Preimplantation embryo metabolism as a biomarker of embryonic viability and health

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Abstract

Worldwide, ~12% of couples suffer from infertility and therefore rely on assisted reproductive technologies to conceive. The current success rate of an initial IVF cycle is ~20%, leaving ~80% of couples unsuccessful after their first cycle. Although success rates increase with subsequent cycles, represented by an increase in cumulative pregnancy rates, IVF treatment is expensive and can have a significant impact on the psychological wellbeing of couples. It is therefore imperative that research is focused on increasing the success rate of initial IVF cycles to reduce the time to pregnancy.

The success of an IVF cycle significantly relies on the ability to select the most competent embryo from a patient's cohort that has the greatest chance of establishing a viable pregnancy. Current embryo selection methods focus on the morphological and/or the morphokinetic development of the preimplantation embryo and preimplantation genetic testing can be utilized to ensure the embryo transferred is genetically normal. However, despite an embryo being regarded as high quality based on these selection methods, its success post-transfer is not guaranteed.

Blastocyst metabolism is a key regulator of embryo development and through metaboloepigenetic interactions, embryonic health, and its assessment represents an additional biomarker that may improve the accuracy of embryo selection. A comparison between blastocyst metabolism, morphology, time-lapse annotations, artificial intelligence, chromosomal status and transfer success was conducted. High glucose uptake and high amino acid consumption were found to be associated with human blastocysts of high viability according to current methods of selection. Further, glucose uptake was significantly higher in human blastocysts that established a viable pregnancy.

Genetically abnormal, or aneuploid, human embryos developed slower and were assigned lower viability scores. Additionally, blastocyst amino acid utilization appeared to be perturbed due to aneuploid associated stress. An analysis of vitrified mouse and human blastocyst pyruvate and/or glucose uptake post-warm was unable to provide a measure of viability. However, a morphological assessment of human blastocyst re-expansion post-warm revealed blastocysts with a greater degree of re-expansion were associated with higher live birth rates. Finally, using a mouse model to demonstrate how changes in embryo culture media can impact blastocyst metabolism and health, the addition of antioxidants (acetyl-*L*-carnitine, *N*-acetyl-*L*-cysteine and α-lipoic acid) to embryo culture media, individually and in combination, was investigated. A reduction in oxidative stress, regulation of blastocyst carbohydrate metabolism, lower NADH levels and improved blastocyst development were identified.

Together the data presented in this thesis provides a comprehensive analysis of human blastocyst physiology and lay the foundation for the development of an algorithm incorporating morphological, morphokinetic and metabolic biomarkers which could assist in the identification of the most viable and healthy blastocyst for transfer. Such an algorithm may also be used to validate future advances in embryo culture conditions. Therefore, these data provide an opportunity to significantly improve human IVF success rates and reduce the time to pregnancy.

Declarations

This is to certify that:

1. This thesis comprises only of my original work towards the Doctor of Philosophy

2. Due acknowledgement has been made in the text to all other material used

3. This thesis is fewer than 100 000 words in length, exclusive of tables, maps, bibliographies and appendices.

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Preface

Laura Ferrick (L.F) was the primary author of all manuscripts and chapters within this thesis. These were written with assistance and editorial changes from supervisors Prof. David Gardner (D.K.G) and Dr. Lisa Lee (Y.S.L.L). Further editorial changes in chapter 4 were provided by Dr. Sharyn Stock-Myer (S.S.M). Study designs were conceived and developed by L.F, D.K.G. and Y.S.L.L. L.F performed all experimental work and analyzed all data used within the thesis. Y.S.L.L, embryologists and scientists at Melbourne IVF assisted in human embryo culture and coordination of clinical spent media sample collection. Amino acid analyses were performed by Metabolomics Australia and S.S.M and Virtus Diagnostics Melbourne team performed PGT-A.

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Table of contents

List of tables

List of figures

Abbreviations

A3 – combination of ALC, NAC and ALA

ADP – adenosine diphosphate

AI – artificial intelligence

ALA – α-lipoic acid

ALC – acetyl-*L*-carnitine

ANOVA – one-way analysis of variance

AQC – 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate

ART – assisted reproductive technologies

ATP – adenosine triphosphate

BSA – bovine serum albumin

DOHaD – developmental origins of health and disease

DTT – dithiothreitol

EGA – embryonic genome activation

FADH2 – flavin adenine dinucleotide

FET – frozen embryo transfer

FSH – follicle stimulating hormone

GnRH – gonadotropin-releasing hormone

GSH – glutathione

H_2O_2 – hydrogen peroxide

HA – hyaluronan

hCG - human chorionic gonadotrophin

HSA – human serum albumin

ICM – inner cell mass

ISCI – intracytoplasmic sperm injection

IU – international units

IVF – in vitro fertilization

IVM – in vitro maturation

LBR – live birth rate

LDH – lactate dehydrogenase

LQ-MS - liquid chromatography-mass spectrometry

MAS – malate aspartate shuttle

MCT – monocarboxylate transporter

MEM – minimal essential medium

MMP – mitochondrial membrane potential

mTOR – the mammalian target of rapamycin

NAC – *N*-acetyl-*L*-cysteine

NAD – no abnormality detected

NAD+/NADH – nicotinamide adenine dinucleotide

Na^+,K^+-ATPases – sodium-potassium adenosine triphosphatase

NGS – next generation sequencing

niPGT – non-invasive preimplantation genetic testing

OHSS – ovarian hyper stimulation syndrome

OXPHOS – oxidative phosphorylation

PBS – phosphate-buffered saline

PDH – pyruvate dehydrogenase

PF1 - peroxyfluor1

PFK – phosphofructokinase

PGT-A – preimplantation genetic testing for aneuploidy

PMSG – pregnant mare's serum gonadotrophin

PN – pronucelate

PPP – pentose phosphate pathway

ROS – react oxygen species

RT – room temperature

SBT – single blastocyst transfer

SEM – standard error of the mean

SET – single embryo transfer

STAR – single embryo transfer trial

tB – time to blastocyst

TCA – tricarboxylic acid

TE – trophectoderm

tEB – time to expanded blastocyst

tH – time to hatching blastocyst

tM – time to morulae

tSB – time to start of blastulation

t2 – time to 2-cell cleavage

t3 – time to 3-cell cleavage

t4 – time to 4-cell cleavage

t5 – time to 5-cell cleavage

t6 – time to 6-cell cleavage

t7 – time to 7-cell cleavage

t8 – time to 8-cell cleavage

UMF – ultramicrofluorescence

ZP – zona pellucida

Chapter 1. General Introduction

Preimplantation embryo physiology

Fertilization of a mature oocyte by a competent spermatozoon initiates the preimplantation period whereby the fertilized oocyte undergoes a series of dynamic morphological and physiological changes leading to the development of a blastocyst. The extrusion of a second polar body reflects fertilization has occurred and the development of the pronucleate oocyte was successful (Edwards, et al., 1969). It is at this stage that the male and female nuclei fuse (syngamy) and the newly formed zygote undergoes mitosis. Cellular divisions of the subsequent cleavage stage embryo form equal sized blastomeres and results in an increase in the overall cell number of the embryo, yet the overall size of the embryo remains the same (Edwards, et al., 1970, Watson, et al., 1992).

During the cleavage stage, the embryo is predominantly supported by maternally derived transcripts and proteins stored during oocyte maturation (Becker, et al., 2005, Latham, 1999, Watson, 2007). It is not until the maternal-zygotic transition that there is rapid decline in maternally inherited transcripts (Clegg, et al., 1983, Pikó, et al., 1982) as the embryonic genome is activated (EGA). As opposed to a single transcriptional event, EGA occurs in a series of transcriptional bursts (Lee, et al., 2014). The first major wave of transcription occurs in the mouse at the 1-2 cell stage (Golbus, et al., 1973) and in the human at the 4-8 cell stage (Braude, et al., 1988). The second major EGA wave occurs around the morula stage in both species (Xue, et al., 2013). EGA requires genome-wide demethylation (Rougier, et al., 1998) and concomitantly, chromatin remodeling (Wu, et al., 2016). Following EGA and compaction, an assessment of physiology is more indicative of embryonic quality, despite some evidence for persisting maternally derived factors

(Barron, et al., 1989, Braude, et al., 1988, Taylor, et al., 1987, West, et al., 1989). In contrast, cleavage-stage embryo physiology is more indicative of oocyte quality.

Compaction begins at the 8-16 cell stage where the blastomeres of the cleavage stage embryo begin to flatten and increase the interblastomeric contact obscuring the distinct cellular boundaries as the embryo forms one cellular mass referred to as the morula (Ducibella, et al., 1975). These cells become polarized with defined apical and basolateral sides (Ziomek, et al., 1981, Ziomek, 1987) and the establishment of gap junctions then mediates interblastomeric cellular communication (Enderasn, et al., 1965, Nishi, et al., 1991) which are prerequisites for blastocyst formation.

The polarity and orientation of the inner and outer cells of the morula, formed during compaction, initiate the differentiation of two distinct cell types; the inner cell mas (ICM) and the trophectoderm (TE) (Ziomek, 1987). The establishment of gap junctional communication and the expression of ion transporters, such as Na^+, K^+-ATPases, and aquaporins on the basolateral plasma membrane of TE cells mediates the formation of the first transporting epithelium within the embryo (Barcroft, et al., 2003, Watson, et al., 1992, Watson, et al., 1990). Active transport of ions across this semi-permeable epithelium subsequently creates an osmotic gradient and initiates the development of a fluid-filled blastocoel in a process called cavitation, assisting with cellular differentiation and separation of the ICM and TE cells (Biggers, et al., 1988). Due to the development of the first transporting epithelium within the TE cells, while the blastocyst is influenced by the uterine environment it also has the ability to manipulate its surroundings and create a unique microenvironment (Gardner, et al., 2015).

During cavitation the blastocyst undergoes a period of expansion which involves an increase in blastocoel size, resulting in an increase in the overall size of the embryo. This energetically expensive process of expansion initiates thinning of the zona pellucida (ZP) and, in addition to the secretion of embryonic proteases, assist TE cells to hatch from the protection of the ZP and become free to initiate contact with the endometrial wall in the uterus (Cole, 1967, Leonavicius, et al., 2018). The timing and duration of each of these morphological events is species specific. The mouse is often used as a model of human embryology, and its duration of the preimplantation period is 4.5 days with compaction initiated on day 3. The human preimplantation period is similar, being 6 days in duration and compaction is initiated late on day 3 (Gardner, et al., 2016). The formation of ICM and TE are vital for the future development of the embryo as the ICM undergoes a series of cellular differentiation stages post-implantation leading to the development of the fetus, while TE cells differentiate into distinct protective extraembryonic tissue, such as the placenta, supporting development of the fetus during and post-implantation.

Maternal reproductive tract environment

The oviduct and the uterus each provide a unique environment which supports the morphological and physiological changes that occur during preimplantation embryo development. The carbohydrate composition of oviduct fluid has been assessed in the mouse (glucose 5.19 mM, pyruvate 0.14 mM, lactate 4.26 mM) (Gardner, et al., 1990) and human (glucose 0.5 mM, pyruvate 0.32 mM, and lactate 10.5 mM) (Gardner, et al., 1996) and mammalian studies have identified that the oxygen concentrations within the oviduct is around 5-10% (Fischer, et al., 1993, Mastroianna, et al., 1965, Ros, et al., 1974), well below the oxygen present in the atmosphere (~20%). This environment provided by

the oviduct fluid supports oocyte transport and maturation, sperm transport and maturation, fertilization, and early or pre-compaction embryonic development (Leese, 1988) until the embryo reaches the uterus on day 4 of development (Croxatto, et al., 1972).

The uterus supports the development of the post-compaction embryo during a period of dynamic and energetically demanding morphological changes. Glucose concentration in the human uterine fluid was more than six-fold higher than oviduct fluid, and both pyruvate and lactate were less concentrated (3.15 mM, pyruvate 0.1 mM, lactate 5.87 mM) (Gardner, et al., 1996). Further, the oxygen concentration of the mammalian uterine environment is as low as 1-5% oxygen (Fischer, et al., 1993, Mastroianna, et al., 1965, Ros, et al., 1974). Amino acids are also abundant within both the oviduct and uterine fluids, and concentrations have also been found to significantly differ throughout the reproductive tract (Harris, et al., 2005, Miller, et al., 1987) and during different stages of the menstrual cycle (Casslén, 1987). It is such metabolite and oxygen gradients within the reproductive tract that ensure biosynthetic and energy requirements of the embryo are met during each developmental stage (Ng, et al., 2018).

The complexity of reproductive fluids extends further than carbohydrate, amino acids and oxygen gradients. Proteomic studies of reproductive fluids have identified over 800 proteins, such as cytokines and growth factors, which are important for embryo development (Boomsma, et al., 2009, Casado-Vela, et al., 2009, Hannan, et al., 2011, Scotchie, et al., 2009) and it is the establishment of the maternal-fetal dialogue through this microenvironment that ensures the receptivity of the endometrium at the time of

implantation (Bhusane, et al., 2016, Salamonsen, et al., 2016). Additionally, preimplantation embryos are exposed to a comprehensive antioxidant system which includes a multitude of antioxidants both enzymatic and non-enzymatic, such as superoxide dismutase, glutathione (GSH) and cysteine (Agarwal, et al., 2012, El Mouatassim, et al., 2000, Gupta, et al., 2009). Exposure to such antioxidants throughout the reproductive tract help sequester reactive oxygen species (ROS), a major source of oxidative stress, which are unavoidably produced during the preimplantation period.

Preimplantation embryo metabolism

Underpinning the dynamic morphological changes during the preimplantation period, the embryo undergoes marked changes in nutrient utilization, as summarized in Figure 1.1.

Carbohydrate metabolism

The cleavage stage embryo exhibits low energetic activity due to the low biosynthetic requirements of blastomere divisions and can be considered relativity quiescent when compared to the blastocyst. Low levels of exogenous pyruvate are consumed and transported across the mitochondrial membrane where it enters the tricarboxylic acid (TCA) cycle producing NADH and FADH2 which undergo oxidative phosphorylation (OXPHOS) thus leading to ATP production (Biggers, et al., 1967). The low energetic activity of the cleavage stage embryo, and hence buildup of ATP, leads to a high ATP:ADP ratio that allosterically inhibit phosphofructokinase (PFK), a rate-limiting glycolytic enzyme, and therefore restricting glucose consumption (Barbehenn, et al., 1974). For over 30 years it was thought that the early embryo had an absolute require for pyruvate to

complete the first division (Biggers, et al., 1967, Whittingham, 1969, Whittingham, et al., 1967), and lactate supported development from the two-cell stage (Brinster, 1965). However, the discovery of the malate-aspartate shuttle (MAS) found in the absence of pyruvate, lactate and aspartate were capable of supporting development through the first division due to the regenerate cytosolic nicotinamide adenine dinucleotide (NAD^+) via MAS (Lane, et al., 2005). The conversion of pyruvate to lactate also regenerates cytosolic NAD^+. As the Gibbs free energy of lactate dehydrogenase (LDH) is zero (Schmid, et al., 1976), the direction of this reaction is regulated by the relative ratio of pryuvate:lactate in the surrounding environment. This subsequently regulates the redox state (NAD^+:NADH) which controls the flux of nutrients through mitochondrial and cytosolic energy generating pathways (Lane, et al., 2000).

Development of the blastocyst involves compaction of blastomeres, development of the first epithelium, development and maintenance of the blastocoel, and exponential cellular growth; processes which are all energetically demanding (Biggers, et al., 1988, Watson, et al., 1990, Watson, et al., 1992, Ziomek, et al., 1981, Ziomek, 1987). The utilization of ATP results in an overall drop in the cellular ATP:ADP ratio, allowing the allosteric inhibition of PFK to be released and glucose to be readily consumed, becoming the predominant metabolite. The increasing levels of energy required for blastocyst formation is supported by an increase in oxygen consumption and OXPHOS. However, almost 100% of the glucose consumed by ICM cells and ~50% of the glucose consumed by TE cells is converted to lactate through aerobic glycolysis even in the presence of sufficient oxygen (Gardner, et al., 2015, Hewitson, et al., 1993), a metabolic process first

described in cancer cells by Otto Warburg, known as the Warburg effect (Gardner, et al., 1990, Gott, et al., 1990, Warburg, 1956).

Lactate is released into the blastocoel and the surrounding environment of the blastocyst creating a microenvironment characterized by high lactate and low pH, which has been hypothesized to have a signaling role mediating the maternal-fetal dialogue and ensuring endometrium receptivity for successful implantation (Gardner, 2015). Glucose-6-phosphate, a rate-limiting glycolytic substrate, also supports carbon flux through the pentose phosphate pathway (PPP). The PPP involves the production of biosynthetic precursors, such as triacylglycerols and phospholipids, in addition to the generation of ribose moieties required for DNA and RNA synthesis supporting exponential cellular growth (Gardner, 1998). The PPP further generates NADPH which is required for the synthesis of complex molecules and the reduction of intracellular GSH, which has been identified as a key antioxidant which supports preimplantation embryo development (Reitzer, et al., 1980). Hence, a high glucose flux through glycolysis ensures that the biosynthetic pathways have sufficient carbon moieties at all times.

