Incubator type affects human blastocyst formation and embryo metabolism: a randomised controlled trial.

Running title: Embryo metabolism and culture regimen

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Abstract

Study question Does the type of incubator used to culture human preimplantation embryos affect development to the blastocyst stage and alter amino acid utilisation of embryos in assisted reproduction?

Summary answer Culturing embryos in a time lapse system (TLS) was associated with a higher day 5 blastocyst formation rate and altered amino acid utilisation when measured from day 3 to day 5 compared to the standard benchtop incubator.

What is known already Culture environment is known to be important for the developing preimplantation embryo. TLSs provide a stable milieu allowing embryos to be monitored *in situ* whereas embryos cultured in standard benchtop incubators experience environmental fluctuations when removed for morphological assessment.

Study design, size, duration A prospective clinical trial randomising 585 sibling embryos to either the TLS (289 embryos) or the standard bench top incubator (296 embryos) over a 23-month period in a UK University Hospital Fertility Clinic.

Participants/materials, setting, methods Participants were aged 42 years or under, had an antral follicle count of ≥12 and ≥ 6 2 pronucleate zygotes. Zygotes were cultured individually in 25µl of medium. Randomised embryos were graded and selected for transfer or cryopreservation on day 5. For those embryos produced by women who underwent stimulation with recombinant FSH injections and were triggered with hCG, spent medium was collected on day 5 for amino acid analysis by high pressure liquid chromatography (HPLC). Clinical pregnancy was defined as the presence of a fetal heart beat on ultrasound scan at 7 weeks.

Main results and the role of chance Overall, blastocyst formation rate on day 5 was significantly higher in embryos cultured in the TLS (55%) compared to the standard incubator (45%; p=0.013). Similarly, there was an increase in the number of blastocysts suitable for cryopreservation in the TLS

(31%) compared to the standard incubator (23%; p=0.032). There was a significant difference in the utilisation of 12 amino acids by blastocysts cultured from day 3 to day 5 in the TLS compared to the standard incubator. Embryos cultured in the TLS displayed an increased total amino acid utilisation (P<0.001) and reduced amino acid production (P<0.001) compared to those in the standard incubator. Irrespective of incubator used, embryos fertilised by ICSI depleted significantly more amino acids from the medium compared to those fertilised by conventional IVF. There was no difference in the mean score of blastocysts transferred, or the clinical pregnancy rate after transfer of embryos from either of the incubators.

Limitations, reasons for caution The study was not powered to discern significant effects on clinical outcomes.

Wider implications of the findings The metabolism and development of preimplantation embryos is impacted by the type of incubator used for culture. Further research is required to investigate the long-term implications of these findings.

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Key words: Culture incubator, time lapse, amino acid metabolism, developmental competency, blastocyst.

Introduction

Successful IVF necessitates the culture, selection and transfer of developmentally competent embryos that will implant and give rise to healthy offspring. To ensure the best possible outcome for each individual embryo, every component of the culture environment requires optimisation. Currently, embryos are cultured in two broad categories of incubator: a standard bench top incubator, or a time lapse system (TLS). One of the major drawbacks with using a standard bench top incubator is the inability to maintain a continuous, stable culture environment due to the requirement for repeatedly removing embryos for morphological assessment under a microscope. This exposes the developing embryo to detrimental fluctuations in temperature, oxygen and CO_2 which adversely affect the pH and osmolality of the culture medium (Swain, 2010). It is therefore important when using a standard benchtop incubator to minimise the number of times that an embryo is exposed to the environment outside the incubator.

In contrast to standard benchtop incubators, TLSs have been developed to provide a more stable milieu where embryos can develop to the blastocyst stage without being disturbed to assess viability by morphological parameters. Furthermore, the incorporation of time lapse imaging, which records development *in situ,* facilitates additional morphokinetic analysis to aid embryo selection. There have been several reports of culture in the TLS providing beneficial clinical outcomes (Rubio *et al.*, 2014; Barrie *et al.*, 2017; Kalleas *et al.*, 2020), whilst other studies have shown no improvement (Cruz *et al.*, 2011; Barberet *et al.*, 2018; Armstrong *et al.*, 2019) over conventional incubators. Thus, both bench top incubators and TLSs continue to be used in IVF laboratories.

