing

Cryopreserved embryos were slow-cooled in glass ampoules in commercially available freezing media containing 1,2-propanediol and sucrose according to the manufacturer's instructions (Medicult UK Ltd, Redhill, Surrey, UK). Embryos were re-warmed by exposure to air for ~30 s, followed by immersion in a water bath at 30°C until thawing had occurred. The contents of the ampoule were expelled into a 30 mm Petri dish (Nunc, Roskilde, Denmark) and embryos were transferred through sequential dilutions of cryoprotectants in the thawing solutions (Medicult UK Ltd) at room temperature according to the manufacturer's instructions, to progressively remove cryoprotectant agents. Embryos were then transferred to an embryo culture medium at 37°C for ~1 h to facilitate full rehydration.

Embryo culture

All embryos were scored using established morphological criteria and cell number (Houghton *et al.*, 2002). Embryos were then incubated in 4 μl drops of a defined culture medium with a composition based on that of human tubal fluid (Tay *et al.*, 1997; Houghton *et al.*, 2002) under washed, equilibrated paraffin oil (Medicult UK Ltd), at 37°C, and 5% CO_2 in air. After 24 h of culture, embryos were scored and were then either continued in culture in a fresh drop of medium up to the blastocyst stage or fixed and prepared for chromosome analysis. All drops of spent culture media were immediately frozen at −80°C until amino acid analysis.

Amino acid analysis

Following thawing, a 2 μl aliquot of culture media was removed from embryo culture drops and diluted 1:12.5 in HPLC-grade water. Embryo-free control drops incubated alongside the embryo drops were treated in the same manner to allow for any non-specific amino acid degradation/appearance. Analysis of 18 amino acids was performed using HPLC as described previously (Houghton *et al.*, 2002).

Mitochondrial activity

5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Invitrogen, Paisley, UK) is a commonly used probe for the assessment of mitochondria membrane potential (Reers *et al.*, 1995; Jones *et al.*, 2004; Van Blerkom and Davis, 2006). Upon interaction with mitochondria, the probe exists as a monomer in the presence of low potential difference (low activity) and fluoresces green, whereas greater membrane potential (high activity) causes dimerization of the probe which then fluoresces orange. By measuring the ratio of green to orange fluorescence, it is therefore possible to quantify membrane potential ($\Delta\psi m$) which reflects mitochondrial activity. Embryos were incubated in 1 μg/ml JC-1 in Hanks-balanced salt solution (HBSS containing 1 mg/ml BSA and 0.01% dimethylsulphoxide) for 30 min at 37°C and washed four times in HBSS. Embryos were placed in drops of holding medium under oil on a stage heated to 37°C, whereas whole-oocyte green and orange fluorescence was measured using a conventional Zeiss Axioplan epifluorescence microscope (Van Blerkom *et al.*, 2002) with photon multiplier tube and photometer attachments (Photon Technology International, Ford, W. Sussex, UK). Data were processed using Felix 32 software (Photon Technology International). The JC-1 ratio was calculated from the measured orange and green fluorescence values.

Embryo chromosome preparation and fixation

One-cell embryos (2PN stage)

Embryos were incubated overnight in embryo culture media containing 0.15 μg/ml podophyllotoxin and 0.15 μg/ml vinblastine sulphate to induce cell cycle arrest (Angell *et al.*, 1988; Rosenbusch *et al.*, 2001). Embryos were then incubated in 0.1% (w/v) hypotonic sodium citrate for 15–20 min at room temperature before transfer to ice-cold 3:1 methanol:glacial acetic acid for ~3 s before being spread on to a Superfrost Plus slide (VWR, Lutterworth, UK).

Cleavage-stage embryos

The embryos were stripped of their zona pellucida by brief exposure to acidified Tyrode's saline solution (pH 3, room temperature). Each embryo was incubated in 0.1% (w/v) hypotonic sodium citrate for 30 min at 4°C before being transferred to Dulbecco's phosphate-buffered saline (Ca^{2+} and Mg^{2+}-free) for 5 min. Finally, the embryo was transferred to ice-cold 3:1 methanol:glacial acetic acid for ~3 s before being spread onto a Superfrost Plus slide. The slide was observed under phase-contrast microscopy and, where necessary, small droplets of 5:4:1 methanol:glacial acetic acid:water were added to assist cell spreading. All slides were aged overnight before dehydration through a series of ethanol solutions (70%, 90% and 100%, 5 min in each). Slides were stored on desiccant at 4°C until FISH analysis.

Cytogenetic analysis and evaluation

Cytoplasmic debris was removed by a 0.5–5 min incubation in 0.01% (w/v) pepsin in 0.01 M HCl at 37°C prior to hybridization. Simultaneous FISH analysis for five chromosomes (13, 18, 21, X and Y) was conducted on all blastomeres according to the methodology described by Clyde *et al.* (2001) using a multicolour kit (PGT, MultiVysion PGT, Abbot, Maidenhead, UK) containing a premixed cocktail of locus-specific probes for chromosomes 13 and 21, and centromeric probes for chromosomes 18, X and Y. The chromosomal constitution of each blastomere nucleus was classified as normal (diploid) and abnormal (haploid, aneuploid, mosaic or chaotic) for the chromosomes analysed according to the definitions described by Ziebe *et al.* (2003). On the basis of the results of sibling blastomeres, each embryo was then classified as uniformly genetically normal (100% normal with all blastomeres diploid for all chromosomes), overall normal (embryos with >50% of analysed cells having normal nuclei for all chromosomes), overall abnormal (≥50% of analysed cells having abnormal nuclei) or uniformly genetically abnormal (0% normal blastomeres). A subset of fresh, cleavage-stage embryos were re-analysed with telomeric probes specific for the p and q arms of chromosome 19 (Telvysion, Abbot, Maidenhead, UK).

Statistical analysis

The data were confirmed as being normally distributed using the Anderson–Darling test. Statistical analyses were conducted using the χ^2, Student's *t*-test or Mann–Whitney *U*-test, as appropriate. A *P*-value of <0.05 was considered to be significant.