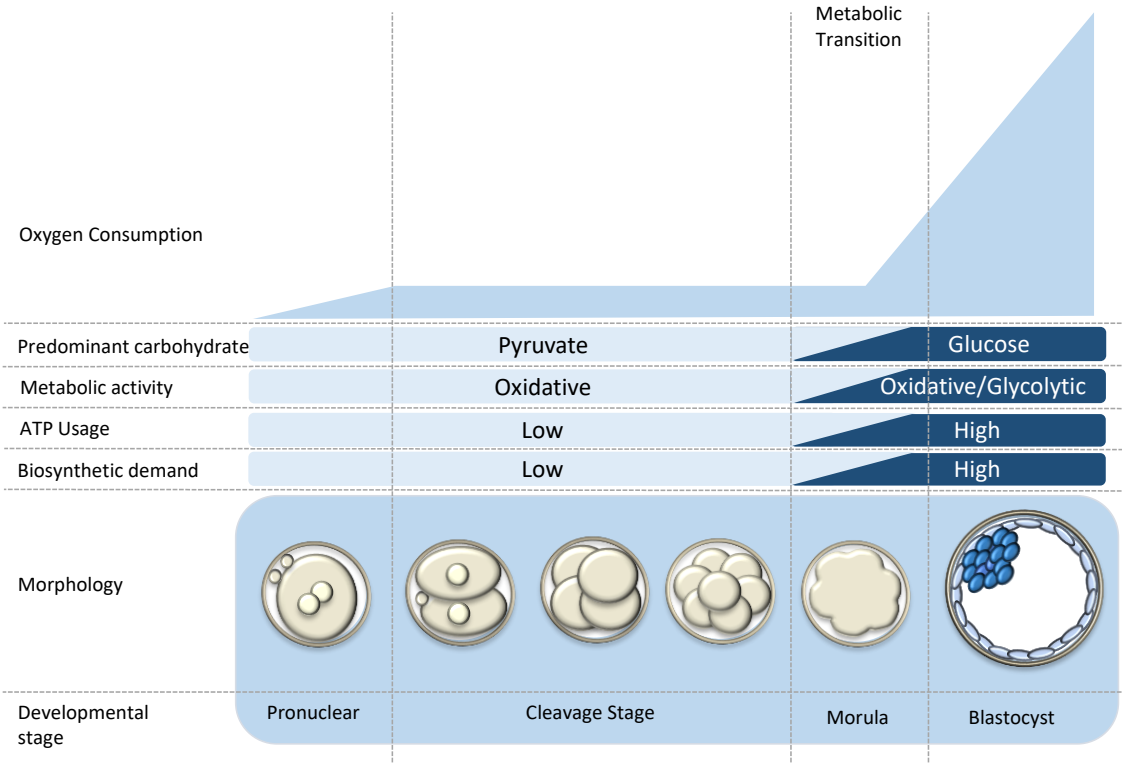


Figure 1.1 Metabolic changes during mammalian preimplantation development.

Prior to compaction, the pronucleate oocyte and cleavage stage embryo consume pyruvate predominantly and through oxidative metabolism produce appropriate levels of ATP required to support cellular division. After compaction, the embryo exhibits exponential growth, requiring higher levels of ATP synthesis and biosynthetic precursors. This increase in activity is supported by an increase in the overall oxidative capacity of the embryo and also a transition to a highly glycolytic metabolism, whereby higher levels of glucose are consumed and converted to lactate in the presence of oxygen.

Amino acid utilization

Amino acid transporters are present on the mammalian preimplantation embryo (Epstein, et al., 1973, Gardner, et al., 1989, Rieger, et al., 1992, Van Winkle, 1988) and amino acids are readily metabolized by the embryo during the preimplantation period creating endogenous pools within the embryo (Schultz, et al., 1981). Amino acids are utilized as an energy source for the production of ATP (Rieger, et al., 1992), for example, aspartate can be utilized via the MAS to regenerate cytolytic NAD^+ (Gardner, 1998, Lane, et al., 2005, Mitchell, et al., 2009), therefore are key regulators of both mitochondrial and cytosolic metabolism. Additionally, amino acids have been shown to be regulators of many cellular functions throughout the preimplantation period including pH and osmotic regulators (Edwards, et al., 1998, Van Winkle, et al., 1990), maintaining cellular homeostasis (González, et al., 2012) and protection from oxidative stress via antioxidant properties (Liu, et al., 1995). Furthermore, amino acids have been recognized as important signaling molecules (Martin, et al., 2003, Wu, et al., 1998) and have a role in blastocyst differentiation (Martin, et al., 2001) and promoting TE invasion of the endometrium (Gangloff, et al., 2004, González, et al., 2012, Guertin, et al., 2006, Martin, et al., 2003). Therefore, in addition to supporting viable preimplantation development, amino acids have a role in ensuring successful implantation.

The preimplantation embryo is able to utilize amino acids throughout the preimplantation period, however, the range and level of amino acid utilization by the embryo is distinctly different between cleavage stages and the blastocyst stages whereby blastocysts exhibit an increase in amino acid utilization, as observed in the mouse (Wale, et al., 2012), cow (Partridge, et al., 1996, Steeves, et al., 1999) and human (Houghton, et

al., 2002) preimplantation embryos. Ammonium is released by the preimplantation embryo as a by-product of amino acid metabolism (Gardner, et al., 1993, Gardner, et al., 2001) and the accumulation of ammonium in the surrounding environment has been shown to be detrimental to the developing mouse embryo (Lane, et al., 2003) and fetus (Lane, et al., 1994). Due to the dynamic nature of the in vivo environment, the production of ammonium may be neutralized by oviduct and uterine epithelial cells (Gardner, et al., 1993) or enzymatic pathways such as the conversion to glutamate via glutamate dehydrogenase (Lane, et al., 1995). Indeed, glutamate was found to be the fourth most abundant amino acid in the mammalian female reproductive tract (Miller, et al., 1987).

Metaboloepigenetics

A link between the levels of specific metabolic intermediates, a reflection of metabolic state and fate, and the epigenome has recently been established (Donohoe, et al., 2012, Gardner, et al., 2015), and has been termed "metaboloepigenetics". The levels of metabolites can directly influence the epigenome. For example, the metabolic co-factor, NAD^+, consumed and produced during glycolysis and replenished via the MAS, is linked to histone acetylation by regulating the activity of sirtuins, a class of histone deacetylases (Imai, et al., 2014, Ryall, et al., 2015), which regulate chromatin configuration. Through this link, the levels of NAD^+ can directly affect embryonic gene expression (Fig. 1.2) (Gardner, 2015, Harvey, et al., 2016, Houtkooper, et al., 2010, Latham, et al., 2012, Moussaieff, et al., 2015). Lactate concentration has been shown to affect the redox potential of embryos through its effect on NAD^+ availability, and as such helps to explain the importance of the relative activity of glycolysis, and hence cytosolic NAD^+, in determining blastocyst viability (Gardner, et al., 2015, Gardner, et al., 2000, Harvey, et

al., 2016). Furthermore, lactate itself is capable of remodeling the epigenome through lactylation (Zhang, et al., 2019). As postulated by the Developmental Origins of Health and Disease (DOHaD) hypothesis, changes to the embryonic environment including nutritional state and hence metabolite availability, is capable of altering gene expression and influencing the developmental programming of the embryo and subsequent fetus (Barker, 1995, Fleming, et al., 2015). Importantly, these perturbations have been found to influence long-term health and increase the risk of adult onset diseases (Feuer, et al., 2012, Feuer, et al., 2016). Hence, an analysis of metabolite utilization provides not only a measure of viability but plausibly a measure of subsequent health.

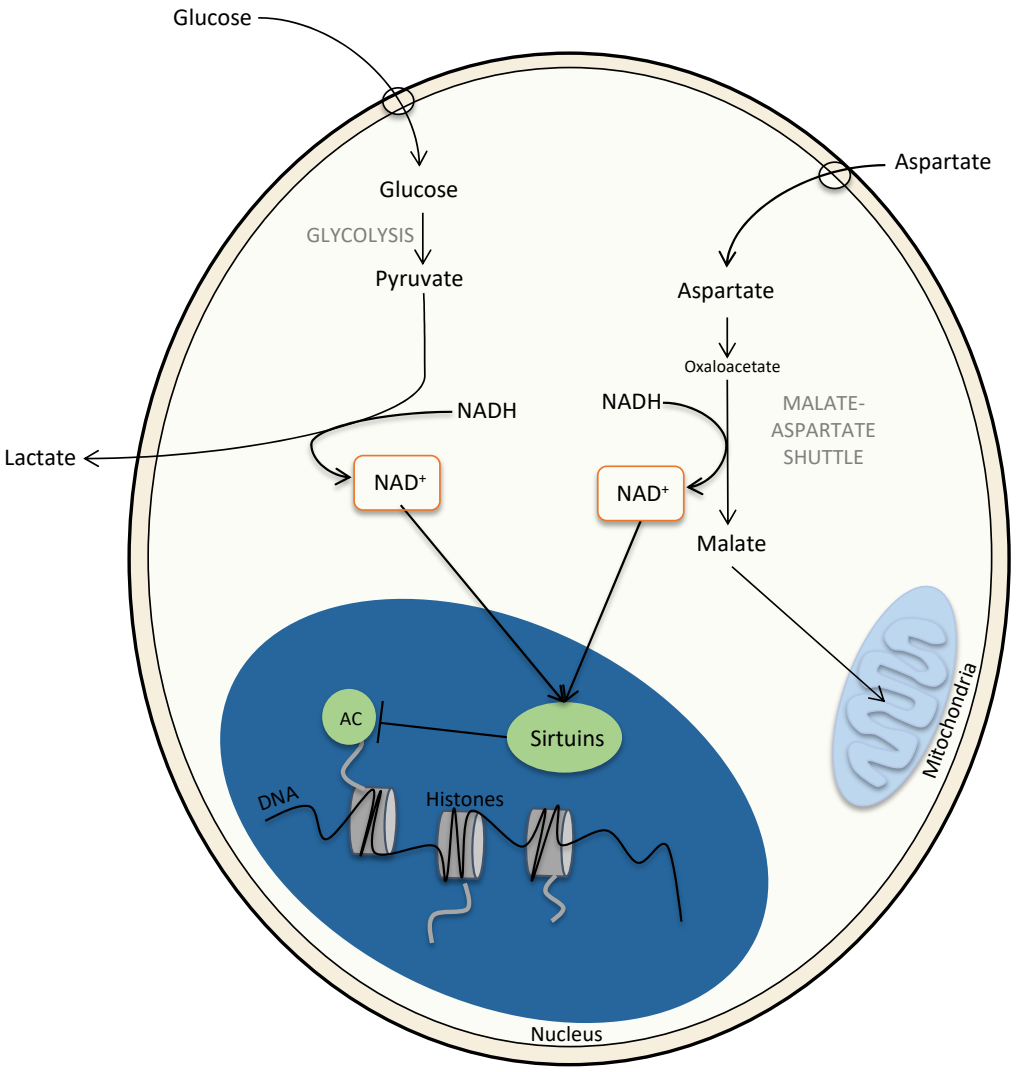


Figure 1.2 The metaboloepigenetic link between cytosolic NAD^+ and the acetylation state of cells within the blastocyst.

Carbohydrate and amino acids can influence the cytosolic levels of NAD^+ through the relative activities of glycolysis and the malate-aspartate shuttle (MAS) respectively. Cytosolic NAD^+ is an activator or sirtuins (a family of deacetylase proteins), which when activated removes acetyl groups (AC) from the tail of histone proteins.

Reproductive aging

Maternal fertility peaks at 25 years and significantly declines from 35 years (Baird, et al., 2005, van Noord-Zaadstra, et al., 1991). This is largely due to a decrease in oocyte quality (Bentov, et al., 2013). While the exact mechanisms of this decrease in oocyte quality remain unresolved, mitochondrial function, which has an important role throughout the preimplantation period, is believed to play a central role (Igarashi, et al., 2015). Indeed, aged oocytes have been found to have lower levels of ATP (Simsek-Duran, et al., 2013, Van Blerkom, et al., 1995) and also an increase in the accumulation of ROS (Fissore, et al., 2002, Takahashi, et al., 2003, Thouas, et al., 2005) which could induce perturbations in many cellular components and subsequent functions (Igarashi, et al., 2015). This increase in ROS is probably directly due to mitochondrial dysfunction, however, aged oocytes are also associated with a decreased ability to provide protective mechanisms against ROS leading to an imbalance between pro-oxidants and antioxidants inducing oxidative stress (Lord, et al., 2013). Indeed, when compared to oocytes from younger mice, the gene expression profile of aged oocytes was significantly different and included the down regulation of a group of genes associated with protection from oxidative stress (Hamatani, et al., 2004).

The exposure to such oxidative stress is believed to be responsible for cytoplasmic perturbations leading to abnormal spindle formation, oocyte chromosome segregation and subsequent genetic stability (Desler, et al., 2007, Eichenlaub-Ritter, et al., 2004, Schon, et al., 2000). It is likely that aberrations in genetic stability associated with aged oocytes results in a significant increase in aneuploidy rates of preimplantation embryos with maternal age (Duncan, et al., 2012, Franasiak, et al., 2014). Such alterations in the

embryonic karyotype can impact gene expression, and subsequently both the embryonic proteome and metabolome which have the potential to significantly compromise preimplantation embryo development, a concept in somatic cells referred to as aneuploid stress (Zhu, et al., 2018). Therefore, the oxidative stress associated with reproductive aging may induce aneuploid stress leading to perturbed embryonic physiology and potential health. Aneuploidy in embryos is the leading cause of pregnancy loss (Hassold, et al., 2001) and consequently, is associated with poor implantation rates (Bartmann, et al., 2004). As people are leaving childbirth till later in life due to social and culture influences, this has a great impact on couples' fertility and therefore the demand for assisted reproductive technologies (ART).

Infertility and assisted reproductive technologies

Infertility, defined as the inability to achieve a clinical pregnancy after 12 months or more of regulated unprotected sexual intercourse, impacts the lives of around 12% of couples worldwide (Ombelet, et al., 2008). ART gives infertile couples hope in conceiving through scientific intervention. Unfortunately, infertility and ART bears significantly negative impacts to the health and psychological state of couples. Not only does infertility and ART take couples on what has been described as 'an emotional roller-coaster', but the financial burden associated with ART on both the couple and society is substantial (De Sutter, et al., 2002, Luke, et al., 1996, Showstack, et al., 1985). It has been reported that up to 26% of patients discontinue treatments due to the psychological burden and further 15% due to divorce, which clearly indicates the negative impact infertility has on couples emotionally (Olivius, et al., 2004). Since the introduction of in vitro fertilization (IVF) nearly 50 years ago (Edwards, et al., 1969), and the birth of the first 'test-tube' baby,

Louise Brown, in 1978 (Steptoe, et al., 1978), significant money, time and knowledge has been employed to understand the preimplantation embryonic development process in vivo, and therefore how this can be replicated in an in vitro setting to ensure infertile couples the best chance at conceiving. Understanding the maternal environment and embryonic changes that occur during what is one of the most dynamic processes in human development has led to significant improvements in in vitro culture systems, in particular the embryo culture media to enable routine blastocyst development. Such improvements to in vitro culture during the preimplantation period has seen a rise in IVF success rates (Calhaz-Jorge, et al., 2017, Fitzgerald, et al., 2017, Sunderam, et al., 2019), however, additional improvements to IVF success rates could further reduce the burdens associated with ART.

Preimplantation in vitro culture

Culturing preimplantation embryos to the blastocyst stage in vitro is possible in simple media consisting of a basic salt solution, yet the quality of these embryos and the blastocyst rates are not optimal (Brinster, 1965, Whitten, 1957, Whitten, et al., 1968, Whittingham, 1971). The addition of amino acids and the creation of more complex media alleviated cellular blocks in all mammalian species and subsequently improved the quality of embryos cultured in vitro (Bavister, et al., 1990, Gardner, 1994, Gardner, et al., 1993, Lamb, et al., 1994, Lane, et al., 1997, Mehta, et al., 1990, Van Winkle, et al., 1996, Van Winkle, et al., 1990). In the past 30 years, two embryo culture media systems have been developed based on human reproductive tract metabolite compositions and are both used in human IVF clinics today. These media take on two very different rationale; (1) sequential media which takes on a ‘back to nature’ rationale and (2) monophasic

medium that takes on a 'let the embryo decide' rationale. The sequential system not only ensures the embryo is exposed to the right metabolite concentrations at the appropriate preimplantation developmental stage, it also enables the removal of built up toxins such as ammonium (Gardner, et al., 2001, Gardner, et al., 2003). Monophasic medium, whereby the embryo is culture in one type of medium for the entirety of the preimplantation period or until transfer, limits the requirement to interfere with the embryo during culture by removal from the incubator (Biggers, et al., 2002, Ho, et al., 1995, Lawitts, et al., 1993). Subsequently, both systems are advantageous in some way (Gardner, et al., 1990, Gardner, et al., 1998, Schoolcraft, et al., 1999).

Oxygen

Approximately 75% of human IVF clinics and laboratories worldwide culture preimplantation embryos in the presence of atmospheric (20%) oxygen for all (38%) or part (34%) of preimplantation embryo culture (Christianson, et al., 2014), despite these concentrations being significantly higher than the in vivo environment and the beneficial effects of culturing mammalian embryos at physiological (5%) oxygen were reported as early as 1969 (Whitten, 1969). Further, even a brief exposure to 20% oxygen can be detrimental to the developing embryo (Gardner, et al., 1996, Pabon Jr, et al., 1989, Truong, et al., 2017). Culturing embryos at 20% oxygen can lead to perturbations in the development of the preimplantation embryo and the viability of the embryo post-transfer (Gardner, 2016, Katz-Jaffe, et al., 2005, Kirkegaard, et al., 2013, Rinaudo, et al., 2006, Wale, et al., 2010) and has been shown to directly impact embryo metabolism (Kelley, et al., 2019, Lane, et al., 2005, Wale, et al., 2012) and embryonic epigenome (Ghosh, et al., 2017). Therefore, current scientific literature suggests culturing embryos

at 20% oxygen not only negatively impacts embryo viability but plausibly the health of the resulting offspring. Furthermore, in an attempt to reduce oxidative stress associated with in vitro culture the addition of antioxidants to embryo culture medium, both individually and in combination, has been investigated and resulted in improved embryo development (Ali, et al., 2003, Castillo-Martín, et al., 2014, Choe, et al., 2010, Dos Santos, et al., 2019, Heydarnejad, et al., 2019, Kim, et al., 2013, Kim, et al., 2018, Kitagawa, et al., 2004, Lane, et al., 2002, Manjunatha, et al., 2009, Silva, et al., 2015, Truong, et al., 2016).