Reports investigating the effect of different incubator types on blastocyst development and implantation rate have often been confused due to morphokinetic characteristics being used for embryo selection in the TLS. Although information obtained through morphokinetic algorithms may be a useful indicator of future development, it is important to assess the impact of different culture incubator environments on the underlying physiology and biochemistry of the embryo. One criterion that has been largely overlooked is the impact of environmental conditions on embryo metabolism. This is surprising since metabolism is intrinsic to embryo viability and perturbed when embryos become stressed (Houghton and Leese, 2004; Lane and Gardner, 2005). Embryo metabolism is known to be altered by several environmental factors including oxygen tension (Wale and Gardner, 2012) and whether cultured in groups or individually (Kelley and Gardner, 2019). Hence, fluctuations in temperature, oxygen and CO_2 caused by the requirement to remove embryos from standard benchtop incubators for observation may also induce metabolic changes in the embryo.

There has been growing interest in the use of metabolic markers as indicators of embryo quality (Thompson *et al.*, 2016). Several metabolic parameters have been suggested over the last 20 years as potentially useful for predicting embryo viability including glucose, amino acids and broader metabolomics (Gardner *et al.*, 2001; Houghton *et al.*, 2002; Brison *et al.*, 2004; Seli *et al.*, 2007; Stokes *et al.*, 2007; Scott *et al.*, 2008). Human embryos which develop to the blastocyst stage deplete less amino acids and produce more alanine from day 2 to day 3 of development than those which arrest prior to blastocyst formation (Houghton *et al.*, 2002). This led to the 'quiet embryo' hypothesis which postulates the benefit of a low level of embryo metabolism (Leese, 2002). Amino acid profiling has also been used to distinguish between developmentally competent embryos of the highest morphological grade (Stokes *et al.*, 2007). A high amino acid turnover at the cleavage stage of development was found to correlate positively with DNA damage (Sturmey *et al.*, 2009) supporting the benefit of a quiet metabolism. Retrospective data have demonstrated that amino acid utilisation has the potential to be used to select developmentally competent embryos (Brison *et al.*, 2004). Moreover, blastocysts deemed to be of higher quality using morphological and morphokinetic grading criteria were associated with a decreased total amino acid production than those of lower quality (Ferrick *et al.*, 2020). Together, these studies highlight that metabolism and specifically amino acid profiling, reflect the intrinsic health of the developing embryo beyond morphology alone.

Combining embryo metabolism and day 5 blastocyst grade may enhance our understanding of whether the stable environment of the TLS is more beneficial for the developing embryo compared to the standard benchtop incubator. Consequently, the aim of this study was to ascertain whether the TLS improves day 5 blastocyst formation rates compared to the standard incubator and if the type of incubator used for culture affects how embryos utilise amino acids.

Material and Methods

A Prospective Randomised trial cOmparing embryo Metabolism and develOpmenT in the standard versus the Embryoscope incubator (PROMOTE) was conducted. Ethical approval for this study was granted by the South Central - Berkshire Research Ethics Committee (14/SC/1260) via the Integrated Research Application System (IRAS). Eighty-one couples who were undergoing IVF or ICSI were recruited to the trial (38 IVF, 41 ICSI, 1 combination, 1 no oocytes to fertilise). Female participants were required to be under the age of 42 years, with an antral follicle count (AFC) of greater than 12 or an anti-müllerian hormone (AMH) of greater than 10 pmol/L. Couples were excluded if either partner had a previous diagnosis of Human Immunodeficiency Virus (HIV) infection or Hepatitis B or C.

Women underwent ovarian stimulation with hMG or recombinant FSH (recFSH) and co-treated with either GnRH agonist or antagonists to prevent premature luteinisation, according to the centre's standard protocols. To trigger oocyte maturation, a single subcutaneous dose of hCG or GnRH agonist was given 36 hours prior to oocyte retrieval. IVF or ICSI was performed in accordance with standard protocols depending on the semen quality. Couples were recruited prior to egg retrieval; couples gave written informed consent. Data on the couples' demographic and clinical history were collected on a standard study proforma.