Results

A total of 66 fresh and 122 cryopreserved embryos were analysed from 59 patients of mean age 33.9 ± 0.6 years (range 24–42 years).

Amino acid turnover of cryopreserved and fresh embryos

On Days 2–3 post-fertilization, certain amino acids (glutamate, asparagine, glycine and arginine) were metabolized differentially by fresh embryos and those that had been cryopreserved (Fig. 1). On Days 2–3 of culture, embryos that had been cryopreserved on Day 2 had a significantly greater production of glutamate compared with either embryos cryopreserved on Day 1 or fresh embryos. Turnover of asparagine and glycine was significantly different between embryos cryopreserved on Day 1 or 2 post-fertilization, but neither was significantly different to fresh, control embryos (Fig. 1). Arginine was consumed at significantly greater rates by embryos cryopreserved on Day 2 (Fig. 1). On Days 2–3, embryos cryopreserved at the 1-cell (2PN) stage did not have significantly different amino acid profiles compared with fresh controls (Fig. 1).

Blastomere damage and cell loss occurred on thawing in some Day 2 cryopreserved cleavage-stage embryos. The effect of cell loss and developmental progression (progressing or arrested) on amino acid turnover by embryos on Day 2–3 post-fertilization was investigated (Fig. 2). The differences in amino acid turnover on Days 2–3 observed in cleavage-stage embryos cryopreserved on Day 2 appeared largely to be associated with cell loss and/or arrested development. Embryos cryopreserved on Day 2 that lost cells upon thawing were also significantly ($P < 0.05$) more likely to arrest during culture (58% arrested compared with embryos that did not lose cells of which 9% arrested). Cell loss was associated with altered turnover of asparagine, glycine and arginine compared with Day 1 embryos, Day 2 intact embryos and fresh controls (Fig. 2b–d). In this group, embryos that arrested during the Days 2–3 of culture had increased glutamate turnover compared with Day 1 embryos, Day 2 intact embryos and fresh controls (Fig. 2a).

On Day 3–4 post-fertilization, the only amino acid affected by prior embryo cryopreservation was arginine. Embryos cryopreserved on Day 2 showed differential arginine consumption according to whether embryos had thawed intact (2.2 ± 1.3 pmoles/h; $n = 11$) or had suffered loss of one or more cells (3.8 ± 0.92 pmoles/h; $n = 11$; $P = 0.03$). No other embryos cryopreserved on Day 1 or 2 had altered amino acid turnover during Days 3–4.

It has been hypothesized that metabolically 'quiet' human and mammalian embryos have better developmental potential (Leese, 2002; Baumann *et al.*, 2007; Leese *et al.*, 2007; Leese *et al.*, 2008). There was no significant difference between net amino acid production between different embryo groups, but embryos cryopreserved on Day 2 which subsequently lost cells upon thawing and underwent developmental arrest during Days 2–3 had consistently and significantly ($P < 0.05$) higher net amino acid consumption, net amino acid activity and net turnover than any other tissue group.

Effect of maternal patient age on embryo amino acid turnover

When maternal age was accounted for, significant differences in turnover of several amino acids were seen in embryos cultured on Days 1–2 (Fig. 3). Aspartate, glutamine, threonine, arginine, tyrosine, valine and isoleucine were consumed in greater quantities by embryos from patients under 37 years of age, whereas lysine was consumed at greater rates by embryos donated by patients aged 37 years or over. The data from fresh embryos and embryos cryopreserved at the PN stage were pooled for the analysis of the effect of maternal age. Embryos that had been cryopreserved on Day 2 were excluded from further analysis due to the impact of cryopreservation on amino acid turnover in this group. On Days 2–3, aspartate (0.13 ± 0.07 versus -0.11 ± 0.05 pmoles/h for embryos from patients under 37 years and 37 years and older, respectively) and glutamate (0.15 ± 0.09 versus -0.27 ± 0.15 pmoles/h for embryos from patients under 37 years and 37 years and older, respectively) were consumed in greater quantities by embryos donated from patients of advancing maternal age. Tyrosine was produced in greater quantities by embryos on Days 2–3 that were derived from younger patients (0.08 ± 0.03 versus -0.01 ± 0.02 pmoles/h for patients under 37 years and 37 years and older, respectively). On Days 3–4 of culture, there were no significant effects of patient age on amino acid turnover.

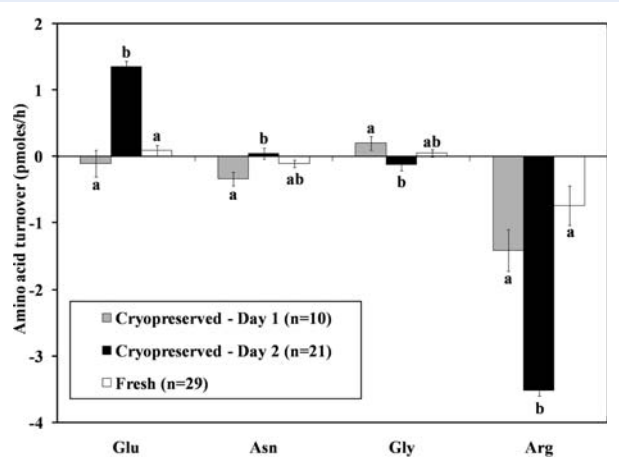

Figure 1 Turnover of glutamate (Glu), asparagine (Asn), glycine (Gly) and arginine (Arg) by embryos on Days 2–3 post-fertilization. Embryos were either cryopreserved on Day 1 (2PN stage) or Day 2 (cleavage stage) or were not cryopreserved (Fresh). Values are mean amino acid turnover per embryo ± SEM for the number of replicates shown in parentheses. Negative values indicate amino acid consumption, whereas positive values indicate production. Values with different letters are significantly different ($P < 0.05$) for the number of analyses shown in parentheses.