Single blastocyst transfer

Prior to the late 1990s, it was not feasible to culture the human embryo to the blastocyst stage due to suboptimal culture conditions, and out of necessity the transfer of cleavage stage embryos asynchronously to the uterus became the norm, consequently exposing them to an environment they would not otherwise see in vivo. Of note, asynchronous embryo transfer has been shown to compromise pregnancy and result in negative fetal outcomes in domestic and laboratory mammals (Barnes, 2000, Walker, et al., 2015). Subsequently, cleavage stage embryo transfers led to low implantation and pregnancy rates in clinical ART. In an attempt to improve transfer outcomes, it was typical to transfer several embryos to the uterus in a given IVF cycle (Kupka, et al., 2016), leading to an increase in high-order multiple gestations carrying significant risk to both mother and babies (Adashi, et al., 2003). The ability to culture human embryos throughout the preimplantation period to the blastocyst stage, which can be attributed largely to extensive studies on embryo metabolism and maternal reproductive tract physiology (Gardner, et al., 1997), has in recent years led to a trend towards single blastocyst transfer (SBT) (Gardner, et al., 2000, Gardner, et al., 1998, Harbottle, et al., 2015, Penzias, et al.,

2017, Takeshima, et al., 2016). Single blastocyst transfers have seen a drop in multiple pregnancies associated with IVF, and therefore a reduction in costs and health implications of ART.

Cryopreservation

Cryopreservation of gametes and embryos has become a routine procedure in clinical IVF. Cryopreservation has facilitated SBT whereby viable sibling blastocysts can be preserved and transferred in a subsequent frozen embryo transfer (FET) and has subsequently contributed to cumulative pregnancy rates (Tiitinen, et al., 2004). Since the first reported pregnancies and live births from cryopreserved embryos (Mukerji S., et al., 1978, Trounson, et al., 1983, Zeilmaker, et al., 1984), the methods of cryopreservation have dramatically changed. Slow freezing was initially adopted to cryopreserve embryos and involves exposing embryos to low concentrations of cryoprotectants and the gradual drop in temperatures over a 2 h period, however, this results in the formation of ice crystals which can be of detriment to embryos (Nagy, et al., 2017). The introduction of vitrification, an ultra-rapid cooling method, eliminated the formation of ice crystals and significantly increased the survival rate of embryos when compared to slow freezing (~95% versus ~85%, respectively) (Balaban, et al., 2008, Rezazadeh Valojerdi, et al., 2009, Stehlik, et al., 2005) and live birth rates following transfer (Debrock, et al., 2015). While vitrification has been proven to be clinically safe (Balaban, et al., 2008, Gook, et al., 2016, Rezazadeh Valojerdi, et al., 2009, Wei, et al., 2019), it is important to recognize that this is an artificial procedure which forces the embryo to undergo a series of morphological changes. Exposure to high concentrations of cryoprotectants during vitrification promotes the dehydration and shrinking of the embryo and warming involves the

removal of cryoprotectants and subsequently swelling of the embryo. Such chemical, mechanical and thermal stress can potentially impact embryonic cellular function (Nagy, et al., 2020, Smith, et al., 2004).

Biomarkers of embryo viability

Improving culture systems to better replicate the maternal environment still remains a key research area and identifying new tools and/or methods of embryo selection has become a key focus of research. These embryo selection methods aim to improve our ability in identifying the most competent embryo in a cohort for transfer. To ultimately improve the overall success rate per cycle and, of significant importance, reduce the time to pregnancy which will consequently limit both the emotional and financial toll for patients undergoing IVF.

Blastocyst morphology

For over 40 years, morphology has been the key determinant in selecting which human embryo(s) to transfer. Key morphological features such as pronuclear appearance, blastomere symmetry and number, degree of fragmentation, and ICM and TE development have been analyzed and related to subsequent embryo viability leading to the development of numerous morphological grading systems (Cummins, et al., 1986, Gardner, et al., 2000). Until recently, grading the morphology of preimplantation embryos required embryos to be removed from the incubator, exposing embryos to variations in oxygen, temperature and pH, all of which are detrimental to development and subsequent viability (Sun, et al., 2004, Wale, et al., 2012, Wang, et al., 2001, Zander-Fox, et al., 2010). Furthermore, such morphological grading only provides a “snap-shot”

of preimplantation embryo development at a given time and consequently limits the frequency of observations, when in actuality embryonic development is capable of markedly changing within a matter of hours (Meseguer, et al., 2011, Mio, et al., 2008).

Morphokinetics

The introduction of time-lapse incubation systems has enabled continuous non-invasive assessment of embryo development, including the timing of cleavage divisions and other visible developmental events, referred to as morphokinetics. Morphokinetic parameters, such as the timing of the first cleavage division, have been measured and linked to embryo viability post-transfer (Edwards, et al., 1984, Lundin, et al., 2001, Sakkas, et al., 2001, Salumets, et al., 2003). Of interest, these more frequent observations have revealed novel morphological events, such as direct cleavage, involving the immediate cleavage of one blastomere into three daughter cells, and reverse cleavage, where either daughter cell fuses after cleavage division (Liu, et al., 2014, Meseguer, et al., 2011, Rubio, et al., 2012), both associated with poor implantation potential, and hence are valuable parameters to consider during embryo selection. Further, morphokinetic data has facilitated the development of algorithms in an attempt to predict embryo development and implantation potential (Milewski, et al., 2016, Motato, et al., 2016, Petersen, et al., 2016, Rubio, et al., 2014). The Known Implantation Data Score (KIDScore) morphokinetic de-selection algorithm was developed using a data set of 3275 embryos transferred on day 3 from 24 different clinics with varying culture conditions, creating the world's largest morphokinetic database of known implantation data (Petersen, et al., 2016). Unlike previously designed algorithms, the KIDScore is able to be used within different clinics, independent of their culture conditions. The algorithm was later broadened to analyze

implantation potential on day 5 prior to a SBT (D5 KIDScore algorithm). This algorithm involves two morphological parameters; pronucleate oocyte and TE quality, as well as, five morphokinetic annotations; t2-5 (time from insemination to 2-5 cell stage and tB (time from insemination to blastocyst stage). The KIDScore algorithm provides each embryo with a morphokinetic score (KIDScore) as a relative measure of implantation potential and ultimately positive pregnancy outcome (Reignier, et al., 2019), allowing embryologists to rank blastocysts for transfers based on implantation potential. However, debate continues as to whether such algorithms are capable of predicting embryo viability (Armstrong, et al., 2015, Campbell, et al., 2013, Pribenszky, et al., 2018). Additionally, annotating morphokinetic parameters for each individual embryo is time consuming and a level of inter- and intra-observer subjectivity exists which can impact overall grading success (Storr, et al., 2017).

The recent creation of extensive digital libraries of embryo development, made possible through time-lapse analysis, has created the opportunity to employ artificial intelligence (AI) to predict embryo viability (Curchoe, et al., 2019, Miyagi, et al., 2019, Thirumalaraju, et al., 2019, Tran, et al., 2019). The incorporation of AI to embryo selection methods is two-fold, as it has the opportunity to standardize and automate selection, eliminating subjectivity. Additionally, it employs deep-learning and neural networks, capable of identifying characteristics that are associated with positive transfer outcomes with no preset assumptions. Currently, the use of AI in clinical IVF awaits validation in prospective randomized controlled trials. Although time-lapse and AI technologies offers the ability to increase our knowledge of morphological events, and ultimately assist in improving embryo selection, thereby decreasing time to pregnancy, such analyses of images do not

provide direct quantification of embryo physiology or genetic composition that are more closely associated with embryo health.

DNA analysis and genomic composition

Preimplantation genetic testing (PGT) is widely utilized to identify aneuploid embryos and is also able to diagnose specific gene defects prior to embryo transfer and implantation. Using fluorescent in situ hybridization technologies it was possible to identify common aneuploidies associated with chromosomes X, Y, 18, 13 and 21 (Munné, et al., 1993), however, with the development of next-generation sequencing (NGS), it is now possible to screen the entire genome for whole and partial chromosome duplications and deletions (Gutiérrez-Mateo, et al., 2011, Treff, et al., 2010, Wells, et al., 2014, Zheng, et al., 2015, Zimmerman, et al., 2018). These methods remain the most reliable test for ploidy status of embryos, however, require highly skilled embryologists to perform biopsy procedures and NGS tests are expensive to run. Further, in order to perform PGT on blastocysts, TE biopsy is required and currently achieved in one of two ways; (1) forming an artificial hole in the ZP at the cleavage stage which promotes TE cells to herniate by day 5, thereby assisting biopsy (McArthur, et al., 2005, Schoolcraft, et al., 2010) and/or; (2) at the blastocyst stage the ZP is opened and TE cells are removed for biopsy from the opposite side to the ICM (Capalbo, et al., 2014), both of which are invasive procedures.

The recent discovery of cell-free DNA in both embryo culture medium and blastocoel fluid has prompted investigations of non-invasive PGT (niPGT) (Palini, et al., 2013, Stigliani, et al., 2013). Noninvasive PGT utilizes this cell-free DNA from spent culture media and subsequently eliminates any risks associated with the invasive nature of TE biopsy. The

concordance of niPGT utilizing spent culture media with PGT has resulted in a range of success (Leaver, et al., 2020). Several studies reporting concordance of 80-95% (Huang, et al., 2019, Liu, et al., 2017, Rubio, et al., 2020, Rubio, et al., 2019, Xu, et al., 2016), while others have reported 50% and lower (Ho, et al., 2018, Vera-Rodriguez, et al., 2018). The inconsistencies of concordance with PGT is possibly due to one of three reasons; (1) amplification of appropriate DNA concentrations, (2) sensitivity of technologies and (3) interference from cumulus cell DNA. Research in this area is ongoing with the aim to optimize procedures, increase the concordance with PGT and subsequently provide a means of assessing embryo ploidy status non-invasively. Of significance, in the context of embryo selection, it is important to understand that embryos that are considered genetically "normal" are not guaranteed to be viable, and subsequently result in the development of a healthy fetus.

Carbohydrate metabolism

Utilizing carbon dioxide production as a measure of metabolic activity, a correlation between embryo metabolism and the ability to develop in vitro was established (Menke, et al., 1970). This led a number of laboratories to investigate the concept of metabolic normality as a biomarker of embryo viability and conversely, identifying embryos that exhibit aberrant physiology. Renard and colleagues demonstrated that high glucose uptake by day 10 bovine embryos was associated with higher pregnancy rates, indicating glucose metabolism was a biomarker of embryo viability (Renard, et al., 1980). Subsequent development of ultramicrofluorescence (UMF), capable of accurately analyzing nano- and pico-liter volumes of spent culture media, enabled the rate of carbohydrate metabolism to be non-invasively measured throughout the entire

preimplantation period (Leese, et al., 1984). It was subsequently determined that glucose consumption is higher in mouse blastocysts exhibiting greater developmental potential post-transfer (Gardner, et al., 1987).

Analysis of carbohydrate metabolism by human preimplantation embryos has confirmed that metabolic activity is associated with both embryo developmental potential in vitro and viability post-transfer (Conaghan, et al., 1993, Gardner, et al., 2001, Gardner, et al., 2011, Hardy, et al., 1989). Human embryos that developed to the blastocyst stage exhibited significantly higher levels of pyruvate and glucose uptake than embryos that arrest during earlier stages of development (Gardner, et al., 2001). Furthermore, glucose uptake by morphologically identical embryos from the same patient revealed that metabolism is independent of morphology yet is correlated to developmental potential. It was subsequently determined that glucose uptake by human blastocysts on both day 4 and 5 of embryo development was significantly higher in those blastocysts that went on to establish a viable pregnancy (Gardner, et al., 2011). Of note, during preimplantation development there is a finite period when both X chromosomes are active resulting in differences in cellular phenotypes between male and female embryos. Specifically, glucose-6-phosphate dehydrogenase, a rate-limiting enzyme in the metabolism of glucose, is X-linked, and an analysis of glucose uptake revealed uptake was higher in female mouse embryos (Gardner, et al., 2010). These results were subsequently repeated in the human, whereby day 4 female embryos consumed 28% more glucose than males (Gardner, et al., 2011) and suggests that, where possible, embryonic sex should be accounted for when identifying potential metabolic biomarkers.

The significance of measuring glycolytic activity is that not only is the amount (rate) of metabolite consumption or production important but also the pathway (fate) that is active when utilizing that metabolite. Together, these observations gave rise to the "Rate and Fate" hypothesis (Gardner, et al., 2013). For example, lactate production from glucose reflects the relative rate of glycolysis. A high glycolytic rate, calculated on the basis that 2 mol lactate is produced per mol of glucose consumed, exists when an embryo converts majority of glucose to lactate, irrespective of the amount of glucose consumed. A conversion of ~50% of glucose to lactate in the mouse blastocyst is associated with the development of viable embryos (Gott, et al., 1990, Lane, et al., 1996). Mouse embryos in culture exhibit a wide range of glycolytic rates. It was determined that those embryos in the lowest 15% of the glycolytic distribution (<88% conversion) exhibited higher implantation rate (80%) compared to embryos in the top 15% of glycolytic distribution (>160%; resulting in a mere 6% implantation rate) (Lane, et al., 1996). When embryos were randomly selected for transfer, implantation rates were 20%, four-fold lower than when selecting embryos based on their glycolytic rate, indicating selection of embryos based on embryo physiology is more accurate than morphology alone. Importantly, blastocysts calculated to have a glycolytic rate greater than 100% indicate the use of endogenous substrates, such as glycogen (Lane, et al., 1996). This premature utilization of glycogen could compromise implantation, during which the embryo is transiently exposed to an avascular and relatively anaerobic environment (Rogers, et al., 1982), which requires the use of endogenous glucose stores, such as glycogen, metabolized through glycolysis. Should the implantation process be impaired through compromised metabolic function, there is a potential that placental function downstream could be compromised if a pregnancy ensues.

Amino acid utilization

Amino acid turnover of human embryos has been assessed (Houghton, et al., 2002) and found to significantly differ not only between developmental stages, in particular cleavage stage compared to blastocyst stage, but also as a factor of embryo quality. Houghton and colleagues determined that metabolism of alanine, arginine, glutamine, methionine, and asparagine on day 2–3 of human embryo development was associated with blastocyst formation (Houghton, et al., 2002). A follow up study, examining the relationship between pronucleate oocyte amino acid use and fate post-transfer on day 2 (Brison, et al., 2004), found that a lower amino acid turnover was associated with a higher implantation potential, clinical pregnancy rates and live births. The utilization of amino acids by mouse preimplantation embryos cultured at 5% and 20% oxygen were found to be significantly different (Kelley, et al., 2019, Wale, et al., 2012). Culturing embryos in the presence of 5% oxygen improved development, as measured by total blastocyst cell numbers and blastocyst formation rates, and blastocysts were found to utilized asparagine, aspartate, glutamate, threonine, lysine, tyrosine, methionine, valine, isoleucine, leucine phenylalanine and tryptophan differently when compared to lower quality embryos culture at 20% oxygen (Kelley, et al., 2019, Wale, et al., 2012). As human studies analyzing blastocyst amino acid utilization have been recorded under atmospheric (20%) oxygen, analyses need to be repeated under physiological (5%) oxygen concentrations.

Gender differences have also been observed in amino acid metabolism of human cleavage stage embryos. Male embryos exhibit higher consumption rates of tryptophan

and leucine than female embryos, which consume higher levels of asparagine (Picton, et al., 2010). A microarray gene expression profile of in vitro cultured bovine blastocysts revealed male and female embryos have very distinct transcription profiles, and may explain these gender differences (Bermejo-Alvarez, et al., 2010). Furthermore, the amino acid profile of chromosomally normal embryos were found to be significantly different to those that suffered from abnormalities (Picton, et al., 2010) suggesting in addition to being a measure of many cellular processes in which amino acids are involved in, amino acid profiles may provide a means of assessing chromosomal status of embryos.

Combination of biomarkers

To date, techniques for assessing embryo viability have been used in isolation however, it is plausible that more parameters measured concomitantly could lead to the development of a viability algorithm that facilitates greater accuracy in diagnosis. To this end, a comprehensive mouse study utilizing time-lapse microscopy and analysis of spent media samples was undertaken, which enabled the assessment of morphokinetics and both carbohydrate and amino acid metabolism within individual preimplantation embryos (Lee, et al., 2015). Embryos were grouped into “fast” or “slow” developing embryos, according to the time of the first cleavage division to the two-cell stage. “Fast” embryos exhibited a significant increase in ICM and TE cell numbers and higher levels of glucose uptake and lactate production. The relative proportion of glucose that was converted to lactate was lower in “fast” embryos, which is expressed as a lower overall glycolytic rate of 49.6%, compared to “slow” embryos exhibiting a mean glycolytic rate of 59.7%. This study is consistent with previous studies demonstrating that a high glucose uptake, combined with a glycolytic rate of ~50% is associated with higher implantation

potential in preimplantation embryos. Amino acid analysis revealed significant differences in profiles between both groups of preimplantation embryos, and a significantly lower overall amino acid turnover by “fast” embryos. The transfer of “fast” preimplantation embryos also resulted in a greater developmental potential post-transfer. These data support the combined use of morphological, morphokinetic, and physiological parameters. However, to investigate the possibility of using such biomarkers in conjunction for the selection of human embryos in the IVF clinic, a comprehensive assessment of the relationship between such biomarkers to provide an analysis of embryo viability and health in human blastocysts is required.

Investigation into the timing of blastocoel expansion (tEB-tSB), has recently been linked to euploidy whereby the euploidy rate was 47.7% in embryos completing blastocoel expansion within 13 h. In contrast, embryos whose blastocoel expansion took longer than 13 h exhibited a reduced euploidy rate of 31.5% (Desai, et al., 2018). As blastocoel expansion requires a considerable amount of energy, it is plausible that morphokinetic analysis of blastocoel expansion (tEB-tSB) reflects the metabolic activity of the embryo. An analysis of a combination of current morphokinetic parameters and metabolic biomarkers could provide a measure of the chromosomal status of embryos and is yet to be established.