Oocytes were fertilised in GIVF+ medium, transferred to G1+ medium (Vitrolife, Sweden) and cultured at 5% O_2, 6% CO_2 with the remainder N_2 immediately following ICSI, or after the day 1 fertilisation check for IVF embryos. All inseminated oocytes were cultured in MINC benchtop incubators (Cook Medical, USA) until the fertilisation check. Following fertilisation, if less than six, 2 pronuclei (PN) embryos were obtained, they were excluded from the trial. If six or more 2PN embryos resulted, they were divided into two groups; alternate sibling embryos were placed in culture in either the TLS or a standard incubator (Figure 1). Embryos assigned to the TLS were cultured in an EmbryoScope (Vitrolife, Sweden), while those placed in a standard incubator were cultured in a MINC benchtop incubator.

All embryos were cultured individually in 25 µl drops of medium overlaid with Ovoil (Vitrolife, Sweden) in either the TLS or the standard incubator. Embryos were cultured in IVF sequential media; G1+ from day 1 to day 3 and G2+ (Vitrolife, Sweden) from day 3 until day 5. Embryos in the TLS were only removed on day 3 for media change but otherwise were observed from the time lapse images. Embryos cultured in the standard incubator were removed on day 2, 3 and 5 for scoring and on day 3 for a media change.

Blastocysts for single embryo transfer were selected based solely on a validated morphological scoring system (Gardner *et al.*, 2000). No morphokinetic analysis of embryos cultured in the TLS was used for this purpose. Any remaining high quality embryos (>3BB) were cryopreserved by vitrification. An ultrasound scan was performed at seven weeks gestation to confirm a clinical pregnancy.

Amino acid analysis

The G2+ spent embryo medium from each day 5 embryo along with control medium incubated in the absence of an embryo was stored at -80 °C prior to analysis. The concentrations of amino acids in the media were analysed using reverse phase HPLC (Agilent 1100) and calculated relative to a known concentration of amino acids. Pre-column derivatisation was achieved via the automated reaction of 10 µl sample and 10 µl *o*-phthaldialdehyde (OPA) reagent containing 0.2% β2-mercaptoethanol. Amino acids were eluted using an elution gradient. Buffer A was comprised of 15 ml tetrahydrofuran, 200 ml HPLC grade methanol and 800 ml sodium acetate (83 mM, pH 5.9) and buffer B 200 ml sodium acetate (83 mM, pH 5.9) and 800 ml HPLC grade methanol (Christensen *et al.*, 2014). This method allowed the separation and analysis of 18 amino acids, including essential amino acids: histidine (His), glutamine (Gln), arginine (Arg), threonine (Thr), tyrosine (Tyr), methionine (Met), valine (Val), tryptophan (Trp), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), and lysine (Lys); and non-essential amino acids; aspartic acid (Asp), glutamate (Glu), asparagine (Asn), serine (Ser), glycine (Gly), and alanine (Ala). This method did not allow the measurement of proline and cysteine. Total amino acid production, depletion and turnover (sum of depletion and production) were calculated.

Sample size and power considerations

Blastocyst formation rates are key to clinical outcome and hence were selected as the primary outcome measure. To show, with 80% power and a significance level of 0.05, an increase in blastocyst formation rate from 30% observed in our centre using standard incubators to 45% proposed for the TLS, 176 embryos were required in each arm. To generate sufficient embryos to address the research question, 80 couples were to be recruited. This number was anticipated to be sufficient to generate at least 180 embryos for analysis in each arm, even when 25% of subjects did not generate 6 or more 2PN embryos, necessitating exclusion from the study.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, NY, USA) and GraphPad Prism 8.4.3 (GraphPad software, San Diego, USA).

A generalised linear mixed model was used to compare the numbers of embryos that formed blastocysts and were suitable for cryopreservation from each type of incubator (including the parental origins of the embryos as a random effect), accounting for the non-independent nature of the embryos. This approach was also used to compare clinical pregnancy outcomes. Results were reported as odds ratios (OR) with accompanying 95% confidence intervals (95% CI).

The amino acid consumption and production of the embryos was compared, examining those inseminated by IVF and by ICSI and the differing culture environments. Data were tested for normality utilising the Kolmogorov-Smirnov test. Non-parametric data were log transformed allowing parametric analysis. Data were analysed using Linear mixed models with parental origins as a random effect, allowing the non-independent nature of the embryos to be taken into account. P values <0.05 were considered statistically significant.