Cytogenetic analysis

Clear FISH signals were obtained from 544 cells (Fig. 4) harvested from 112 embryos of which 85% had corresponding amino acid profile data. No significant differences between different-grade embryos were observed. The demography of the FISH data showed that there were no statistically significant differences in the number of male and female embryos analysed, such that 53% of embryos were female and 47% were male. For those embryos with one or more abnormal cells, 33% of the chromosome abnormalities involved only one of the six chromosomes tested, 20% involved two chromosomes and the remainder (47%) involved three or more chromosomes. Hypoploidies and hyperploidies accounted for 54% and 46% of the chromosomal abnormalities, respectively. Numerical

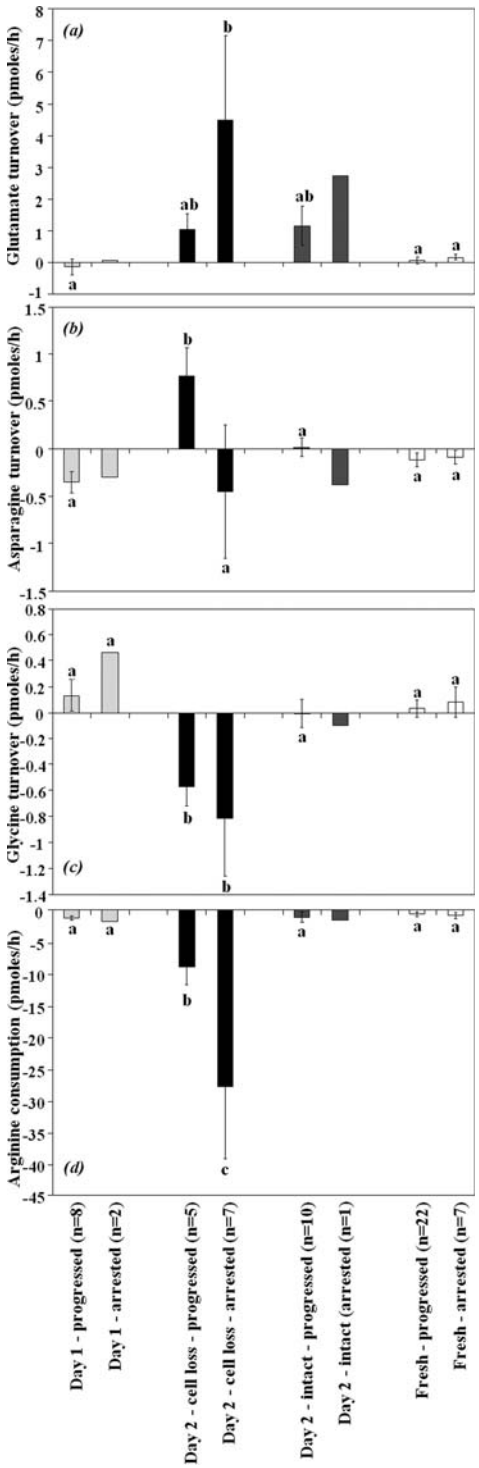

Figure 2 Turnover of glutamate (a), asparagine (b), glycine (c) and arginine (d) by progressing and arrested embryos on Days 2–3 post-fertilization that were cryopreserved on Day 1 (2PN stage) or Day 2, compared with fresh embryos. Values are means ± SEM for the number of embryos shown in parentheses, adjusted for any cell loss. Statistical significance is identified by differences in the histogram labels such that bars labelled with different letters are significantly different ($P < 0.05$) for the number of analyses shown in parentheses. Conversely, bars labelled with common labels are not significantly different.

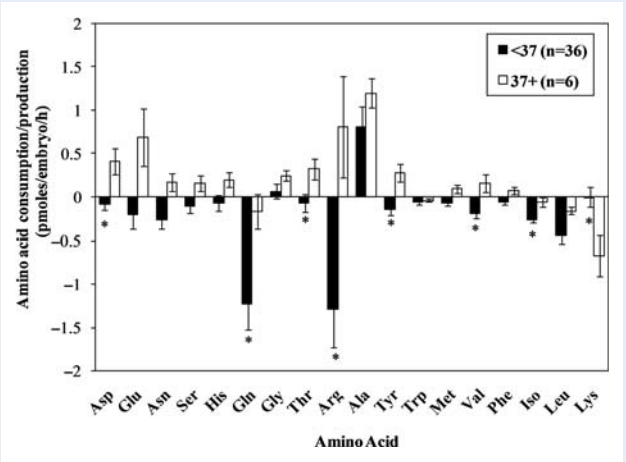

Figure 3 Effect of maternal age on amino acid turnover by embryos cultured from Days 1–2. Values are means ± SEM for the number of embryos shown in parentheses. Significant differences are indicated by an asterisk ($P \leq 0.05$).

abnormalities in chromosomes 13 and 21 were the most prevalent, accounting for 28% and 25% of the genetic errors, respectively. Abnormalities in chromosome 18 accounted for 15% of errors, whereas errors in X and/or Y accounted for 32%. A small subset of the fresh embryos underwent a second round cytogenetic evaluation for chromosome 19. On Day 3, 100% of the embryos that had normal counts for chromosomes 13, 18, 21 and X/Y also had normal, diploid, counts for chromosome 19 ($n = 5$). Of all the embryos analysed for chromosome 19 on Days 3–5, 17.4% had one or more cells with an abnormal count for chromosome 19.

Effect of cryopreservation on incidence of chromosome abnormalities

There was no significant difference in the frequency of chromosome abnormalities on Day 3 (after culture from Days 2–3) in embryos that had been cryopreserved at the PN stage compared with fresh embryos. Cell loss post-thaw did not affect the incidence of chromosome abnormality in embryos cryopreserved on Day 2 that were processed for FISH on Day 4.