Summary

Improvements in embryo culture have led to the introduction of single blastocyst transfer. Morphological and morphokinetic analyses of the human embryos has assisted in the exclusion of embryos exhibiting abnormal phenotypes, while PGT has been of value

in identifying euploid embryos for transfer. However, preimplantation embryos with identical morphology can have distinct physiologies (Gardner, et al., 2001, Gott, et al., 1990, Lane, et al., 1996, Renard, et al., 1980). Subsequently, a functional analysis of embryo physiology would assist in a more detailed analysis of embryo viability and plausibly chromosomal status, thereby increasing the accuracy of embryo selection and assisting reducing the time to pregnancy. Of further significance, given the relationship between metabolic activity, such as cytosolic NAD^+ availability, and the regulation of the epigenome through metaboloepigenetics (Canto´ C, et al., 2015, Donohoe, et al., 2012, Harvey, et al., 2016), metabolic biomarkers may not only relate to embryonic viability but also to the epigenetic state of the embryo during development. Therefore, conveying information regarding developmental programing and subsequently the health of resulting children. Importantly, however, the conditions in which embryos are cultured can also have a profound effect on cellular physiology, hence on the very biomarkers one may be assessing. This is especially true for metabolism, where oxygen concentration, among other factors, can significantly affect the relative activity of metabolic pathways and the epigenome (Chason, et al., 2011, Lane, et al., 2005, Wale, et al., 2012, Wale, et al., 2013).

Hypothesis and aims

Hypothesis:

Non-invasive analysis of individual blastocyst metabolism is related to embryonic development, chromosomal status, viability post-warm, and transfer success. In turn, metabolic biomarkers will facilitate selection of a single blastocyst for transfer in order to reduce time to pregnancy and further, assist in validating advances in embryo culture conditions.

Aims:

To determine how human blastocyst glucose and amino acid metabolism, as a measure of embryonic viability and health, relates to embryonic developmental parameters and transfer outcomes when human embryos are cultured at physiological oxygen

To establish whether human blastocyst morphology, morphokinetic parameters and viability scores (KIDScore and AI EmbryoScore) and blastocyst metabolism, can provide a means of assessing chromosomal status in human blastocysts.

To analyze the carbohydrate metabolism of vitrified mouse and human blastocysts post-warm and prior to transfer and relate this to blastocyst morphology and implantation potential.

To investigate the addition of antioxidants to embryo culture and its effect on the regulation of blastocyst metabolism and reducing reactive oxygen species, subsequently to establish how changes to embryo culture medium can influence embryonic health

Chapter 2. Materials and methods

Ethics

Faculty of Science Animal Ethics Committee, University of Melbourne approved mouse experimentation (1814430.1/1914891.1). For experiments involving human embryos, ethics application was approved by Melbourne IVF Human Research Ethics Committee (53/117-MIVF) and patient consent was obtained during clinical consultation.

Media preparation

All chemicals were supplied by Sigma Aldrich (St Louis, USA), with the exception of essential amino acids (minimal essential medium [MEM] Cellgro; Corning Life Sciences, Massachusetts, USA) or unless otherwise specified, and prescreened using a mouse embryo assay (Gardner, et al., 2005).

G-series media were prepared in the laboratory (Gardner, et al., 2014) or, where specified were supplied by Vitrolife (AB, Sweden). To prepared stock concentrations, chemicals were weighed using an analytical balance (Quintix 124-1S, Sartorius Lab Instruments, Germany) and dissolved in ultra-pure (MilliQ) water. Culture medium was prepared from stock solutions (Appendix A) in a 14 ml round bottom tube (Falcon, BD Biosciences, Australia), with final concentrations, pH and osmolarity outlined in Table 2.1 (Gardner, et al., 2014). The pH of medium was adjusted with 2 M sodium hydroxide (NaOH) solution and measured using a pH meter (Orion Versa, ThermoFisher, Australia). Osmolarity was tested using an osmometer (Osmo1, Advanced Instruments, USA). Media and stocks were prepared in a laminar flow hood, filtered through 0.2 µM filter (Pall Corporation, Australia) and stored at 4^{o}C.

Table 2.1 Final concentrations of components in in-house media

Component	G1 (mM)	G2 (mM)	Modified G2 (mM)	G2-MOPS (mM)	Modified EmbryoGlue (mM)
NaCl	90.08	90.08	90.08	90.08	90.08
KCl	5.5	5.5	5.5	5.5	5.5
NaH_2PO_4	0.25	0.25	0.25	0.25	0.25
$MgSO_4.7H_20$	1	1	1	1	1
$NaHCO_3$	25	25	25	2	25
$CaCl_2$	1.8	1.8	1.8	1.8	1.8
Glucose	0.5	3.15	0.5	3.15	0.5
Sodium L-Lactate acid	10.5	5.87	-	5.87	5.87
Sodium pyruvate	0.32	0.1	-	0.1	0.1
EDTA (Titriplex)	0.01	-	-	-	-
Alanyl-glutamine	0.5	0.5	0.5	0.5	0.5
L-Alanine	0.1	0.1	0.1	0.1	0.1
L-Aspartic acid	0.1	0.1	0.1	0.1	0.1
L-Asparagine-H_2O	0.1	0.1	0.1	0.1	0.1
L-Glutamic acid	0.1	0.1	0.1	0.1	0.1
Glycine	0.1	0.1	0.1	0.1	0.1
L-Proline	0.1	0.1	0.1	0.1	0.1
L-Serine	0.1	0.1	0.1	0.1	0.1
Taurine	-	0.1	0.1	0.1	0.1
Arginine	-	0.6	0.6	0.6	0.6
Cystine	-	0.1	0.1	0.1	0.1
Histidine	-	0.3	0.3	0.3	0.3
Isoleucine	-	0.4	0.4	0.4	0.4
Leucine	-	0.4	0.4	0.4	0.4
Lysine	-	0.4	0.4	0.4	0.4
Methionine	-	0.1	0.1	0.1	0.1
Phenylalanine	-	0.2	0.2	0.2	0.2
Threonine	-	0.4	0.4	0.4	0.4
Tryptophan	-	0.5	0.5	0.5	0.5
Tyrosine	-	0.2	0.2	0.2	0.2
Valine	-	0.4	0.4	0.4	0.4
D-Ca Pantothenate	-	0.0042	0.0042	0.0042	0.0042
Pyridoxal-HCl	-	0.0049	0.0049	0.0049	0.0049
Thiamine-HCl	-	0.0030	0.0030	0.0030	0.0030
Riboflavin	-	0.0003	0.0003	0.0003	0.0003
G-MM	-	-	-	-	2.5 mg/ml
Human Serum Albumin (HSA)	5 mg/ml	5 mg/ml	5 mg/ml	-	-
Hyaluronan (HA)	0.125 mg/ml	0.125 mg/ml	0.125 mg/ml	0.125 mg/ml	0.25 mg/ml

Gentamicin	0.01 mg/ml	0.01 mg/ml	0.01 mg/ml	0.01 mg/ml	0.01 mg/ml
MOPS	-	-	-	23	-
pH (un-gassed)	7.8 ± 0.05	8.0 ± 0.05	8.0 ± 0.05	2.3 ± 0.05	7.8 ± 0.05
Osmolarity (mOsmo/kg)	273 ± 5	270 ± 5	256± 5	273 ± 5	268 ± 5

Dish preparation

Mouse embryo culture dishes were prepared in 60 mm Easy-Grip Falcon dish (Falcon) (Gardner, et al., 2014, Gardner, et al., 2019). Group culture dishes contained a 20 µl wash drop and 20 µl culture drops of culture media and were prepared by placing 10 µl of culture medium down with a rinsed sterile 20 µl filtered pipette tip, followed immediately by 7 ml of paraffin oil (Ovoil; Vitrolife), then the final 10 µl of culture medium was added to each drop. Individual culture dishes contained a 20 µl wash drop and 2 µl culture drops created using a multi-channel pipette (Voyager, Integra, Australia) and were covered immediately with 7 ml Ovoil. Metabolic culture dishes were prepared using 5 µl eVol® XR Digital Analytical Syringe (SGE Analytical Science, Australia) to place 1 µl culture drops directly under 7 ml Ovoil to minimize evaporation. Dishes were preequilibrated at least 4 h before culture in a humidified multi gas incubator (Sanyo MCOK-5M[RC], Japan) (37°C, 6% CO_2, 20% O_2 or 37°C, 6% CO_2, 5% O_2, 89% N_2).

Animal and stimulation protocol

F1 (C57BL/6 x CBA) mice were bred by Florey Institute of Neuroscience and Mental Health animal facility (Melbourne, Australia), and were obtained when 3 to 4 weeks old. Mice were housed at the animal research facility, School of Biosciences, University of Melbourne in individual ventilated cages (Optimice; Animal Care Systems, USA), under a 12 h light/dark photoperiod (6am–6pm) with food and water *ad libitum* and controlled temperature (21°C).

Female mice 3 to 4 weeks old were superovulated via intraperitoneal injection with 5 IU pregnant mare's serum gonadotrophin (PMSG; Folligon; Intervet, Australia) followed by an injection of 5 IU human chorionic gonadotrophin (hCG; Chorulon; Intervet) 48 h later and mated to a male of the same strain. The following morning the presence of a vaginal plug was used as an indicator of successful mating.

Mouse pronuclear (2PN) oocyte collection

Female mice were sacrificed via cervical dislocation, the abdominal was disinfected with 80% ethanol (EtOH) and an incision was made in both the skin and peritoneum using fine surgical scissors to expose the reproductive tract. Watchmaker forceps (size 5) were used to hold the uterine horn taught and the oviduct was dissected out. Oviducts were placed in a 35 mm Easy-Grip collection dish (Falcon) containing 2 ml G-MOPS PLUS medium (Vitrolife) prewarmed to 37°C, then individually transferred to a 60 mm center well organ culture dish (Falcon) containing warmed 500 µl G-MOPS PLUS. Under a SMZ 1500 dissection microscope (Nikon Instruments, USA) containing a heated stage (37°C), watchmaker forceps were used to tear open the swollen ampulla of the oviduct releasing the cumulus mass. The oviduct was removed from the organ well dish and 500 µl prewarmed hyaluronidase solution (300 IU/ml) was added for maximum of 1 min till cumulus cells had dissociated. Denuded pronucleate (2PN) oocytes were collected using a hand-pulled borosilicate glass pasteur pipette with a diameter slightly larger than the 2PN oocytes and washed three times with G-MOPS PLUS medium prior to being pooled before allocation for culture.

Mouse preimplantation embryo culture

For standard culture, 2PN oocytes were allocated to culture dishes individually or as groups to 2 µl or 20 µl G1 medium, respectively, in a humidified incubator at either high (37°C, 6% CO_2, 20% O_2) or low oxygen conditions (37°C, 6% CO_2, 5% O_2, 89% N_2). After 48 h, embryos were washed in G2 medium before being transferred to G2 culture drop for a further 48 h of culture. For continuous culture, 2PN oocytes were allocated individually to 10 µl G-TL medium (Vitrolife) and cultured uninterrupted for 74 h in low oxygen conditions. For metabolic analyses, embryos were washed in modified G2 medium before being transferred to a metabolic culture dish and cultured in groups of 5 regardless of the initial embryo density and oxygen conditions remained the same.

Mouse blastocyst vitrification and warming

Embryos were vitrified using RapidVit^{TM} Blast kits (Vitrolife) with a cryo-loop as the cryo-device (Lane, et al., 1999) on day 4, after 74 h of culture. A 4-well Nunc dish (Thermo Fisher Scientific, Australia), containing all three vitrification solutions (Vitri 1 Blast, Vitri 2 Blast and Vitri 3 Blast) was prewarmed on a heated stage for 20 min to 37°C. Embryos were transferred to 1 ml Vitri Blast 1 for 5-20 mins, then to 1 ml Vitri Blast 2 for 2 mins and lastly to 20 µl Vitri Blast 3 for 45 secs. Embryos were pipetted onto cryo-loop and placed into cryo-vial which is then submerged in liquid nitrogen.

Mouse embryos were warmed using RapidWarm^{TM} Blast kits (Vitrolife). A 4-well Nunc dish (Thermo Fisher Scientific), containing all three warming solutions (Warm 1 Blast, Warm 2 Blast and Warm 3 Blast) was prewarmed on a heated stage for 20 min to 37°C.

The cryo-loop with vitrified embryo(s) was dipped into Warm 1 Blast and incubated for 2 min. Embryos were then transferred to Warm 2 Blast for 3 min followed by Warm 3 Blast for 5-10 min. Embryos were washed once in modified EmbryoGlue (0.5 mM glucose) and immediately transferred to 0.5 µl modified EmbryoGlue, overlaid with 3.5 ml Ovoil, for 4 h until transferred to outgrowth assay.

Mouse embryo viability assessment

Morphological assessment

On days 2-5 of culture, culture dishes were removed from the incubator and morphological development was assessed using a dissecting microscope with a heated stage (37°C). The morphology of cleavage stage embryos was defined by the number of blastomeres present. Post compaction, the developmental stages were defined as: morula, loss of membrane definition between blastomeres; early blastocyst, the presence of blastocoel cavity occupying less than half of the embryo volume; blastocyst, blastocoel cavity occupies at least half of the embryo volume; expanded blastocyst, blastocoel cavity occupies the majority of the embryo volume and the zona pellucida (ZP) beings to thin; hatching, cells have begun to protrude the ZP; fully-hatched, the embryo has escaped the ZP completely (Gardner, et al., 2014, Gardner, et al., 2019).

Blastocyst total cell number

To determine the total cell number of blastocysts, after 96 h of culture expanded and hatching blastocysts were incubated in 0.1 mg/ml bisbenzimide (Hoechst 33258) in 10% EtOH and G-MOPS (Vitrolife) at 37°C for 20 mins. Blastocysts were washed once in G-

MOPS PLUS (Vitrolife) and mounted onto glass microscope slides (SuperFrost Plus, Thermo Scientific, Germany) in 100% glycerol and imaged using a Nikon Eclipse Ts100 inverted fluorescent microscope equipped with a Nikon digital sight DS-Fi camera (Nikon Instruments) at x200 magnification (Fig. 2.1). Total blastocyst cell number was obtained using FIJI image analysis software (ImageJ 2.0.0, www.imagej.net).

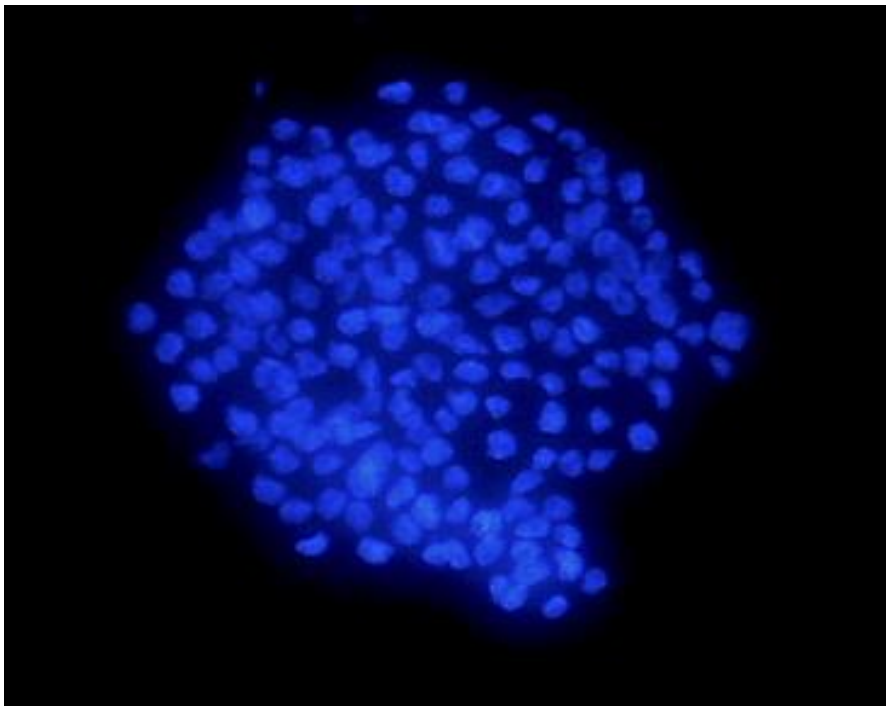


Figure 2.1 Fluorescent microscope image of day 5 mouse blastocyst after bisbenzimide staining.

Blue nuclei represent both trophectoderm (TE) and inner cell mass (ICM) cells, magnification x200.

Outgrowth assay

To determine the implantation potential of blastocysts (Binder, et al., 2015), individual wells of a microwell plate (Nunc MicroWell MiniTrays, Thermo Fisher Scientific, Australia), were rinsed with sterile phosphate-buffered saline (PBS) then incubated with 10 µg/ml fibronectin solution (Corning, USA) for 1 h at room temperature (RT). Wells were subsequently washed 3 times with PBS and incubated with 2.5 µl of bovine serum albumin solution (BSA; 4 mg/mL, MP Biomedicals, Australia), for 1 h at RT creating an appropriate surface for embryos to attach (Hannan, et al., 2011). Wells were washed thoroughly 3 times with PBS and 5 µl G2 medium containing 5% fetal calf serum (FCS; Invitrogen, Thermo Fisher Scientific) was added to each well followed immediately by 5 µl Ovoil and preequilibrated to 37°C, 6% CO_2, 5% O_2 and 89% N_2 for 4 h. Blastocysts were individually transferred from culture dish to outgrowth plate and incubated for 84 h. Blastocysts were imaged at 48 h, 60 h, 72 h, and 84 h post-culture using an inverted microscope with a heated stage (Ti-U eclipse; Nikon Instruments) with a CoolSNAP HQ camera (Photometrics, USA). Images were captured using NIS Elements BR 3.00, SP7 Laboratory Imaging software (Nikon Instruments) and area of outgrowths were calculated using FIJI ImageJ software (ImageJ 2.0.0, www.imagej.net) (Fig 2.2).