Results

Participant characteristics

The average age of the women recruited to the trial was 32.6 years ± 3.8. Reasons for requiring IVF or ICSI were representative of the wider population seeking fertility treatment: 22% suffering from female infertility (polycystic ovarian syndrome (PCOS) 12/81 and tubal factors 6/81), 36% male infertility (29/81), 28% unexplained (23/81), and in 11 of the 81 cases (14%) there was more than one cause recorded.

The number of oocytes collected on the day of oocyte retrieval varied between 1 and 42 with a mean value of 15.1. Six couples were excluded due to having fewer than six oocytes. After excluding these six couples, the mean number of mature oocytes collected was 14.6 with a range of between 2 and 42. A further six couples were excluded due to having less than six mature oocytes. Following exclusion of these six couples, the mean number of 2PN zygotes was 9.5 ± 5.5 with a range of 0 and 27. Eighteen couples produced fewer than six 2PN embryos and were therefore excluded at this point. The sibling zygotes from the remaining 51 couples were randomised between the TLS or the standard incubator (Figure 2).

Incubator type affects rate of blastocyst formation and embryo quality

A total of 289 zygotes were cultured in the TLS compared to 296 embryos in the standard incubator. The primary endpoint for the study was the percentage of normally fertilized oocytes forming a blastocyst on day 5 (Table I). Significantly more embryos developed to the blastocyst stage on day 5 in the TLS (55%) compared to the standard incubator (45%; OR 1.518, 95% CI 1.094 – 2.106, p=0.013). When embryos were separated according to the mode of fertilisation, significantly more embryos generated through IVF developed to the blastocyst stage on day 5 in the TLS (59%) compared to the standard incubator (48%; OR 1.595, 95% CI 1.048 – 2.427, p=0.029). In contrast, there was no difference in development to the blastocyst stage on day 5 between ICSI embryos cultured in either the TLS or the standard incubator.

The number of embryos suitable for cryopreservation on day 5, defined as a blastocyst which scored 3BB and above using the Gardner scoring system for the quality of the inner cell mass and trophectoderm, was analysed according to incubator used. Significantly more embryos were deemed suitable for cryopreservation when cultured in the TLS (90 out of 289 embryos, or 31%), compared to the standard incubator (69 out of 296 embryos, or 23%; OR 1.497, 95% CI 1.036 – 2.163, p=0.032). When these were separated according to method of fertilisation, significantly more embryos fertilised by IVF and incubated in the TLS were suitable for cryopreservation (38%) compared to those in the

standard incubator (26%; OR 1.785, 95% CI 1.143 – 2.788, p=0.011). No such difference was observed for embryos fertilised by ICSI.

A number of regimens were used for follicular stimulation and triggering of oocyte maturation. Three women underwent downregulation prior to ovarian stimulation and were therefore excluded from further analysis. Seventy-five % (36/48) of women underwent stimulation with recFSH followed by an hCG trigger, 8/48 (17%) had recFSH followed by a GnRH agonist trigger and 4/48 (8%) had hMG for stimulation with either hCG (3/48; 6%), or GnRH agonist (1/48; 2%) as the trigger. To negate any potential confounding effect of the different drug regimens used to stimulate and trigger oocyte maturation, further analyses included only those patients who received recFSH and hCG. A total of 203 embryos produced as a result of recFSH stimulation followed by an hCG trigger were placed into the TLS and 203 in the standard incubator. Significantly more embryos formed a blastocyst on day 5 in the TLS (108/203) compared to the standard incubator (84/203; OR 1.639, 95% CI 1.103 – 2.435, p=0.015). Similarly, an increased number of embryos fertilised by IVF formed a blastocyst on day 5 in the TLS compared to the standard incubator (78/137 vs. 62/140; OR 1.798, 95% CI 1.089 – 2.969, p=0.022). In contrast, the type of incubator used to culture embryos generated by ICSI did not affect the day 5 blastocyst rate (OR 1.520, 95% CI 0.716 – 3.225, p=0.273).