Stage of embryo development

Following FISH, the proportion of embryos with chromosome abnormalities at different stages of development was analysed (Fig. 5). Pronuclear-stage embryos had an abnormality frequency of 23% ($n = 26$). The percentage of genetically abnormal embryos increased with successive days of development (Fig. 5). Further analysis revealed that this was due to an increasing occurrence of embryos with a mixture of normal and abnormal cells (mosaic) as development progressed (Table I). The proportion of uniformly abnormal embryos did not change significantly.

Association between amino acid profile and chromosome abnormalities

Amino acid turnover on Days 2–3 and 3–4 post-fertilization was compared between embryos that were uniformly normal (no

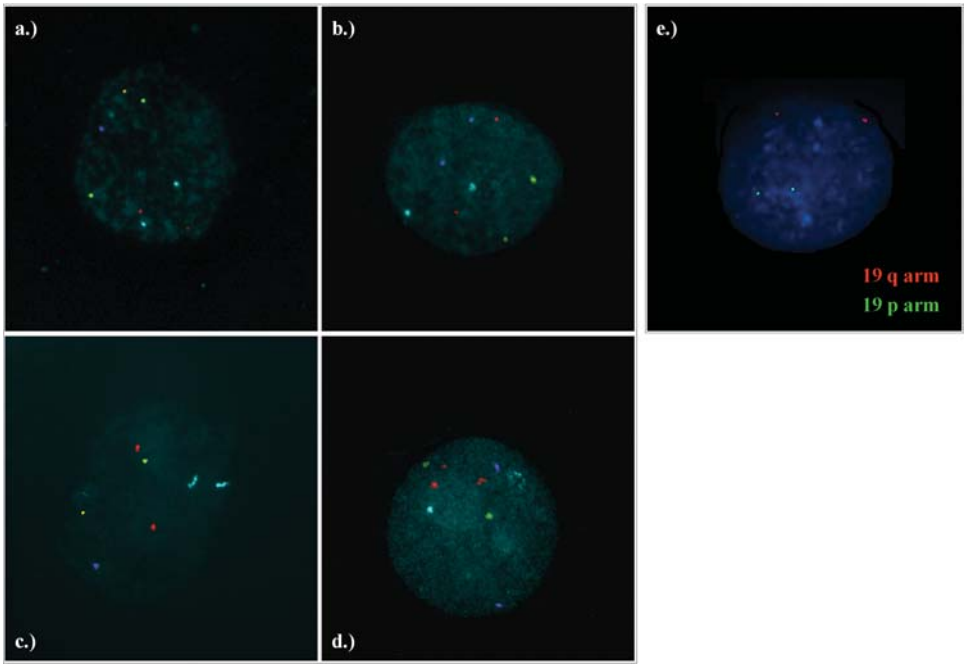

Figure 4 Interphase FISH of embryo blastomeres for chromosomes: 13 (Spectrum Red™), 18 (Spectrum Aqua™), 21 (Spectrum Green™), X (Spectrum Blue™), Y (Spectrum Gold™) and 19 (key shown in image). (a) Nuclei were counterstained with Cot-1 DNA labelled with Spectrum Aqua™. Images (a) and (b) are nuclei from male and female embryos, respectively, with normal number for the chromosomes analysed. (c) A male nucleus with monosomy 21 and (d) a female nucleus with trisomy 13. (e) Identification of chromosome 19 using telomeric probes for the p (green) and q (red) arms, counterstained with DAPI.

abnormal cells) and uniformly abnormal (no normal cells) (Fig. 6). On Days 2–3, turnover of asparagine, glycine and valine was significantly different between normal and abnormal embryos (Fig. 6a). The differences observed in tyrosine and lysine approached significance (Fig. 6a). As development progressed, there was a shift in the observed difference such that on Days 3–4, turnover of serine, leucine and lysine was significantly different between normal and abnormal embryos (Fig. 6b). On Days 2–3 and 3–4, embryos containing a mixture of normal and abnormal cells did not have significantly different amino acid turnover relative to uniformly normal or uniformly abnormal embryos.

Relationship between embryo sex and amino acid turnover

The influence of embryo sex, as determined by FISH for chromosomes X and Y, was investigated. On Days 1–2, no significant differences in amino acid turnover were observed between female ($n = 5$) and male ($n = 6$) embryos. In contrast, on Days 2–3, tryptophan was consumed at greater rates by male embryos (-0.06 ± 0.02 pmoles/h, $n = 12$) compared with female embryos (0.002 ± 0.01 pmoles/h, $n = 16$; $P = 0.04$). Similarly, leucine was consumed at greater rates by male embryos (-0.38 ± 0.06 pmoles/h) compared with female embryos (-0.18 ± 0.05 pmoles/h; $P = 0.014$). By Days 3–4 of culture, only asparagine was differentially metabolized by female and male embryos (-0.14 ± 0.07 pmoles/h, $n = 10$ versus 0.20 ± 0.11 pmoles/h, $n = 13$ for female and male embryos, respectively, $P = 0.025$).

Mitochondrial activity

Mitochondrial activity, as assessed by JC-1 ratio, was analysed in a subset of embryos. A high value for JC-1 ratio indicates a high proportion of mitochondria with low $\Delta\psi m$ and therefore low activity which presumably produce reduced ATP. Conversely, a low JC-1 value indicates a low proportion of inactive mitochondria and a high proportion of active mitochondria. Relative mitochondrial activity changed significantly during early development (Table II). One-cell embryos had a significantly greater proportion ($P < 0.05$) of active mitochondria compared with all subsequent stages of development. In contrast, cleavage-stage embryos that had arrested had proportionally more inactive ($P < 0.05$) mitochondria compared with their counterparts that were still actively progressing. A similar finding was observed in blastocysts ($P < 0.05$) (Table II). Embryos cryopreserved on Day 2 that were subsequently thawed, cultured and assessed for mitochondrial activity on Day 4 had differences in JC-1 ratio that approached significance according to whether the thawed embryos were cellularly intact or whether they had lost one or more cells post-thaw. Embryos that thawed intact had a lower JC-1 ratio of 1.7 ± 0.3 ($n = 10$) and therefore a higher number of active mitochondria compared with embryos with cell loss (2.9 ± 0.6; $n = 9$; $P = 0.066$) which contained proportionally more inactive mitochondria. Mitochondrial activity was also significantly correlated with aspartate ($P = 0.04$, Pearson $= -0.32$) and glutamine turnover ($P = 0.041$, Pearson $= 0.321$). At each stage of embryo development, the proportion of inactive mitochondria was