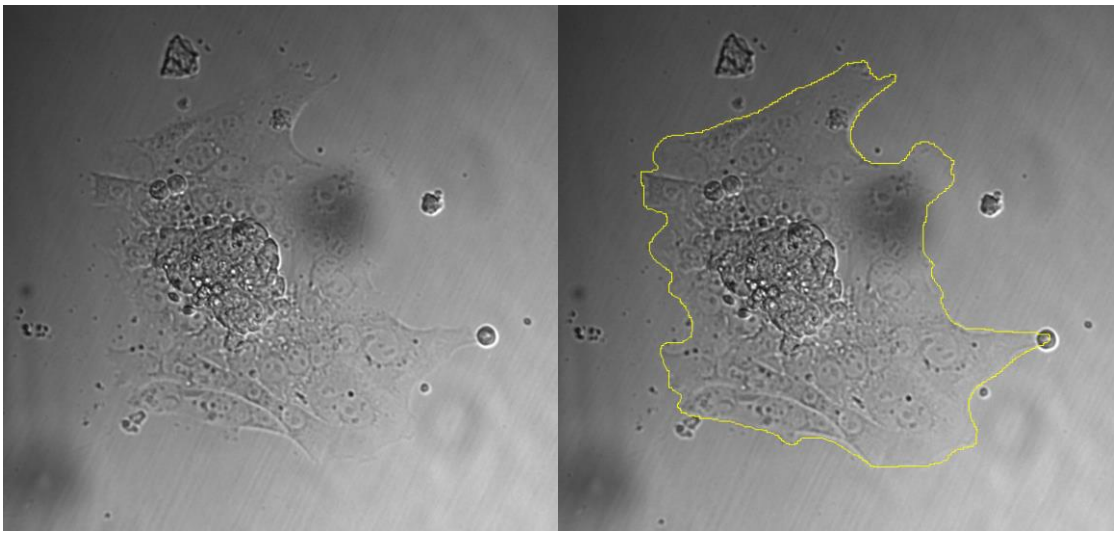


Figure 2.2 Microscope image of vitrified and warmed mouse blastocyst outgrowth area.

Yellow line represents circumference of outgrowth area as calculated using Fiji ImageJ software, magnification x100.

Human stimulation protocol

Women underwent a standard gonadotropin-releasing hormone (GnRH) antagonist stimulation protocol. Ovarian stimulation began on the second day of the menstrual cycle with recombinant follicle stimulating hormone (FSH) injections (Gonal F, Merck, Australia). A GnRH antagonist (Cetrotide, Merck) was administered on day 4-5 of stimulation to prevent premature ovulation. Cycle progress was monitored using transvaginal ultrasound and serum estradiol levels. Upon leading follicles reaching ~17 mm in size, a trigger hCG injection (Ovidrel, Merck) was administered and oocyte collection performed 36 h post-trigger.

Human preimplantation embryo insemination and time-lapse culture

Two hours following oocyte retrieval oocytes were denuded of surrounding cumulus cells using a hyaluronidase solution (SynVitro Hyadase, CooperSurgical, USA) and inseminated via ICSI or IVF. Injected oocytes were immediately transferred to individual culture wells of preequilibrated culture dish. Fertilization assessment was performed 16 h postinsemination and 2PN embryos were transferred to an EmbryoSlide® culture dish (Vitrolife). EmbryoSlide® wells were prepared with 20 µl G-TL media per culture well (Vitrolife) and immediately covered with 1.4 ml Ovoil (Vitrolife) and preequilibrated for at least 4 h prior to culture at 5% O_2, 6% CO_2, 89% N_2 at 37°C in a humidified multi gas incubator (Origio, planer benchtop incubator, CooperSurgical). Embryos were cultured in an EmbryoScope® time-lapse incubator (Vitrolife) at 5% O_2, 6% CO_2, 89% N_2 at 37°C. At 90 h post insemination, embryos were washed in G-TL medium and transferred to a new preequilibrated EmbryoSlide® culture dish containing 15 µl G-TL medium in each well

overlaid with 1.4 ml Ovoil (Vitrolife). Embryos were cultured to day 5-7 when they reached blastocyst stage and could be transferred, vitrified or preimplantation genetic testing (PGT) conducted. Embryos that did not reach the blastocysts stage were discarded accordingly.

Human blastocyst vitrification and warming

Embryos were vitrified on either day 5 or 6 of development using RapidVit™ Blast kits (Vitrolife) and Rapid-i Vitrification System (Vitrolife) (Desai, et al., 2013). A multi-well dish containing 1 ml of Vitri 1 Blast, Vitri 2 Blast and Vitri 3 Blast and was prewarmed to 37°C. Embryos were transferred through vitrification solutions as previously described in the mouse vitrification section. Embryos were pipetted onto Rapid-i device and placed into Rapid-I straw precooled in liquid nitrogen and the straw was immediately sealed with an ultrasonic sealer. The Rapid-i straw was then placed into cane for storage.

On the day of transfer, embryos were warmed using RapidWarm™ Blast kits (Vitrolife). A multi-well dish containing 1 ml of Warm 1 Blast, Warm 2 Blast and Warm 3 Blast was prewarmed on a heated stage to 37°C. The Rapid-i straw was cut and the Rapid-i device removed with tweezers and placed into Warm 1 Blast solution. Embryos were then transferred through warming solutions as previously described in the mouse warming section. Following warming embryos were immediately washed twice with G-TL medium and once with EmbryoGlue medium before being incubated in 10 µl EmbryoGlue medium under 2 ml Ovoil awaiting transfer (Vitrolife).

Human embryo viability assessment

Morphological grade

Assessment of human blastocyst morphology was conducted at 115 h post insemination. Blastocyst expansion, and inner cell mass (ICM) and trophectoderm (TE) quality were graded according to the Gardner grading system (Gardner, et al., 2000). The level of blastocyst expansion was demonstrated by the numerical grade from 1-6, these grades can be defined as: 1, the blastocoel is less than half of the volume of the embryo; 2, the blastocoel is equal to or greater than half the volume of the embryo; 3, the blastocoel fills the total volume of the embryo; 4, an increase in embryo volume and thinning of the ZP; 5, TE begins to herniate from the ZP; and 6, the embryo completely escapes the ZP. The quality of ICM and TE was represented by an alphabetical grade A-C, these grades can be defined as: A, many tightly packed cells; B, few/several loosely grouped cells; and C, very few large disorganized cells.

Blastocyst diameter

Blastocyst diameter was measured 115 h post insemination using the EmbryoViewer® software diameter tool (version 7.5.201.20844, Vitrolife). This was measured as the distance between the outer borders of the TE (Almagor, et al., 2016) (Fig. 2.3).

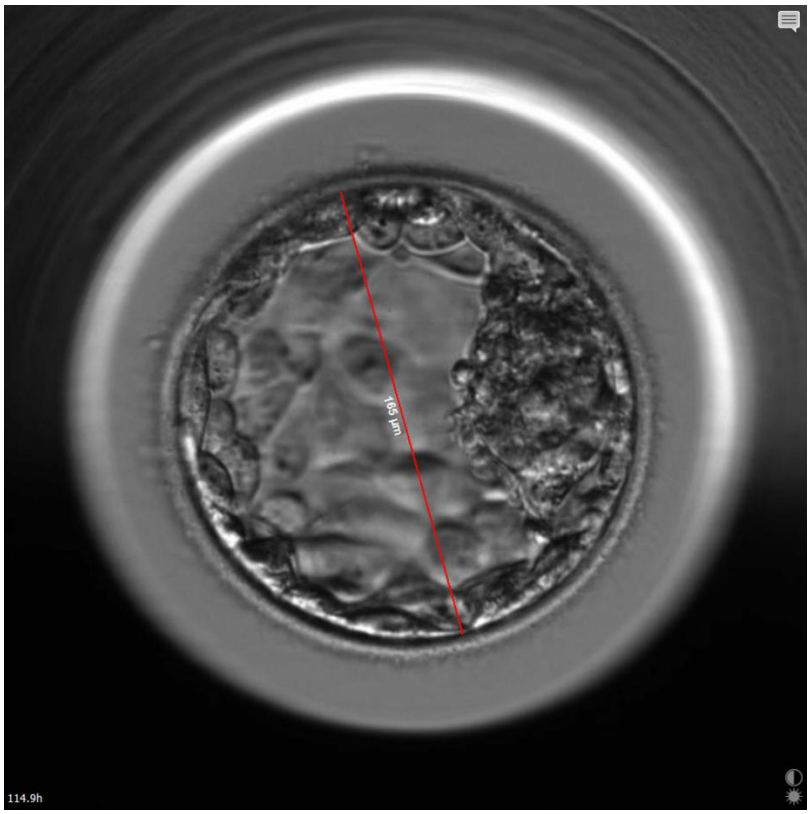


Figure 2.3 Assessment of human blastocyst diameter.

Blastocysts were assessed at 115 h using EmbryoViewer® software diameter tool. As demonstrated by the red line, diameter is calculated as the distance between the outer borders of the trophectoderm layer.

Morphokinetic parameters

Morphokinetic parameters were annotated using EmbryoViewer® software from images captured by the EmbryoScope® time-lapse incubator every 10 minutes in seven focal planes. The times of 2-cell cleavage (t2), 3-cell cleavage (t3), 4-cell cleavage (t4), 5-cell cleavage (t5), 6-cell cleavage (t6), 7-cell cleavage (t7), and 8-cell cleavage (t8) were recorded as the time when new blastomere cell membranes separated (Meseguer, et al., 2011). The notation of morphokinetic development post-compaction of morula (tM), start of blastulation (tSB), blastocyst (tB), expanded blastocyst (tEB) and hatching blastocyst (tH) can be defined as described in the mouse embryo morphological assessment section and as previously described (Desai, et al., 2014), and were recorded as hours post insemination (Fig. 2.4).

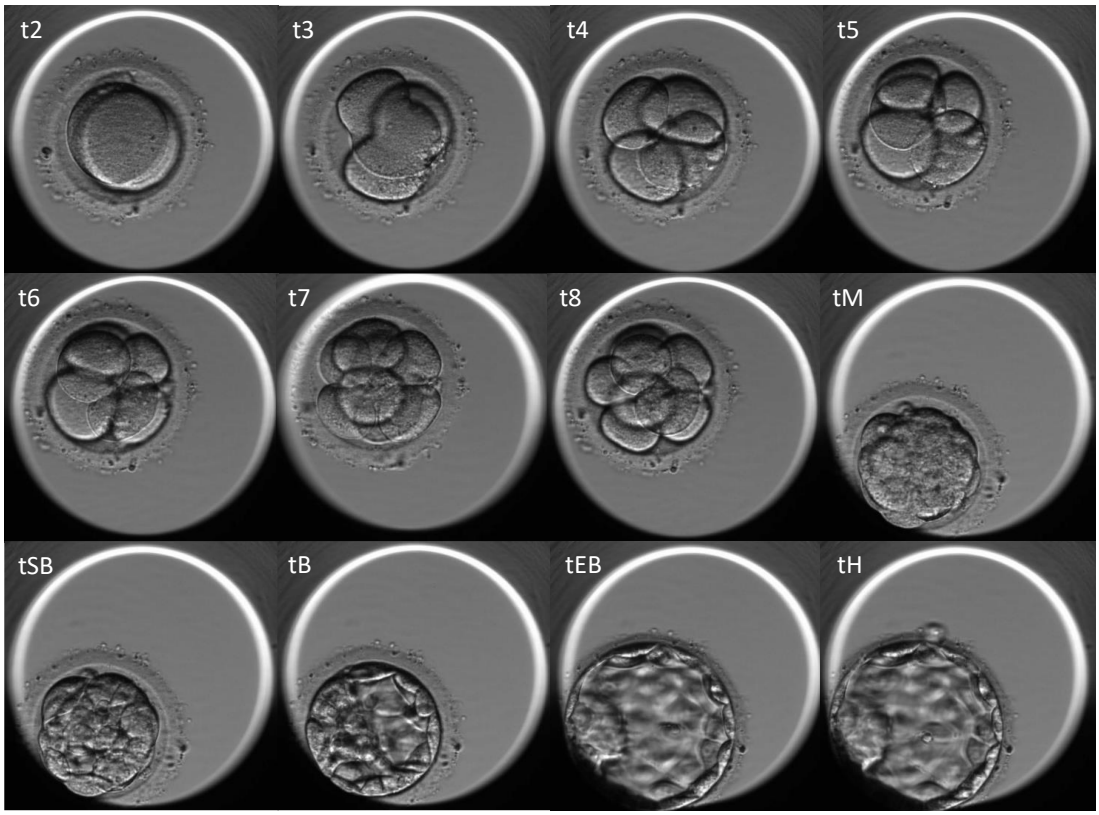


Figure 2.4 Development stages of human preimplantation embryo.

Embryo developmental stages annotated as 2-cell cleavage (t2), 3-cell cleavage (t3), 4-cell cleavage (t4), 5-cell cleavage (t5), 6-cell cleavage (t6), 7-cell cleavage (t7), 8-cell cleavage (t8), morulae (tM), start of blastulation (tSB), blastocyst (tB), expanded blastocyst (tEB) and hatching blastocyst (tH), recorded as h post insemination.

KIDScore

KIDScore values (0 to 9.9) were calculated using EmbryoViewer® software utilizing the D5 KIDScore algorithm (version 2). The KIDScore predicts the probability an embryo will successfully implant based on the assessment of whether the embryo is 2PN, the annotations for t2, t3, t4, t5 and tB and TE quality (Reignier, et al., 2019).

Artificial intelligence (AI) selection

Videos of preimplantation development (0 – 115 h post insemination) were exported from EmbryoViewer® software and uploaded into the AI program IVY (version 1.0.0, Harrison AI, New South Wales, Australia). IVY assesses preimplantation embryo development across a single focal plane of the time-lapse video utilizing no pre-set parameters and calculates an EmbryoScore (%), representing the probability that an embryo will develop a fetal heartbeat (Tran, et al., 2019).

Preimplantation genetic testing for aneuploidy (PGT-A)

Trophectoderm biopsy of 5-10 cells was performed on suitable blastocysts on days 5, 6 or 7 of culture and DNA was amplified using SurePLEX (Illumina, USA). A library of all amplified samples was constructed using VeriSeq PGS Library Prep Kit and sequencing was performed by MiSeq Reagent Kit v3-PGS on Miseq system following the manufacturer's instructions (Illumina). Analysis and copy number variant calling were performed using BlueFuse Multi software (Illumina) (Munné, et al., 2019). Only embryos considered euploid (no abnormality detected), or embryos with uniform chromosome aneuploidies were included for experimentation.

Pregnancy assessment

Pregnancy rates were recorded for both frozen embryo transfers (FET) and fresh single embryo transfers (SET). Biochemical pregnancy was recorded after a hCG blood test (positive pregnancies were hCG levels >5 IU/L), clinical pregnancies were later confirmed at a 6-week ultrasound with observation of a fetal heartbeat, and live birth rates were recorded as well as gender at the time of delivery.

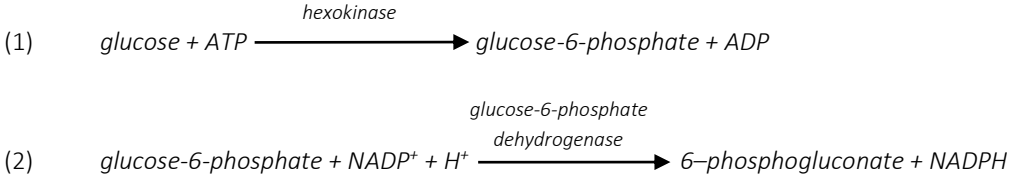
Carbohydrate metabolic analysis

Coupled enzymatic assays were used to analyze carbohydrate metabolism of mouse and human blastocysts from samples of spent medium with fluorescence being quantitated via ultramicrofluorescence (UMF) (Leese, et al., 1984). Quantification of metabolite concentrations were determined by analyzing the change in fluorescence of the co-factor NAD(P)H (excitation 340 nm, emission 459 nm). Fluorescent values lower or higher than controls respectively represented the consumption or production of carbohydrate metabolites (Gardner, et al., 1986). Nanoliter volume micropipettes were prepared from borosilicate glass capillaries pulled over a flame and a microforge was used to place a constriction in the pipette as previously described (Gardner, 2007). Micropipettes were siliconized with Sigmacote prior and between use and reactions were set up using a micro-manipulator. Individual drops of carbohydrate enzymatic cocktail were prepared using micropipettes under Ovoil to minimize evaporation. The initial fluorescent value for cocktail alone was measured then spent media sample was added in a 1:10 ratio of sample:cocktail (glucose and lactate assay), or 1:6 ratio (pyruvate assay). Fluorescent values were measured again and the difference from the initial reading was compared against a standard curve of R^2=0.98 or greater to determine metabolite concentration.

Duplicate fluorescent values were measured during optimization of each carbohydrate assays to confirm replicability and accuracy of assays. Single measurements were measured during experimental work.

Glucose assay

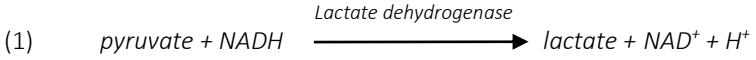
To measure the glucose concentrations of spent medium samples and controls, the following coupled reactions were utilized:



The glucose cocktail contained 0.42 mM dithiothreitol (DTT), 3.08 mM MgSO4, 0.42 mM ATP, 1.25 mM $NADP^+$, 14.11 U/ml hexokinase and 7.06 U/ml glucose-6-phosphate dehydrogenase in EPPS buffer, at pH 8.0. For mouse spent medium (Chapter 6) a standard curve was created using modified G2 medium with glucose concentrations of 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mM. For human spent medium (Chapter 3 and 4) a standard curve was created using G-TL medium with glucose concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM.

Pyruvate assay

To measure pyruvate concentrations of spent medium samples and controls, the following single reaction was utilized:



The pyruvate cocktail contained 0.12 mM NADH and 189.5 U/ml LDH in EPPS buffer, pH 8.0. For mouse and human spent medium (Chapter 5) a standard curve was created using modified EmbryoGlue medium with pyruvate concentrations of 0, 0.02, 0.04, 0.06, 0.08 and 0.1 mM.