More embryos were suitable for cryopreservation when cultured in the TLS (65/203) compared to the standard incubator (43/203; OR 1.768, 95% CI 1.128 – 2.772, p=0.013). These differences were maintained when only embryos fertilised by IVF were examined (54/137 for TLS vs. 36/140 for the standard incubator; OR 1.945, 95% CI 1.155 – 3.275, p=0.013). Incubator type did not impact the suitability of ICSI generated blastocysts for cryopreservation (OR 1.545, 95% CI 0.532 – 4.483, p=0.421).

Incubator type alters embryo metabolism

To investigate whether the type of incubator used to culture preimplantation embryos impacts embryo metabolism when patients received recFSH and hCG, the consumption and production of 18 amino acids by individual blastocysts cultured from day 3 to day 5 in either the TLS or the standard incubator was measured. There was a significant difference in the utilisation of glutamine, arginine, threonine, alanine, tyrosine, methionine, valine, tryptophan, phenylalanine, isoleucine, leucine and lysine between the two incubators (Figure 3a). Embryos cultured in the TLS were found to deplete more amino acids from the medium ($p<0.001$) and to produce fewer amino acids into the medium ($p<0.001$), with no effect on amino acid turnover compared to those cultured in the standard incubator (Figure 3b).

As embryos fertilised by IVF and ICSI were cultured in the TLS and standard incubator, the effect of mode of fertilisation on amino acid metabolism was investigated. There was no difference in the utilisation of individual amino acids between blastocysts cultured from day 3 to day 5 in the TLS and fertilised using IVF or ICSI (Figure 4a) whereas the only difference observed in the standard incubator was in the utilisation of valine ($p=0.048$; Figure 4c). When the sum of amino acid utilisation was investigated, mode of fertilisation did not affect total amino acid production or turnover in either the TLS, or the standard incubator. However, ICSI embryos displayed a significantly increased total amino acid depletion compared to IVF embryos when cultured in either the TLS ($p=0.022$; Figure 4b) or standard incubator ($p=0.013$; Figure 4d).

Discussion

This study investigated the effect of culturing sibling human preimplantation embryos in either a standard bench top incubator or a TLS in terms of development to the blastocyst stage and amino acid metabolism of blastocysts cultured from day 3 to day 5 of development. To the best of our knowledge, this is the first report showing that the incubator used to culture preimplantation embryos alters embryo metabolism.

One of the ongoing challenges associated with clinical IVF is that of identifying which embryos from a cohort are developmentally competent and should be either transferred or cryopreserved for use in a subsequent cycle. Despite current assessment methods, such as embryo morphology, rate of cleavage and morphokinetic analysis, success rates have remained consistently low with at best only 31% of embryos transferred resulting in a live birth (HFEA, 2020). One variable that has been largely overlooked is the effect of incubator type used to culture developing embryos and the potential impact this might have on embryo metabolism. It should be noted that the concern is not with the stability of the environmental conditions of the incubator per se, but rather the requirement to repeatedly remove developing embryos for morphological assessment from the defined conditions of the standard incubator and thus the recurrent exposure of embryos to the atmospheric conditions found in the IVF laboratory. Such variations in culture conditions may be disruptive to the physiology of the embryo and impact its metabolism, something which is fundamental to embryo health.

Our data show improved overall blastocyst development and quality observed on day 5 in the TLS compared to the standard incubator. This suggests that the TLS is beneficial for preimplantation embryo development which is consistent with previous studies (Alhelou et al., 2018; Barberet et al., 2018). The enhanced number of IVF embryos which developed to the blastocyst stage on day 5 and were suitable for cryopreservation in the TLS compared to those fertilised by ICSI was intriguing especially since mode of fertilisation did not affect blastocyst development when cultured in the standard incubator. These data suggest that the more stable environment of the TLS whilst being beneficial for IVF embryos may not be able to negate the impact of the ICSI procedure such as removal of the cumulus cells, physical damage inflicted through injection and the resulting effect on homeostatic mechanisms regulating embryo physiology. However, additional factors such as sperm quality, skill of the ICSI practitioner, the length of time to conduct the ICSI procedure and sample size may also have a role, and hence further research is required.