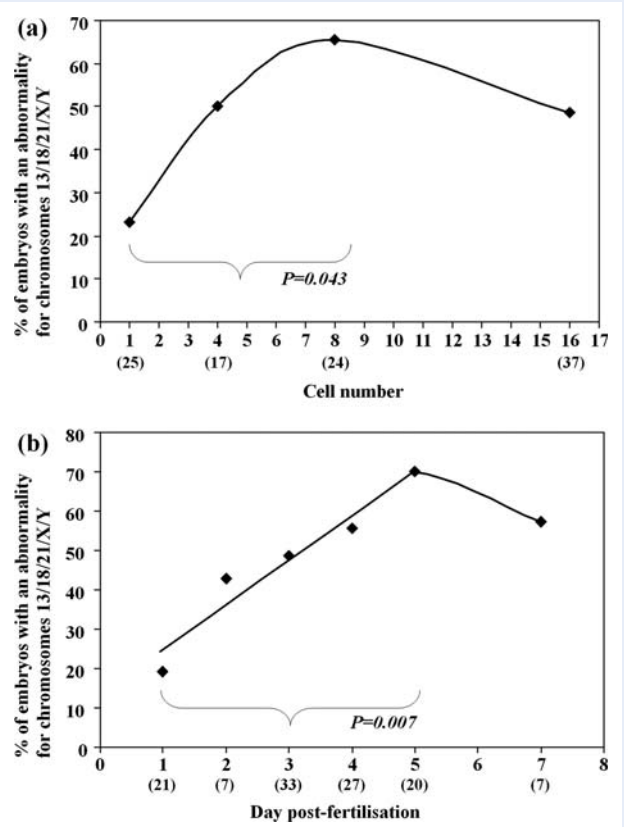

Figure 5 Frequency of embryos with one or more abnormalities for chromosomes 13, 18, 21, X or Y during preimplantation development, with respect to embryo cell number (a) and time post-fertilization (b). The numbers of embryos analysed are shown in parentheses. Brackets indicate significant trends (*P*-value shown in italics).

Table I Incidence of chromosome abnormalities during early preimplantation development.

Day	% uniformly normal	% uniformly abnormal	% mixture normal/ abnormal cells
1	81.0	19.0	0.0
2	57.1	14.3	28.6
3	51.5	15.2	33.3
4	44.4	22.2	33.3
5	30.0	25.0	45.0
P	0.007	0.200	0.042

P-values for trend analysis of each column are shown.

higher in embryos that had ≥50% abnormal cells compared with embryos with >50% normal cells but the differences at each stage were not significant. However, when cleavage-stage embryos that were either uniformly normal or uniformly abnormal were analysed, mitochondrial activity was observed to be significantly higher in normal embryos (1.11 ± 0.16; $n = 4$) compared with uniformly abnormal embryos which had more inactive mitochondria (2.71 ± 0.51; $n = 5$, $P = 0.041$).

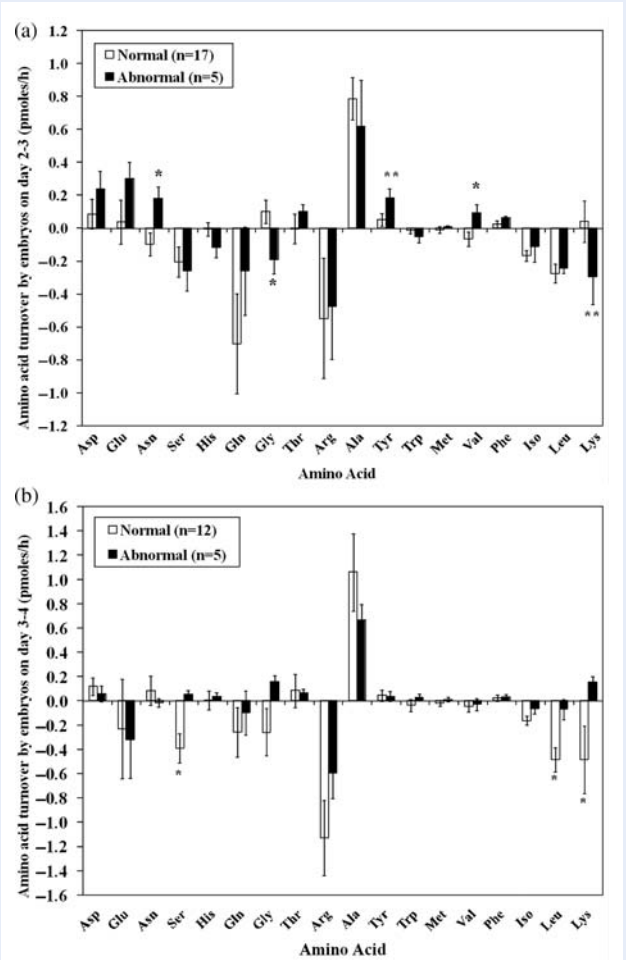

Figure 6 Amino acid turnover by individual embryos in relation to abnormality of chromosomes 13, 18, 21, X and Y on Days 2–3 (a) and Days 3–4 (b). Data from embryos cryopreserved on Day 1 were pooled with data from stage-matched fresh embryos for analyses on Days 2–3, whereas data from embryos cryopreserved on Day 2 and fresh embryos were pooled for analyses on Days 3–4. Embryos that were uniformly chromosomally normal or abnormal are shown. Values are means ± SEM of the number of embryos shown in parentheses. *$P < 0.05$ and a double-asterisk indicates a trend towards significance; $P = 0.099$ for tyrosine and $P = 0.085$ for lysine.