Lactate assay

To measure lactate concentrations of spent medium samples and controls, the following single reaction was utilized:

$$(1) \qquad lactate + NAD^+ + H^+ \xrightarrow{Lactate\ dehydrogenase} pyruvate + NADH$$

The lactate cocktail contained 4.76 mM NAD^+, 195.3 U/ml lactate dehydrogenase (LDH) in glycine hydrazine buffer, at pH 9.4. To measure mouse spent culture media (Chapter 6) a standard curve was created using modified G2 medium with lactate concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1 mM.

Amine analysis

For amine analysis liquid chromatography-mass spectrometry (LC-MS) using an Agilent 1200 LC-system coupled to an Agilent 6420 ESI-QqQ-MS was utilized to measure amino acid concentration when compared against a standard calibrated curve, as performed by (Wale, et al., 2012). A sample of spent culture medium (1 µl) was diluted 1:10 with 50% methanol containing 10 µM IS 15N 13C-valine. The mixture was vortex, after which 70 µl of borate buffer containing 2 µM 13C6-Leucine was added and centrifuged for 1 min. To this, 20 µl of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) was added and immediately vortexed. AQC treated samples were then warmed to 55^{o}C for 10 mins. The

solution was centrifuged for a further 5 mins and once cooled to RT, 45 µl was transferred to glass vials for analysis via LC-MS. Pooled batch quality controls and pooled quality controls were utilized to ensure uniformity within and between batches.

Confocal microscopy

To quantify mitochondrial membrane potential (MMP), mitochondrial superoxide concentration and glutathione (GSH) levels in mouse blastocysts, individual blastocysts were stained with different fluorescent probes and analyzed using confocal microscopy. The fluorescent probes utilized were; 1.5 µM JC-1 (MP30168, Invitrogen, USA), to determine embryonic MMP, 5 µM MitoSOX™ (M36008, Invitrogen), to determine mitochondrial superoxide concentration, and 20 µM ThioTracker™ (T10095, Invitrogen), to measure GSH levels. A microwell plate (Nunc MicroWell MiniTrays, Thermo Fisher Scientific) was used to set up staining solutions. To prevent evaporation, 1 ml G2-MOPS was added to the area of the dish surrounding the wells and the dish was preequilibrated in a humified environment to 37°C.

Expanded or hatching blastocysts were transferred from the culture dish to initial wash drop of 5 µl G2MOPS handling medium then to 5 µl G2MOPS supplemented with a fluorescent probe for 15 mins. Following this, blastocysts were transferred to 5 µl G2MOPS supplemented with fluorescent probe and nuclear stain (10 µM DRAQ5, 62251, Thermo Scientific) for a further 15 mins. Once blastocysts had been washed three times in 5 µl G2-MOPS they were transferred to 0.2 µl of G2-MOPS in 60 mm coverslip bottom dish (FluroDish, World Precision Instruments Ltd, USA) under Ovoil. Individual blastocysts were imaged using a confocal microscope (Nikon A1R, Nikon Instruments) and NIS

Element software version 4.6 (Fig. 2.5). Stain intensities were quantified using Imaris software 8.4.1 (Oxford instruments, Abingdon, UK).

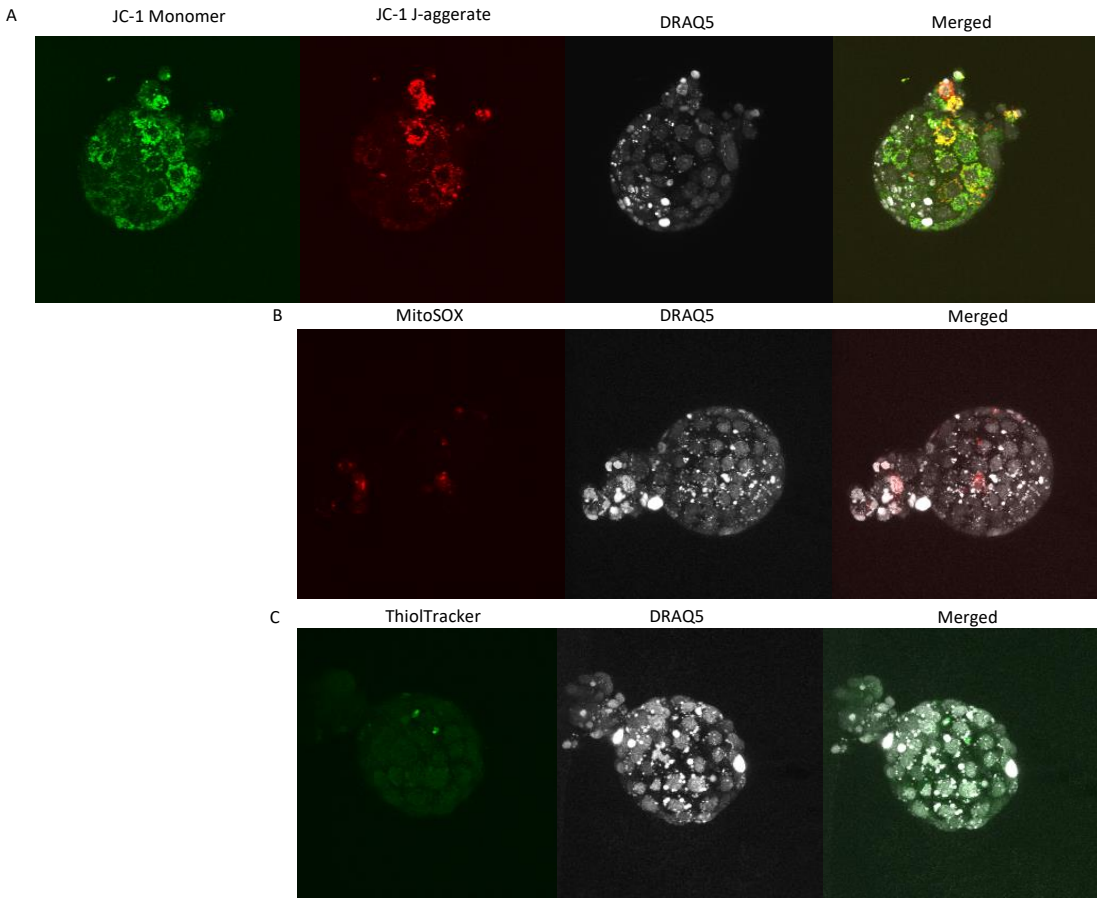


Figure 2.5 Confocal images of mouse blastocysts stained with fluorescent probes.

(A) JC-1 stained hatching blastocyst with DRAQ5 nuclear stain to measure mitochondrial membrane potential. (B) MitoSOX stained hatching blastocyst with DRAQ5 nuclear stain to measure mitochondrial superoxide concentration. (C) ThioTracker stained hatching blastocyst with DRAQ5 nuclear stain to measure GSH levels. All images captured at magnification x600. FIJI ImageJ software was utilized to compress Z-stacks of 3D image and merge channels. Analysis of stain intensities was performed on the 3D image using Imaris software 8.4.1.

Autofluorescence

NADH autofluorescence was quantified as a measure of the redox state of mouse blastocysts that had reached the expanded or hatching stage. Individual blastocysts were transferred to 0.2 µl of G-MOPS in 60 mm coverslip bottom dish (FluroDish, World Precision Instruments Ltd) under Ovoil after 96 h of culture and imaging was achieved using an inverted microscope with a heated stage (Ti-U eclipse; Nikon Instruments) with a CoolSNAP HQ camera (Photometrics) and NIS Elements BR 3.00, SP7 Laboratory Imaging software (Nikon Instruments). To reduce light exposure before imaging each treatment group was transferred to the imaging dish and imaged separately. NADH fluorescent intensities were quantified for individual embryos using FIJI ImageJ software (ImageJ 2.0.0, www.imagej.net)(Fig. 2.6).

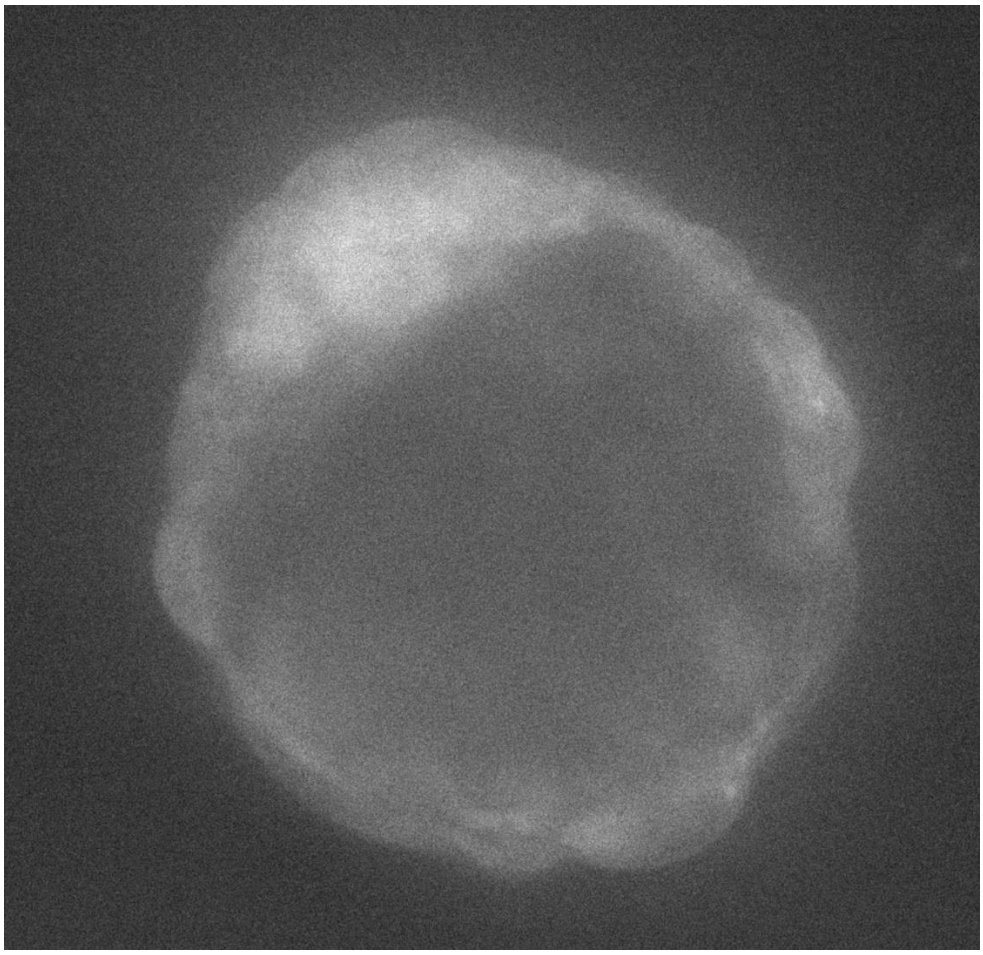


Figure 2.6 An example of NADH autofluorescence of an expanded mouse blastocyst.

Magnification x400.

Hydrogen peroxide levels

Intracellular levels of hydrogen peroxide in blastocysts were quantitated using peroxyfluor1 (PF1) (Centre for Nanoscale BioPhotonics, Australia). Following 96 h of culture, expanded and hatching blastocysts were incubated in G2 medium supplemented with 0.01 µM PF1 for 1 h under the same culture conditions. Following PF1 incubation, blastocysts were transferred to 0.2 µl of G2-MOPS in a 60 mm coverslip bottom imaging dish (FluroDish, World Precision Instruments Ltd) under Ovoil. Fluorescent intensities were captured using inverted microscope with a heated stage (Ti-U eclipse; Nikon Instruments) with a CoolSNAP HQ camera (Photometrics) and NIS Elements BR 3.00, SP7 Laboratory Imaging software (Nikon Instruments) and quantified using FIJI ImageJ software (ImageJ 2.0.0).

Statistical analyses

All analyses were conducted using Prism 8 (version 8.3.0, GraphPad, LLC) and significance threshold was set as *P* value was less than 0.05. In Chapter 4, to determine the relationship between morphological grade and embryonic ploidy status data was subject to a Fisher's exact test. Pearson's correlation coefficient was utilized to determine the correlation between maternal age and rate of blastocyst expansion, KIDScore, EmbryoScore and glucose and amino acid metabolism. In Chapter 5, a Chi-Square test was used to analyze the relationship between live birth rates and the expansion grade of human blastocysts. For all other analyses, normality of data was assessed using Shapiro-Wilk test prior to analysis. If data were normally distributed a comparison was made between two groups using a Student's *t*-test and for analyses with >2 groups, data were

subject to a one-way analysis of variance (ANOVA) and Bonferroni's Multiple Comparison test for multiple comparisons between groups. If data were not normally distributed, data were subject to a nonparametric Mann-Whitney U test.

Chapter 3. Metabolic activity of human blastocysts correlates with their morphokinetics, morphological grade, KIDScore and artificial intelligence ranking

Abstract

Study question: Is there a relationship between blastocyst metabolism and biomarkers of embryo viability?

Summary answer: Blastocysts with higher developmental potential and a higher probability of resulting in a viable pregnancy consume higher levels of glucose and exhibit distinct amino acid profiles.

What is known already: Morphological and morphokinetic analyses utilized in embryo selection provide insight into developmental potential, but alone are unable to provide a direct measure of embryo physiology and inherent health. Glucose uptake is a physiological biomarker of viability and amino acid utilization is different between embryos of varying qualities.

Study design, size, duration: Two hundred and nine human preimplantation embryos from 50 patients were cultured in a time-lapse incubator system in both freeze all and fresh transfer cycles. A retrospective analysis of morphokinetics, morphology (Gardner grade), KIDScore, artificial intelligence (AI) grade (EmbryoScore), glucose and amino acid metabolism, and clinical pregnancies was conducted.

Participants/materials, setting, methods: ICSI was conducted in all patients, who were aged ≤ 37 years and previously had no more than two IVF cycles. Embryos were individually cultured in a time-lapse incubator system, and those reaching the blastocyst stage had their morphokinetics annotated and were each assigned a Gardner grade, KIDScore and EmbryoScore. Glucose and amino acid metabolism were measured via ultramicrofluorescence and liquid chromatography-mass spectroscopy, respectively. Clinical pregnancies were confirmed by the presence of a fetal heartbeat at 6 weeks gestation.

Main results and the role of chance: Glucose consumption was at least 40% higher in blastocysts deemed of high developmental potential using either the Gardner grade (P <0.01, n = 209), KIDScore (P <0.05, n = 207) or EmbryoScore (P <0.05, n = 184), compared to less viable blastocysts and in blastocysts that resulted in a clinical pregnancy compared to those that failed to implant (P <0.05, n = 37). Additionally, duration of cavitation was inversely related to glucose consumption (P <0.05, n = 200). Total amino acid consumption was significantly higher in blastocysts with an EmbryoScore higher than the cohort median score (P <0.01, n = 185). Furthermore, the production of amino acids was significantly lower in blastocysts with a high Gardner grade (P <0.05, n = 209), KIDScore (P <0.05, n = 207), and EmbryoScore (P <0.01, n = 184).

Limitations, reasons for caution: Samples were collected from patients who had ICSI treatment and from only one clinic.

Wider implications of the findings: These results confirm that metabolites, such as glucose and amino acids, are valid biomarkers of embryo viability and could therefore be used in conjunction with other systems to aid in the selection of a healthy embryo.

Key words embryo, glucose, amino acids, non-invasive, selection, viability

Introduction

Historically, transferring multiple embryos in an IVF cycle was performed to attain acceptable success rates. However, transferring two or more embryos creates the possibility of a multiple gestation, which carries significantly greater medical risks to both mother and child (Adashi, et al., 2003, Albrecht, et al., 1996, Luke, et al., 1996, Ombelet, et al., 2005). Improvements in embryo culture systems facilitated single embryo transfers, reducing the incidence of multiple gestations (Gerris, et al., 1999) and in the last 20 years there has been a shift to single blastocyst transfers (Gardner, et al., 2000, Gardner, et al., 1998, Harbottle, et al., 2015, Penzias, et al., 2017, Takeshima, et al., 2016). While pregnancy rates worldwide have increased largely due to improvements in culture conditions, our ability to decrease time to pregnancy is dependent upon our capabilities to select the most viable blastocyst for transfer.

Traditionally, embryos are graded on morphological features leading to the creation of scoring systems for each successive stage of development. Parameters assessed include blastomere number, symmetry, and degree of fragmentation during the cleavage stages (Cummins, et al., 1986, Gerris, et al., 1999, Van Royen, et al., 1999), as well as size of blastocoel, and inner cell mass (ICM) and trophectoderm (TE) differentiation in the case of the blastocyst (Gardner, et al., 2000). Traditional morphological grading requires removal of the embryo culture dish from the incubator for assessment under the microscope, thereby exposing embryos to fluctuations in the environment such as light, temperature and pH, which can adversely impact embryo physiology and developmental potential (Gardner, et al., 2017, Hansen, 2007, Lane, et al., 2005, Wale, et al., 2016, Zander-Fox, et al., 2010). Additionally, such an approach to grading is, by necessity,

restricted to only a handful of discrete time points during development, thereby failing to capture the true dynamic nature of preimplantation embryo development (Montag, et al., 2011).

With the advent of time-lapse systems, preimplantation embryo development can be captured continuously, whilst also providing an uninterrupted culture environment. Time-lapse has subsequently facilitated identification of novel morphological events, such as reverse and direct cleavage, both of which have been linked to decreased embryo viability (Liu, et al., 2014, Rubio, et al., 2012). Annotating the timing of key morphological events and the rate of development (i.e. the time between two morphological events) has in turn led to the development of algorithms for embryo selection incorporating both morphological and morphokinetic parameters (Basile, et al., 2015, Liu, et al., 2016, Milewski, et al., 2015, Petersen, et al., 2016, Reignier, et al., 2019, Rubio, et al., 2014). Annotating morphokinetic parameters for each individual embryo is, however, time consuming and a level of inter-observer and intra-observer subjectivity exists impacting overall grading success (Storr, et al., 2017).