There was a concomitant difference in the utilisation of amino acids by blastocysts cultured from day 3 to day 5 between the two types of incubator. Embryos cultured in the TLS had an increased requirement for amino acids compared to those maintained in the standard incubator which likely reflects the higher quality of these blastocysts and the increased protein synthesis that occurs as the blastocyst expands (Sellens *et al.*, 1981). Moreover, embryos that cavitate earlier have been shown to display a significantly decreased rate of aneuploidy (Minasi *et al.*, 2016; Desai *et al.*, 2018). This, combined with the increased day 5 blastocyst rate in the TLS, suggests that a more stable culture environment may be beneficial. In cell lines, aneuploidy is known to induce metabolic stress (Zhu *et al.*, 2018) and to alter energy metabolism and amino acid utilisation (Williams *et al.*, 2008; Stingele *et al.*, 2012). Aneuploid human preimplantation embryos also display altered amino acid utilisation (Picton *et al.*, 2010). Thus, the improved rate of blastocyst formation and quality, together with differential amino acid utilisation profile suggest that the TLS may provide a preferential environment to support the development of euploid embryos compared to the standard incubator.

The increased total amino acid depletion observed by embryos cultured in the TLS compared to the standard incubator contrasts with the quiet embryo hypothesis (Leese, 2002), which suggests metabolically quiescent embryos are more developmentally competent. However, in terms of amino acid utilisation, the quiet embryo hypothesis was based on embryos cultured at atmospheric oxygen for 24 hours. In contrast, embryos in the current study were cultured at 5% oxygen and amino acid utilisation of blastocysts assessed from day 3 to day 5 allowing the first two morphological events of development, compaction and cavitation to be incorporated in the metabolic assessment. Culture at 5% oxygen is known to be beneficial for human embryo development yielding higher implantation, pregnancy and live birth rates compared to atmospheric oxygen (Meintjes *et al.*, 2009; Kasterstein *et al.*, 2013; Ruiz *et al.*, 2020). Thus, the quiet embryo hypothesis may not be applicable for the improved conditions used in the current study.

Embryos cultured in the TLS depleted significantly more arginine, threonine, tyrosine, methionine, valine, tryptophan, phenylalanine, isoleucine, leucine and lysine compared to those cultured in the standard incubator. This increased reliance on largely essential amino acids suggests that embryos in the TLS may be more developmentally competent since the transport of these amino acids has been shown to support the development of more viable embryos after the 8-cell stage (Van Winkle, 2001). Moreover, essential amino acids also increase cleavage rates after the eight cell stage and stimulate development of the inner cell mass of mouse blastocysts (Lane and Gardner, 1997). Leucine and arginine are important for maintaining cellular homeostasis by regulating the mammalian target of

rapamycin (mTOR) pathway (González *et al.*, 2012). Recently, human blastocysts deemed to be of higher viability were found to consume more leucine and arginine than those which were less viable (Ferrick *et al.*, 2020). This suggests that embryos cultured in the TLS are more developmentally competent and better equipped to maintain cellular homeostasis than those cultured in the standard incubator.

The primary difference between the TLS and standard incubator is the culture environment, which is more stable in the TLS, whereas with the standard incubator embryos are repeatedly removed to assess development. This subjects embryos to a temperature shock, exposes them to dramatically increased oxygen and decreased CO_2 levels, which alters the pH and osmolality of the medium resulting in altered amino acid metabolism. It is tempting to speculate that it may be the exposure to atmospheric oxygen that has resulted in the differential amino acid metabolism profiles observed for embryos in the TLS compared to the standard incubator. An increased consumption of asparagine, glutamate, tryptophan, lysine, threonine, tyrosine, methionine, valine, isoleucine, leucine and phenyalanine was observed when mouse post-compaction embryos were cultured at 5% oxygen compared to 20% oxygen (Wale and Gardner, 2012); this was remarkably similar to the amino acid profiles observed in the TLS compared to the standard incubator. Thus, maintenance of an uninterrupted 5% oxygen environment in the TLS may explain the increased amino acid depletion compared to embryos cultured in the standard incubator.