Patient age had a significant influence on mitochondrial activity in PN-stage embryos. Embryos from young patients of 37 years or less had significantly higher mitochondrial activity (0.59 ± 0.05, $n = 8$), compared with embryos from older patients over 37 years (1.03 ± 0.09, $n = 4$; $P = 0.014$). Maternal age did not significantly influence mitochondrial activity at subsequent stages of embryo development.

Discussion

It is now possible to base embryo selection criteria on quantifiable biological characteristics of the developing embryo rather than on subjective measures. The results of this study confirm and extend previous

Table II Mitochondrial activity ratios in human embryos, assessed by JC-1 staining.

Embryo stage	*n*	JC-1 ratio	SEM
1-cell (2PN)	12	0.74[a]	0.08
Cleavage—progressing	18	2.25[b]	0.24
Cleavage—arrested	22	4.95[c]	0.93
Blastocyst—progressing	3	1.84[b]	0.62
Blastocyst—arrested	8	8.89[d]	1.38

Values are means ± SEM of the number of embryos shown. Values with different letters are significantly different ($P < 0.05$). Higher values indicate a greater proportion of inactive mitochondria.

measurements of the turnover of a physiological mixture of 18 amino acids by human preimplantation embryos (Houghton *et al.*, 2002; Stokes *et al.*, 2007). In addition, this is the first report to compare directly the amino acid turnover of fresh and cryopreserved embryos and to link aspects of amino acid profile with cytogenetic assessments of embryo health. Reduced blastocyst formation (Houghton *et al.*, 2002; Stokes *et al.*, 2007) and increased DNA damage (Sturmey *et al.*, 2009) have been linked to greater overall amino acid metabolism which supports the hypothesis that metabolically 'quiet' embryos have better developmental competence (Leese, 2002). The current data on cryopreserved-thawed embryos reinforce this observation, as embryos cryopreserved on Day 2 that subsequently lost blastomeres on thawing had reduced rates of development and a higher overall amino acid consumption and turnover. Furthermore, embryo cryopreservation on Day 2 post-insemination was associated with altered amino acid turnover on Days 2–3 of culture. In marked contrast, embryos that had been cryopreserved on Day 1 (2PN stage) did not have an altered amino acid profile by Days 2–3 of culture, compared with fresh controls. The differential amino acid profile of embryos cryopreserved on Day 2 was associated with shorter recovery time compared with embryos cryopreserved on Day 1 and was also associated with cell loss. This suggests that cryopreservation *per se* does not alter embryo metabolism, but rather that cellular damage or stress associated with blastomere loss does. Additionally, the turnovers of glycine and arginine were exacerbated by developmental arrest.

Amino acids have numerous roles in early embryo development in addition to protein synthesis. Glycine is known to be an osmoregulator, balancing changes in embryo cell volume (Hammer *et al.*, 2000; Baltz, 2001; Steeves and Baltz, 2005). In this context, glycine was consumed in greater quantities by embryos cryopreserved on Day 2 that lost cells during thawing. However, it is unclear from the present study whether the increased glycine consumption occurred in response to cell loss or whether it actually was the cause of cell swelling and subsequent lysis. Arginine consumption has been linked to increased nitric oxide (NO) production by mammalian cells (Cendan *et al.*, 1996). NO is necessary for normal mammalian embryo development (Tranguch *et al.*, 2003; Manser *et al.*, 2004) but elevated NO is associated with induction of apoptosis (Brune *et al.*, 1995, 1999; Nicotera *et al.*, 1995) and poor human embryo quality (Lee *et al.*, 2004). It has previously been shown that higher rates of arginine consumption by human embryos are associated with poorer developmental outcomes (Houghton *et al.*, 2002). In the present study, glutamate was produced in greater quantities by embryos cryopreserved on Day 2 that suffered cell loss and developmental arrest, post-thaw. This might reflect an attempt to neutralize increased ammonium production by these embryos (Lane and Gardner, 1995; Orsi and Leese, 2004). It is pertinent to note that recent preliminary findings suggest that human embryos which are less likely to result in pregnancy have elevated glutamate output (Seli *et al.*, 2008). Regardless of the differences in amino acid turnover between the embryos as each developmental stage, variation in metabolism between the early and late time points may also reflect the transition from maternally sourced mRNA to embryo-driven transcription and translation following embryonic genome activation.

In the current work, cytogenetic evaluations were conducted using published definitions of embryo normality (Bielanska *et al.*, 2002; Ziebe *et al.*, 2003) such that embryos with ≥50% normal diploid blastomeres were considered to be viable. This definition facilitated comparison between embryos which were 100% genetically normal or 100% abnormal with those containing a mixture of cell types. The analysis of chromosomes presented here correlates with the observations of other studies (Ziebe *et al.*, 2003) and shows that the chromosome complement of up to 70% of surplus ART embryos is compromised by non-disjunction events and mosaicism, depending on the stage of development and number of days post-fertilization.

On the first day post-fertilization, prior to the onset of mitotic cell divisions, the incidence of aneuploidy involving one or more of the six chromosomes tested was ~20%, which is within the range of values reported for good quality oocytes: 8–10% (Smitz *et al.*, 2007), 9–14% (Volarcik *et al.*, 1998) and 35% (Sandalinas *et al.*, 2002). The proportion of embryos with one or more chromosomally abnormal cells is known to increase during the first few days post-fertilization, largely due to mosaicism, agreeing with the observations of Bielanska *et al.* (2002). To fully asses the consequences of chromosome alterations on embryo metabolism and developmental potential, it is necessary to conduct full karyotype analyses of the complete compliment of blastomeres within each individual embryo. This goal can be achieved by achieved by multiparameter FISH analysis of metaphase spreads of blastomeres using our previously published protocols (Clyde *et al.*, 2003). However, although the current protocol did support the arrest of blastomeres in metaphase, insufficient cells were arrested at this stage of the cell cycle to permit meaningful analysis and the greater majority of embryo blastomere remained in interphase at the end of embryo culture. Consequently, the current study in line with accepted practices used interphase FISH on a subset of embryo chromosomes (Smith *et al.*, 1998; Marquez *et al.*, 2000; Thum *et al.*, 2008) to analyse all blastomeres and so provide an index of the cytogenetic health of each embryo. Indeed, the chromosome series analysed in the current work contribute to ~95% of all chromosome disorders observed in live births and 20–27% of the abnormalities observed in human metaphase II oocytes (chromosomes 13, 18, 21 and X; S.E.H. SE and H.M.P., unpublished observations; Clyde *et al.*, 2003). In support of the proposed close association between early embryo metabolism and karyotype, we have previously analysed amino acid turnover and energy metabolism in relation to the full karyotype analysis of metaphase II human oocytes and have found these indices of oocyte metabolism to be associated with patient aetiology and the capacity of the oocytes to progress