Artificial intelligence (AI) is relatively new to the field of assisted reproductive technologies and represents a potential means of standardizing and automating embryo selection. AI can be trained to assess embryo viability without pre-set assumptions, thereby removing the subjectivity previously associated with embryo grading (Curchoe, et al., 2019). Such deep-learning has been applied to a single static image during the preimplantation period (Miyagi, et al., 2019, Thirumalaraju, et al., 2019), while other groups have utilized images from across the entire preimplantation period facilitated by

time-lapse microscopy, thereby significantly increasing the amount of data used to identify embryos with the highest viability (Tran, et al., 2019). Such technologies hold great promise and the clinical introduction of AI awaits validation in prospective randomized controlled trials.

While assessment of morphology and morphokinetics is of value in deselection and selection of embryos for transfer, they are unable to provide a direct measure of embryo physiology (Paternot, et al., 2011, Santos Filho, et al., 2012, VerMilyea, et al., 2014). Metabolic regulation is essential for embryo development, and aberrations in preimplantation embryo metabolism are an indicator of lower post-implantation viability (Gardner, 1998, Gardner, et al., 2013). Importantly, embryos that have been assigned the same morphological grade do not necessarily exhibit the same metabolic profile (Gardner, et al., 2001). Analysis of embryo metabolism can therefore provide additional information regarding embryo viability subsequently aiding embryo selection. To date studies on human embryos have focused on the utilization of carbohydrates or amino acids (Gardner, et al., 2001, Gardner, et al., 2011, Gott, et al., 1990, Houghton, et al., 2002). However, other metabolites including lactate, lipids and oxygen (the consumption of which can provide an indirect measure of oxidative metabolism) which have been analyzed in animal models, may also be of value in establishing the metabolic state and health of the human embryo (Lane, et al., 1996, Sturmey, et al., 2009, Thompson, et al., 1996).

The metabolic profile of an embryo can be determined through the non-invasive analysis of changes in metabolite concentrations within spent culture medium (Houghton, et al.,

2002, Leese, et al., 1984). It has been demonstrated that high levels of glucose consumption are associated with higher development and implantation potential in mouse, cow and human, blastocysts (Gardner, et al., 2001, Gardner, et al., 1987, Gardner, et al., 2011, Kelley, et al., 2019, Lane, et al., 1996, Lee, et al., 2015, Renard, et al., 1980). Additionally, amino acid profiles are distinctly different between embryos of different developmental stages and quality (Brison, et al., 2004, Houghton, et al., 2002, Kelley, et al., 2019, Lee, et al., 2015, Picton, et al., 2010, Wale, et al., 2012). These data therefore support the possible use of both glucose and amino acids as biomarkers to evaluate embryo physiology and developmental potential prior to transfer.

Furthermore, metabolism is directly linked to gene expression and developmental programming through the metaboloepigenetic axis, in which metabolites and cofactors regulate the activity of epigenetic modifiers (Donohoe, et al., 2012, Gardner, et al., 2015, Harvey, et al., 2016). As distinct metabolic profiles of an embryo have been shown to manifest as changes in development post-transfer (Lane, et al., 1996, Lane, et al., 1998, Mitchell, et al., 2009), the utilization and analysis of metabolic biomarkers would aid in the selection of embryos with normal preimplantation physiology and subsequently increase the likelihood of healthy offspring (Barker, et al., 1986, Barker, et al., 1989, Ferrick, et al., 2019, Gardner, et al., 2017). It is currently unknown if metabolism is linked to existing embryo viability markers. Consequently, the aim of this study was to determine how blastocyst glucose and amino acid metabolism, as a measure of embryonic viability and health, relates to embryonic developmental parameters and transfer outcomes.

Materials and methods

Patient selection and ovarian stimulation

Fifty patients aged ≤ 37 years, with no more than 2 previous IVF cycles, undergoing an ICSI cycle between January 2018 to July 2019 were included in this retrospective study. Women underwent a gonadotropin-releasing hormone (GnRH) antagonist stimulation protocol. Ovarian stimulation began on the second day of menstrual cycle with recombinant FSH injections (Gonal F, Merck, Australia). A GnRH antagonist (Cetrotide, Merck) was administered on day 4-5 of stimulation to prevent premature ovulation. Transvaginal ultrasound and serum estradiol levels were used to monitor cycles. Upon leading follicles reaching ~17 mm in size, a trigger hCG injection (Ovidrel, Merck) was administered and oocyte collection performed 36 h post-trigger.

Embryo culture

Two hours following oocyte retrieval oocytes were denuded of surrounding cumulus cells using a hyaluronidase solution (SynVitro Hyadase, CooperSurgical, USA) and inseminated via ICSI 2 h later. Injected oocytes were immediately transferred to individual culture wells containing 25 µl G-TL medium overlaid with 1.4 ml Ovoil (Vitrolife AB, Sweden) in a pre-equilibrated EmbryoSlide® culture dish (Vitrolife). Embryos were cultured in an EmbryoScope® time-lapse incubator (Vitrolife) at 5% O_2, 6% CO_2, 89% N_2 at 37 °C and time of ICSI was set as insemination time. At 90 h and 114 h post-insemination, embryos were washed in culture medium and transferred to a new pre-equilibrated EmbryoSlide® culture dish containing 15 µl G-TL medium in each well overlaid with 1.4 ml Ovoil. On day 5 of development, embryos were assessed for transfer or vitrification, or cultured until

day 6 where they were either vitrified or discarded accordingly. The 90 – 114 h culture dishes containing spent media were stored at -20 °C until collection for metabolic analysis. Two hundred and nine embryos that reached the blastocyst stage during culture (i.e. by day 6) were retrospectively analyzed for morphological grade, morphokinetics, metabolism and transfer success.

Analysis of morphology

Morphological assessment was conducted at 115 h post-insemination. Blastocyst expansion, and ICM and TE quality were graded according to the Gardner grading system (Gardner, et al., 2000). Embryos graded higher than BB were considered to be of higher viability than embryos graded BB or less (Gardner, et al., 2004). Blastocyst diameter was measured (115 h post-insemination) using the EmbryoViewer® software diameter tool (version 7.5.201.20844, Vitrolife) and calculated as the distance between the outer borders of the TE (Almagor, et al., 2016).

Analysis of morphokinetics

Morphokinetic analysis was achieved using EmbryoViewer® software to assess images captured by the EmbryoScope® time-lapse incubator every 10 minutes in seven focal planes. The specific timings of 2-cell cleavage (t2), 3-cell cleavage (t3), 4-cell cleavage (t4), 5-cell cleavage (t5), 6-cell cleavage (t6), 7-cell cleavage (t7), 8-cell cleavage (t8), morulae (tM), start of blastulation (tSB), blastocyst (tB), expanded blastocyst (tEB) and hatching blastocyst (tH) were recorded as hours post-insemination (Desai, et al., 2014). Annotations were recorded for embryos which could be accurately assessed. KIDScore values (0 to 9.9) are calculated utilizing the D5 KIDScore algorithm (version 2), which is

integrated into the EmbryoViewer® software and available for use clinically. KIDScores predict the probability that an embryo will successfully implant and are based on the assessment of whether the embryo is 2PN, the annotations for t2, t3, t4, t5 and tB and TE quality (Reignier, et al., 2019). Embryos assigned a KIDScore greater than the median cohort grade (6.9) were considered to be of higher viability than embryos assigned a lower KIDScore. Of the 209 embryos cultured, 2 embryos where annotations could not be accurately assessed, were not included in analysis. Additionally, rate of expansion (tEB-tSB) was calculated and embryos were grouped into fast (≤ 13 hours) or slow (>13 hours) expansion rates as previously described (Desai, et al., 2018). To minimize inter-user variability, only one scientist conducted the annotation assessments.

Artificial intelligence selection

Videos of preimplantation development were exported from EmbryoViewer® software (0 – 115 h post-insemination) and uploaded into IVY (version 1.0.0, Harrison AI, Australia) an AI program, which assesses preimplantation embryo development across a single focal plane utilizing no pre-set parameters and calculates an EmbryoScore (%) which represents the probability that an embryo will develop a fetal heartbeat and can subsequently assist in ranking embryos for transfer (Tran, et al., 2019). Embryos assigned an EmbryoScore greater than the median cohort grade (29%) were considered to be of higher viability than embryos assigned a lower EmbryoScore. Of the 209 embryos cultured, 25 embryos could not be included in analysis as a result of an interference in the time-lapse video, such as the embryo went out of focus or oil bubbles in the culture well. Such factors can impact the overall EmbryoScore.

Metabolic analysis

Day 5 (90-114 h) spent media samples were collected and analyzed for both glucose and amino acid metabolism to provide a detailed metabolic profile for each individual embryo that reached the blastocyst stage. Spent media samples (test) and no-embryo (control) media were collected for each patient and metabolite consumption/production was calculated by subtracting test samples from controls.

Glucose uptake analysis

Ultramicrofluoresence (UMF), a miniaturization of conventional enzymatic analysis, was utilized to quantitate glucose metabolism (Gardner, et al., 2001, Leese, et al., 1984).

Amino acid analysis

Liquid chromatography-mass spectrometry (LC-MS) analysis using an Agilent 6490 Triple Quadrupole LC-MS with iFunnel Technology coupled with an Agilent 1290 liquid chromatography system (Agilent, USA) was utilized to measure amino acid concentrations when compared against a standard calibrated curve (Wale, et al., 2012). Pooled quality controls and pooled batch quality controls were conducted to ensure uniformity within and between batches and Agilent MassHunter Quantitative Analysis software (B.08.0, Agilent) was used to calculate relative abundances (integrated area) of amine-containing metabolites.

Pregnancy assessment

Of the 209 embryos cultured, 37 single blastocysts were transferred in a fresh cycle and the remaining blastocysts were vitrified as part of a freeze-all cycle. The embryo with the

best morphology, followed by the highest KIDScore amongst a cohort was selected for transfer. Only embryos transferred in a fresh cycle were included in pregnancy rate analysis. Clinical pregnancies were confirmed at a 6 week ultrasound with observation of a fetal heartbeat.

Statistical analysis

For morphokinetic analysis blastocysts were grouped according to the median glucose and amino acid utilization. To analyze blastocyst metabolism in comparison to KIDScore and EmbryoScore, embryos were grouped based on the median scores (6.9 and 29%, respectively). Embryos were grouped as per Gardner grade (higher than BB or BB and less) for analysis of blastocyst metabolism and morphological grade. Data is expressed as mean ± SEM and tested for normality using the Shapiro-Wilk test. The difference between two groups was determined using a parametric Student's t-test or nonparametric Mann-Whitney U test and the *P* value threshold was set to 0.05. All analyses were conducted using Prism 8 (version 8.3.0, GraphPad, LLC).

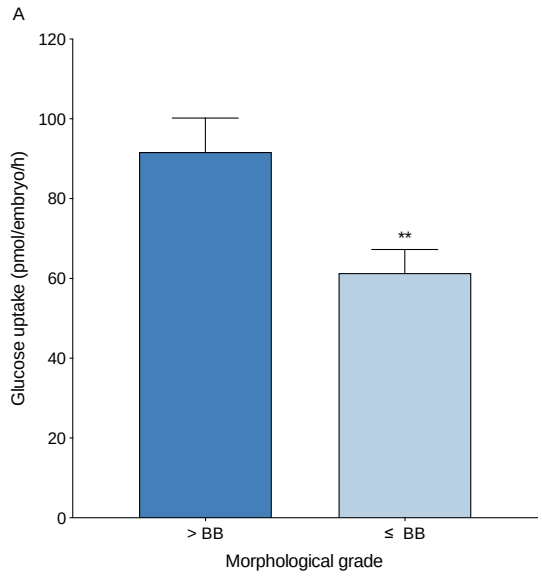
Ethical approval

Ethical approval was obtained from Melbourne IVF Human Research Ethics Committee (53/117-MIVF), patients were recruited during clinical consultation.

Results

Blastocyst metabolism is positively related to embryo viability scores

Three different grading systems were used to retrospectively assess embryo viability prior to transfer: (1) morphological grading using the Gardner grading system, (2) D5 KIDScore algorithm, and (3) EmbryoScores through an AI system (IVY). Regardless of which method embryo viability was measured with, blastocysts with the highest viability consumed at least 40% more glucose than lower quality blastocysts (Fig. 3.1). Blastocysts with a Gardner grade greater than a BB grade (n = 86) consumed significantly more glucose on day 5 of development than less viable blastocysts which scored a BB grade or less (n = 123) (91.7 ± 8.5 versus 61.4 ± 5.9 pmol/embryo/h, P <0.01) (Fig. 3.1A). Similarly, blastocysts that were assigned a KIDScore greater than the median cohort score (n = 100) consumed more glucose on day 5 than lower scoring blastocysts (n = 107) (88.1 ± 7.8 versus 60.9 ± 6.3 pmol/embryo/h, P <0.05) (Fig. 3.1B) and blastocysts with an EmbryoScore greater than the median cohort score (n = 91), (i.e. those with a higher probability of developing a fetal heartbeat) consumed more glucose on day 5 of development compared to less viable blastocysts (n = 93) (87.9 ± 8.1 versus 61.7 ± 7.4 pmol/embryo/h, P <0.05) (Fig. 3.1C). Of those embryos with a high KIDScore and an EmbryoScore (n = 83), 77% had a high EmbryoScore (n = 64). Of these embryos with both a high KIDScore and EmbryoScore, 64% consumed more glucose than the median cohort uptake (n = 41).


A
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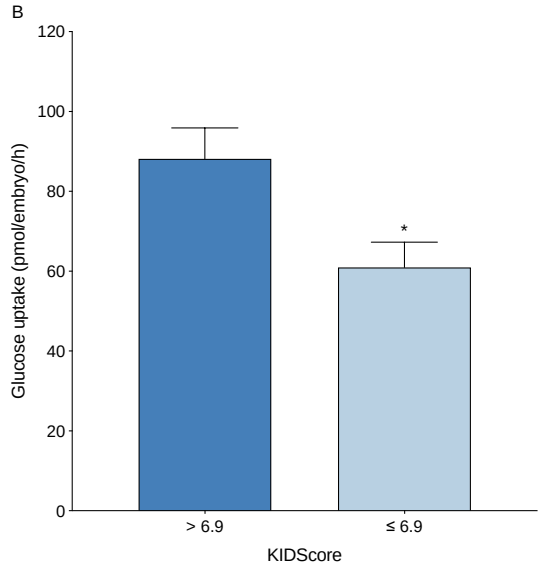
Glucose uptake (pmol/embryo/h)
**
> BB
≤ BB
Morphological grade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Glucose uptake (pmol/embryo/h)
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> 6.9
≤ 6.9
KIDScore

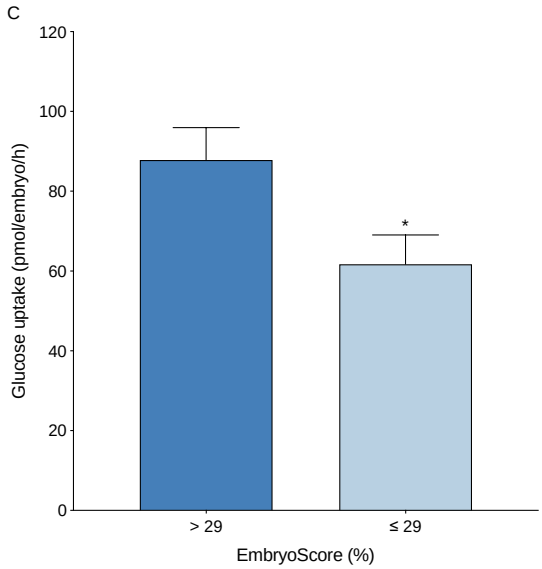

C
120
100
80
60
40
20
0
Glucose uptake (pmol/embryo/h)
*
> 29
≤ 29
EmbryoScore (%)

Figure 3.1 Blastocyst glucose uptake compared to embryo viability.

(A) Gardner grade (>BB, n = 86; ≤BB n = 123), (B) KIDScore (>6.9, n = 100; ≤6.9 n = 107), and (C) EmbryoScore (>29%, n = 91; ≤29% n = 93). Each data set (A-C) is expressed as mean ± SEM (pmol/embryo/h). Significance is measured utilizing Mann-Whitney U test and denoted by asterisks, *P <0.05, **P <0.01.

Blastocyst glucose metabolism is related to clinical pregnancy rates

Blastocysts that resulted in a clinical pregnancy demonstrated a two-fold increase in glucose consumption on day 5 of the preimplantation period than blastocysts that failed to implant or maintain a successful pregnancy (77.4 ± 11.7 versus 38.4 ± 11.2 pmol/embryo/h, P <0.05) (Fig. 3.2). Of note, blastocysts assigned an AA morphological grade which established a clinical pregnancy consumed the highest level of glucose (84.4 ± 17.3 pmol/embryo/h, n = 11). Additionally, while results were not statistically significant, blastocysts assigned an AA morphological grade which failed to establish a pregnancy (n = 7) consumed lower glucose than blastocysts assigned a B grade for ICM and/or TE (AB, BA or BB) that successfully implanted (n = 6) (22.2 ± 25.3 pmol/embryo/h versus 64.5 ± 9.1 pmol/embryo/h, P = 0.12). No significant difference was observed in amino acid utilization patterns between embryos that established a clinical pregnancy and embryos that failed to implant (data not shown).