Consistent with previous studies (Speyer *et al.*, 2019; Sauerbrun-Cutler *et al.*, 2020), more embryos derived from IVF reached the blastocyst stage on day 5 compared to those fertilised by ICSI. Thus, it was important to determine whether the differences observed in the amino acid utilisation between embryos cultured in the two incubators were independent of fertilisation type. The overall pattern of amino acid utilisation between ICSI and IVF embryos was similar within incubator type. However, total amino acid depletion was significantly increased in ICSI embryos compared to those fertilised by IVF in both the TLS and standard incubator. This finding is intriguing and may result from the use of lower quality sperm in ICSI or perturbations associated with the ICSI procedure; either removal of the cumulus cells, injection of the oocyte or a combination of the two. Oocytes use paracrine signalling to promote the uptake of amino acids by cumulus cells (Eppig *et al.*, 2005), which can then be transported via gap junctions to the oocyte. Thus, after ICSI, the cooperativity between cumulus cells and the resultant zygote is lost. This, together with the trauma caused by injection, and the direct exposure of the cytoplasm of the oocyte to the culture medium may account for the increased total amino acid depletion observed with ICSI compared to IVF embryos. The fact that these metabolic differences were

observed at the blastocyst stage suggests that an intervention occurring in the oocyte has lasting effects and may contribute to the lower blastocyst rate observed in oocytes fertilised by ICSI compared to IVF.

In conclusion, our data suggest that the controlled environment of the TLS is beneficial for human preimplantation development compared to the standard bench top incubator. Removing embryos from the standard incubator for morphological assessment is detrimental and delays development. Moreover, the type of incubator used impacts embryo metabolism and specifically amino acid utilisation over the period of development encompassing compaction and cavitation.

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Data availability

All data relevant to this study are included in the article.

Authors' roles

AJK, NSM, PCC and FDH conceived and designed the experiments; AJK and SJW consented the patients; PL collected samples; AJK, IF, DC and KP performed the experimental analysis; AJK and SH analysed the data; AJK and FDH drafted the initial manuscript. All authors critically reviewed the manuscript and approved the final version.

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Conflict of interest

None of the authors declare any conflict of interest in relation to this study.

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Figure legends

Figure 1 Schematic representation of the pathway for embryos in the PROMOTE trial TLS= time lapse system; 2PN=2 pronucleate.

Figure 2 Diagram of recruitment to the PROMOTE trial. 2PN=2 pronucleate

Figure 3 Amino acid utilisation is dependent on the type of incubator used for preimplantation embryo culture.

Amino acid profile of culture medium (a) and total amino acid production, depletion and turnover (b) of individual human blastocysts cultured from day 3 to day 5 in either the time lapse system (TLS) (n=84) or standard incubator (n=68). Positive values represent amino acid production and negative values amino acid depletion. Boxes extend from the 25^{th} to 75^{th} percentiles while the whiskers represent the 10^{th} to 90^{th} percentile. A "+" represents the mean and a horizontal line, the median. *P<0.05, **p<0.01, ***p<0.001. Asp, aspartate; Glu, glutamate; Asn, asparagine; His, histidine; Ser, serine; Gln, glutamine; Arg, arginine; Gly, glycine; Thr, threonine; Ala, alanine; Tyr, tyrosine; Met, methionine; Val, valine; Trp, tryptophan; Phe, phenylalanine; Ile, isoleucine; leucine (Leu), Lys, lysine.

Figure 4 Mode of fertilisation affects total amino acid depletion.

Amino acid profile of the culture medium (a and c) and total amino acid production, depletion and turnover (b and d) of individual human blastocysts cultured from day 3 to day 5 in the TLS (a and b) or a standard incubator (c and d) and fertilised by either IVF or ICSI. For the TLS, n=58 and n=26 for embryos fertilised by IVF and ICSI respectively. For the standard incubator, n=51 and n=17 for embryos fertilised by IVF and ICSI respectively. Positive values represent amino acid production and negative values amino acid depletion. Boxes extend from the 25^{th} to 75^{th} percentiles while the whiskers represent the 10^{th} to 90^{th} percentile. A "+" represents the mean and a horizontal line, the median. *P<0.05. Asp, aspartate; Glu, glutamate; Asn, asparagine; His, histidine; Ser, serine; Gln, glutamine; Arg, arginine; Gly, glycine; Thr, threonine; Ala, alanine; Tyr, tyrosine; Met, methionine; Val, valine; Trp, tryptophan; Phe, phenylalanine; Ile, isoleucine; leucine (Leu); Lys, lysine.