to metaphase II *in vitro* (H.M.P., S.E.H. and K.E. Hemmings, unpublished data). Similarly, in bovine oocytes, the competence of *in vitro* matured oocytes to fertilize and progress to the blastocysts *in vitro* can be predicted by the measurement of metaphase II oocyte metabolism (K.E. Hemmings, H.J.L. and H.M.P., unpublished data). Furthermore, a strong association between zygote and oocyte metabolism is expected as the cytoplasmic inheritance of the fertilized zygote originates from the oocyte. With regard to human embryo quality, the limited cytogenetic data set presented here clearly show that altered metabolic turnover by embryos reflects the common aneuploidies. Furthermore, within the cohort of abnormal embryos analysed in the current work, approximately one-third had abnormalities involving just one chromosome, whereas the remaining abnormal embryos had two or more affected chromosomes. On this basis, abnormal embryos tended to have more than one abnormality, making it more likely that at least one of the abnormal chromosomes will be detected in the current study. It is likely that at least a small proportion of the embryos that we have classified as 'uniformly normal' may have an undetected chromosome abnormality which could potentially affect amino acid turnover; however, a large proportion can be expected to be normal.

The observed increase in post-zygotic genetic errors in human embryos grown *in vitro* is likely to be due to multiple, contributing reasons. Early embryos are also thought to be permissive with respect to mitotic checkpoints (Wells *et al.*, 2005) and spindle defects are also common (Chatzimeletiou *et al.*, 2005), but whether the increase in mosaicism is a natural phenomenon or is related to the suboptimal environment *in vitro* or suboptimal ovarian stimulation protocols is not well understood. Ovarian hyperstimulation is thought to be associated with reduced oocyte quality in humans (Akagbosu *et al.*, 1998) and mice (Van der Auwera and D'Hooghe, 2001), including chromosome abnormalities (Simon *et al.*, 1998; Baart *et al.*, 2006; Dursun *et al.*, 2006). However, even unstimulated cycles produce human embryos with high rates of aneuploidy (Verpoest *et al.*, 2008), suggesting that ovarian hyperstimulation is not the only cause of chromosome aberrations. The effect of advancing maternal age on incidence of oocyte and embryo chromosome abnormalities is well documented (Dailey *et al.*, 1996; Pellestor *et al.*, 2005; Munné *et al.*, 2007). The current study has also highlighted for the first time some notable differences in the turnover of amino acids by embryos donated from younger compared with older patients, although the effects of age on amino acid turnover diminished as embryo development progressed. Finally, human and mammalian embryos are known to be susceptible to the *in vitro* effects of supra-physiological oxygen tensions (Catt and Henman, 2000; Orsi and Leese, 2001). Air containing 5% CO_2 is still the most commonly used culture environment for *in vitro*-produced human embryos, although oxygen tension *in vivo* is in the region of 5% compared with 21% in air. Numerous studies of animal and human embryos have demonstrated an association between oxygen tension and reduced embryo quality, likely due to formation of reactive oxygen species (ROS). Although it is possible in the current study that ROS contributed to the increasing incidence of chromosomal abnormalities which were observed with embryo developmental progression, this does not explain the reduction in chromosome abnormalities recorded after Day 5 of culture.

In oocytes, completion of meiosis involves energy-demanding processes including chromosome congression, segregation, separation and polar body formation. The oocyte and early embryo are thus heavily reliant on mitochondrial respiration of energy metabolites to generate energy for subsequent embryo growth and cell replication (Biggers *et al.*, 1967; Hardy *et al.*, 1989; Wilding *et al.*, 2001; Downs *et al.*, 2002; Johnson *et al.*, 2007). Declining oocyte mitochondrial function has been linked to ageing (Tarin, 1996; Wilding *et al.*, 2001; Eichenlaub-Ritter *et al.*, 2004) and it is thought that this may predispose oocytes to meiotic errors (Schon *et al.*, 2000; Eichenlaub-Ritter *et al.*, 2004). The oocyte is especially reliant on pyruvate, but other substrates, including amino acids, are also metabolized for energy generation via mitochondrial respiration.

This study assessed whether there were any demonstrable links between embryo amino acid profile, mitochondrial activity and aneuploidy. Embryo mitochondrial activity was linked to amino acid turnover; however, although the JC-1 ratio was consistently higher in embryos with chromosomal abnormalities, the differences between overall normal (>50% normal cells) and overall abnormal (≥50% abnormal cells) embryos were not significant. However, when embryos were assessed that had either 100% normal cells or 100% abnormal cells, a significant difference in mitochondrial activity was observed. These data point towards a possible link between chromosome abnormality and the quality of the mitochondrial cohort inherited by the embryo from the oocyte. Increased numbers of embryos need to be analysed to confirm this preliminary finding.

Amino acid turnover has previously been shown to predict human embryo developmental potential (Houghton *et al.*, 2002; Brison *et al.*, 2004). The current paper represents the first report of an association between amino acid turnover and human embryo genetic health. It is of particular interest that differences were detected in asparagine and glycine metabolism when uniformly normal versus uniformly abnormal embryos were compared, as these amino acids have been shown to be of value in the prediction of future *in vitro* embryo developmental to the blastocysts stage and subsequent pregnancy potential following embryo transfer. There are multiple possible explanations for the current observations. With extra or missing copies of chromosomes, nuclear gene dosage becomes perturbed, which can lead to altered regulation of transcription (Rachidi and Lopes, 2007; Veitia *et al.*, 2008). Also, it has previously been reported that chromosomes with altered copy numbers have altered nuclear location (McKenzie *et al.*, 2004). Additionally, in abnormal nuclei, all chromosomes (regardless of copy number) tend to have altered nuclear distribution (McKenzie *et al.*, 2004). Chromosome and nuclear architecture is known to regulate gene expression (Finlan *et al.*, 2008; Simonis and de Laat, 2008), so it is possible that in abnormal cells, transcription from multiple chromosomes may be altered. The different elements of amino acid transport and metabolism are coded by multiple genes on multiple chromosomes (NCBI Entrez Gene website). Alteration of chromosome content can therefore be hypothesized to alter the subcellular machinery and these changes would be manifest by an alteration in blastomere metabolism. In support of this idea, individuals with trisomy 21 have altered plasma amino acid levels (Lejeune *et al.*, 1992; Heggarty *et al.*, 1996), including lysine and serine. Furthermore, in trisomic mice, the activity of certain enzymes involved in amino acid metabolism is affected (Vaisman *et al.*, 1981). In this context, for example, chromosome 19 is known to carry several amino acid transporter genes (NCBI Entrez Gene website) and we have previously shown that ~6% of human metaphase II oocytes

have abnormal copy numbers of chromosome 19 (Clyde et al., 2003). In the current study, 17.4% of cleavage-stage embryos carried one or more cells with abnormal copy number for chromosome 19. Although this is higher than the frequency of abnormality observed in oocytes, it may be explained by the progressive increase in chromosome abnormalities that occur as cells replicate during early embryogenesis, as observed in the current study and by Bielanska et al. (2002). Amino acid profiles were not obtained from a sufficient number of embryos for turnover to be compared with respect to chromosome 19 content. Additional analyses are therefore required to test this hypothesis further.

The data presented clearly show that amino acid consumption/production is predictive of blastocyst potential and the link between amino acid profile and genetic health in wholly normal versus abnormal embryos is of clinical importance. However, amino acid turnover *per se* cannot yet be used as an index to predict the genetic health of embryos composed of a mixture of normal and abnormal cells on Days 2–3 post-insemination. This observation is not altogether surprising as the frame of reference used for classification of embryo normality in the current study was based on the definition used by Ziebe et al. (2003) to characterize which embryos were likely to develop or not. At the time of writing, there is still no accurate data to inform the debate on exactly how many abnormal cells a cleavage-stage embryo can accommodate before developmental competence is lost. Inclusion of the experimental series presented in the present study will help answer this important question but extensive further analyses are required to accurately address this issue. Nevertheless, these results, together with the close correlation between the predicted developmental potential of embryos by amino acid profiling and implantation rates achieved following transfer of embryos typed retrospectively (Brison et al., 2004), suggest: (i) that human embryos can accommodate a proportion of genetically abnormal cells as they develop; and (ii) that the upper ceiling of pregnancy rate following selection based on amino acid profiling will ultimately be set by the genetic health of the embryo.

The effect of embryo genetic sex upon amino acid turnover was examined. This data set provides the first direct evidence to show that amino acids are differentially metabolized by male versus female cleavage-stage human embryos. On Days 1–2, the fact that no significant difference in metabolism was observed between male and female embryos is not surprising as genome activation has not occurred by this time and the cellular metabolism of the zygote is largely determined by the cytoplasmic legacy inherited from the oocyte. In contrast, on Days 2–3 and 3–4, differences in the turnover of 3 of the 18 amino acids analysed, tryptophan, leucine and asparagine, were observed. Recent data have demonstrated that gender-specific differences exist in 7 of the 18 amino acids and 2 of the 18 amino acids analysed between male and female bovine blastocysts derived *in vitro* and *in vivo*, respectively (Sturmey et al., 2010). Furthermore, in this species, the gender association between amino acid uptake and production by bovine blastocysts appeared to be dynamic and changes as blastocyst development advances. The identity of the amino acids which exhibit sex-specific differences appears not only to differ between human and bovine embryos but it also changes as embryo development progresses *in vitro*. The reason(s) for the observed gender-associated differences in amino acid turnover is not clear but the division between metabolism in male and female embryos only becomes apparent around the time of genome activation and may be related to copy number of the X chromosome. Chromosome X carries multiple genes related to amino acid turnover and transport, including *SLC38A5*, a system N transporter which can convey asparagine, one of the amino acids differentially metabolized in female and male embryos.

In conclusion, grossly aneuploid embryos demonstrate altered amino acid turnover *in vitro* relative to their genetically normal counterparts. These data suggest that the non-invasive method of amino acid profiling will be of value as a tool for routine embryo selection across all patient groups, as this technology can provide insight into embryo genetic health. Further research is required to determine how many genetically abnormal blastomeres an embryo can accommodate before the developmental competence of the embryo is compromised and whether it will be possible to improve embryo culture conditions and thus reduce mitotic errors.

Authors' roles

H.M.P.: study design, research lead, grant holder, manuscript preparation. K.E.: supply of resources and comments on manuscript. F.D.H.: measurement of amino acids, collaborator, co-author of manuscript. J.A.H.: experimental work including amino acid analysis and comments on manuscript. A.J.R.: clinical lead, research consent and ethics. J.E.H.: embryology work. H.J.L.: co-applicant on grants which funded study, supervision of York-based personnel, regular meetings to discuss data. S.E.H.: post-doctoral research fellow, embryo culture, embryo FISH, data analysis and manuscript preparation.

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Conflict of interest: H.J.L., Scientific advisor and shareholder in *Novocellus*—a company which is developing embryo diagnostic methods for use in clinical IVF. F.D.H., shareholder in *Novocellus*.

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