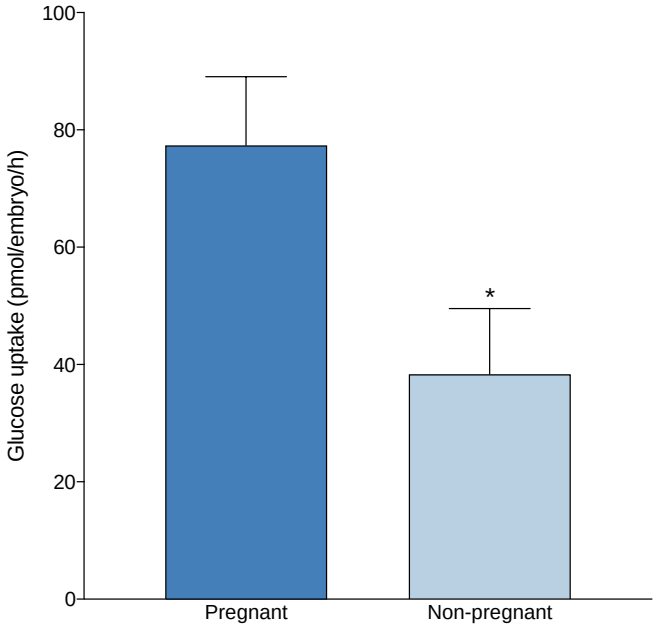
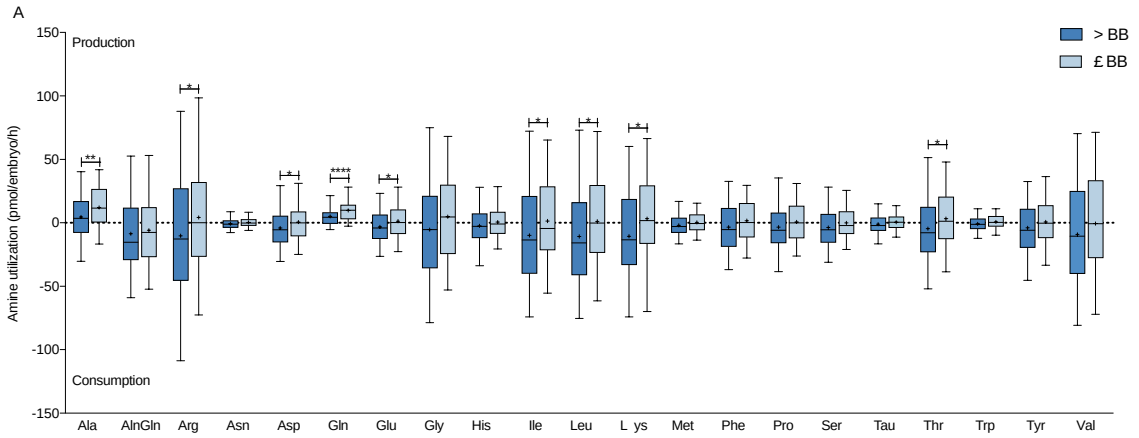


Figure 3.2 Glucose uptake of blastocysts that were transferred in a fresh single blastocyst transfer cycle and ongoing pregnancy rates.

All data is expressed as mean ± SEM (pmol/embryo/h) for pregnant (n = 17) and non-pregnant (n = 20). Significance is measured utilizing Student's t-test and asterisks denote significance, *P <0.05.

Amino acid profiles of blastocysts are distinctly different according to embryo viability scores

Blastocysts with high viability according to their Gardner grade consumed significantly higher levels of arginine (P <0.05), aspartate (P < 0.05), glutamate (P < 0.05), isoleucine (P <0.05), leucine (P <0.05), lysine (P <0.05), and threonine (P <0.05) and produced significantly lower levels or alanine (P < 0.01) and glutamine (P <0.0001), compared to blastocysts assigned a lower Gardner grade (Fig. 3.3A). Similarly, these same amino acids as well as asparagine, phenylalanine, taurine and tryptophan were utilized differently by blastocysts that had a high implantation potential, as assessed by D5 KIDScore algorithm, compared to less viable blastocysts (alanine, P <0.01; arginine, P <0.05; asparagine, P <0.05; aspartate, P <0.05; glutamine, P <0.01; glutamate, P <0.05; isoleucine, P <0.05; leucine, P <0.05; lysine, P <0.05; phenylalanine, P <0.05; taurine, P < 0.05; threonine, P <0.05; tryptophan, P <0.05) (Fig. 3.3B). Furthermore, each amino acid with the exception of alanyl-glutamine, glycine and valine were either consumed at higher levels or produced at lower levels by blastocysts with a higher EmbryoScore than less viable blastocysts (alanine, P <0.001; arginine, P <0.01; asparagine, P <0.01; aspartate, P <0.01; glutamine, P <0.001; glutamate, P <0.001; histidine, P <0.05; isoleucine, P <0.05; leucine, P <0.01; lysine, P <0.01; methionine, P <0.05; phenylalanine, P <0.05; proline, P <0.05; serine, P <0.01; taurine, P <0.01; threonine, P <0.01; tryptophan, P <0.01; tyrosine, P <0.05) (Fig. 3.3C).


A
Production
Consumption
Amine utilization (pmol/embryo/h)
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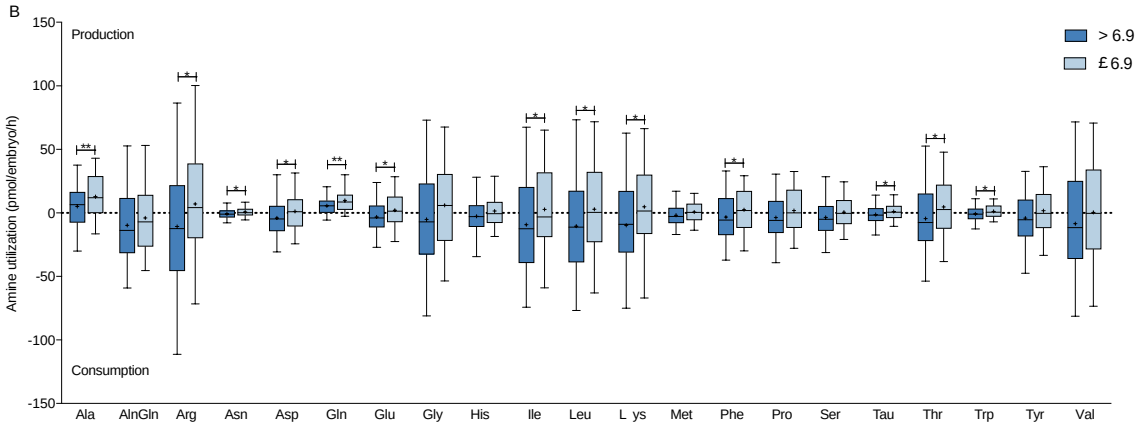
Ala AlnGln Arg Asn Asp Gln Glu Gly His Ile Leu L ys Met Phe Pro Ser Tau Thr Trp Tyr Val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Amine utilization (pmol/embryo/h)
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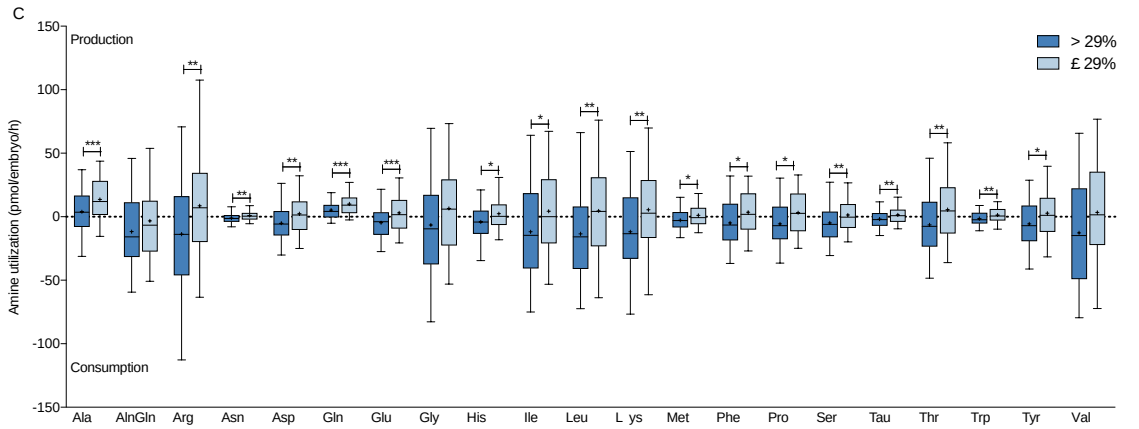
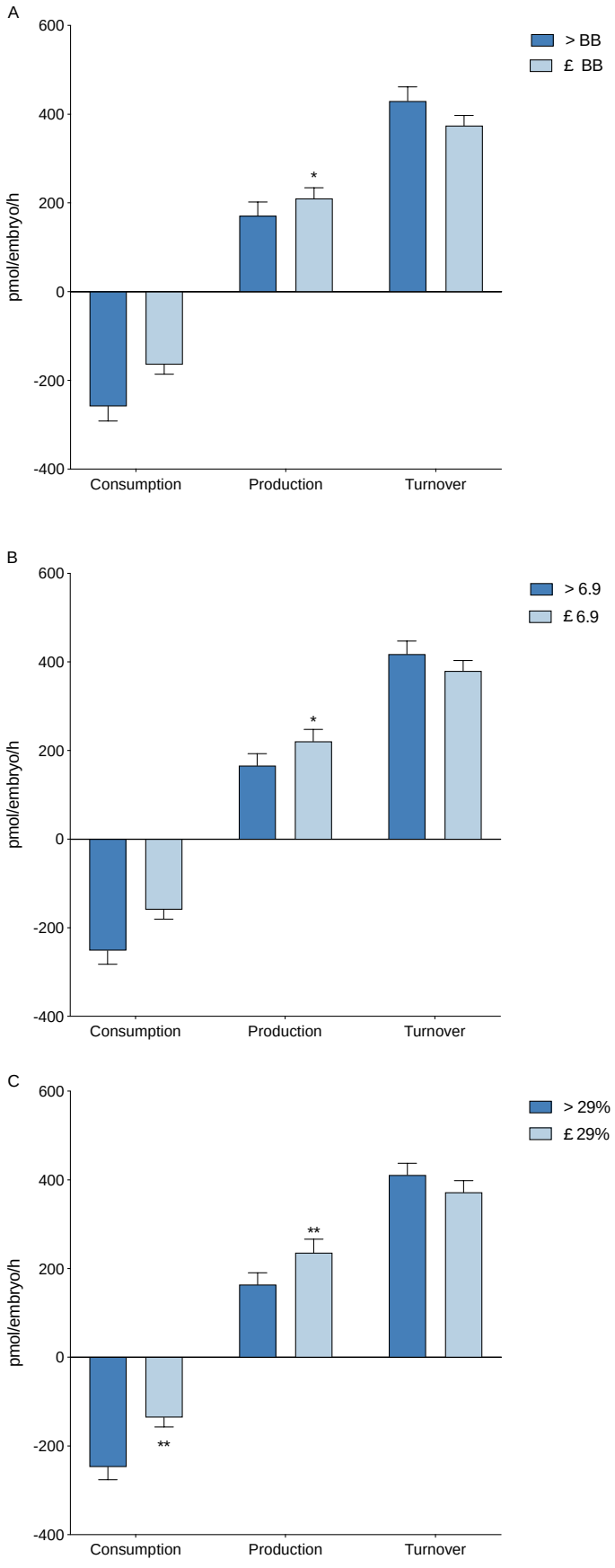

C
Production
Consumption
Amine utilization (pmol/embryo/h)
> 29%
£ 29%
Ala AlnGln Arg Asn Asp Gln Glu Gly His Ile Leu L ys Met Phe Pro Ser Tau Thr Trp Tyr Val

Figure 3.3 Blastocyst amino acid utilization profiles compared to embryo viability.

(A) Gardner grade (>BB, n = 86; ≤BB n = 123), (B) KIDScore (>6.9, n = 100; ≤6.9 n = 107) and (C) EmbryoScore (>29%, n = 91; ≤29% n = 93). Negative values represent amino acid consumption and positive values represent amino acid production. Alanine (Ala), alanyl-glutamine (AlnGln), arginine (Arg), asparagine (Asn), aspartate (Asp), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), taurine (Tau), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). Boxes represent 25th to 75th percentile, whiskers represent 5th to 95th percentile, '+' represents mean, and median represented by horizontal line. Significance is measured utilizing Student's t-test and denoted by asterisks, *P <0.05, **P <0.01, ***P <0.001, ****P <0.0001.

Blastocyst total amino acid consumption and production is related to embryo viability scores

Overall amino acid turnover (sum of consumption and production) was analyzed as a further measure of blastocyst metabolic activity. There was no significant difference in amino acid turnover between blastocysts of high and low viability when assessed using Gardner grade, KIDScore or EmbryoScore (Fig. 3.4). However, the total production of amino acids on day 5 of preimplantation development was significantly lower in blastocysts with high viability, as determined by Gardner grade (171.6 ± 30.5 versus 210.2 ± 24.1 pmol/embryo/h, P <0.05), D5 KIDScore algorithm (166.1 ± 27.0 versus 221.1 ± 26.6 pmol/embryo/h, P <0.05) and EmbryoScore (160.5 ± 26.4 versus 235.9 ± 30.4 pmol/embryo/h, P <0.01) (Fig. 3.4). Additionally, blastocysts with high EmbryoScores also consume significantly more amino acids overall compared to less viable blastocysts (245.2 ± 28.6 versus 134.7 ± 20.6 pmol/embryo/h, P <0.01) (Fig. 3.4C).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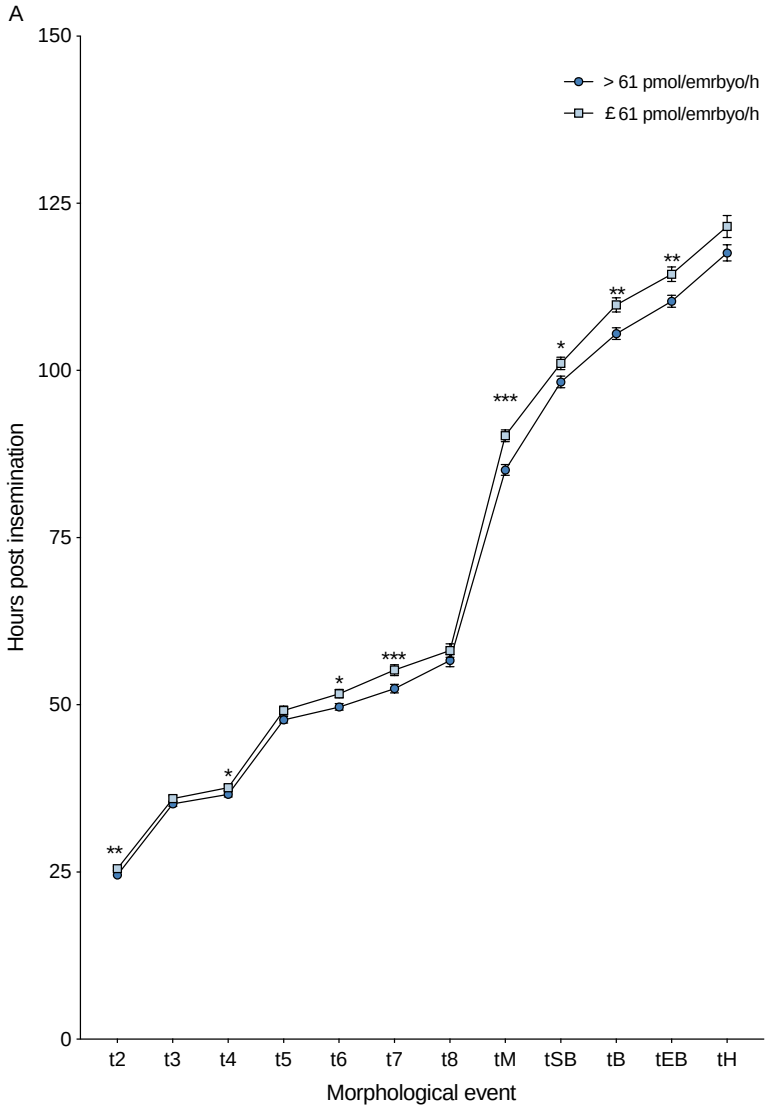
Consumption
Production
Turnover
> 29%
£ 29%

Figure 3.4 Total amino acid consumption, production and amino acid turnover in relation to embryo viability.

(A) Gardner grade (>BB, n = 86; ≤BB n = 123), (B) KIDScore (>6.9, n = 100; ≤6.9 n = 107) and (C) EmbryoScore (>29%, n = 91; ≤29% n = 93). Each data set (A-C) is expressed as mean ± SEM (pmol/embryo/h), n > 85 per group. Significance is measured utilizing Mann-Whitney U test and denoted by asterisk, **P* <0.05, ***P* <0.01.

Fast developing embryos have different day 5 metabolic profiles compared to slow developing embryos

Embryos that consumed more than the median amount of glucose (61 pmol/embryo/h) on day 5 of development developed faster across all morphokinetic parameters (with the exception of t3, t5, t8, and tH) compared to embryos that consumed lower levels of glucose on day 5 of development (Fig. 3.5A). A delay of ~1 h during the early cleavage stages was observed in embryos that consumed lower levels of glucose on day 5, and this delay was extended to 2-4 h by the blastocyst stage (Fig. 3.5A). Furthermore, embryos with an expansion rate (tEB-tSB) of 13 hours or less consumed significantly higher levels of glucose on day 5 of development compared to embryos that took longer than 13 hours to fully expand (85.0 ± 6.9 versus 61.6 ± 9.2 pmol/embryo/h, P <0.05) (Fig. 3.5B). Blastocysts with a diameter greater than the mean of 151 µm (n = 96) consumed significantly more glucose than smaller blastocysts (n = 104) (94.7 ± 8.1 versus 56.2 ± 6.6 pmol/embryo/h, P < 0.001), with a significant linear relationship between blastocyst size and glucose uptake (P <0.001). Consistent with glucose consumption, the amino acid profiles of faster developing embryos were significantly different. Specifically, embryos that consumed higher levels of arginine, asparagine, aspartate, isoleucine, lysine, taurine and/or valine developed faster to tM, tSB, tB and/or tEB (P <0.05) (Table 3.1). Additionally, alanine and glutamate were produced at lower levels by embryos that developed faster to tM, tSB, tB and/or tEB (P <0.05) (Table 3.2). No significant difference was observed between day 5 amino acid profiles and the time between tSB and tEB (i.e. rate of expansion).


A
> 61 pmol/emrbyo/h
£ 61 pmol/emrbyo/h
Hours post insemination
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125
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tSB
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tH
Morphological event

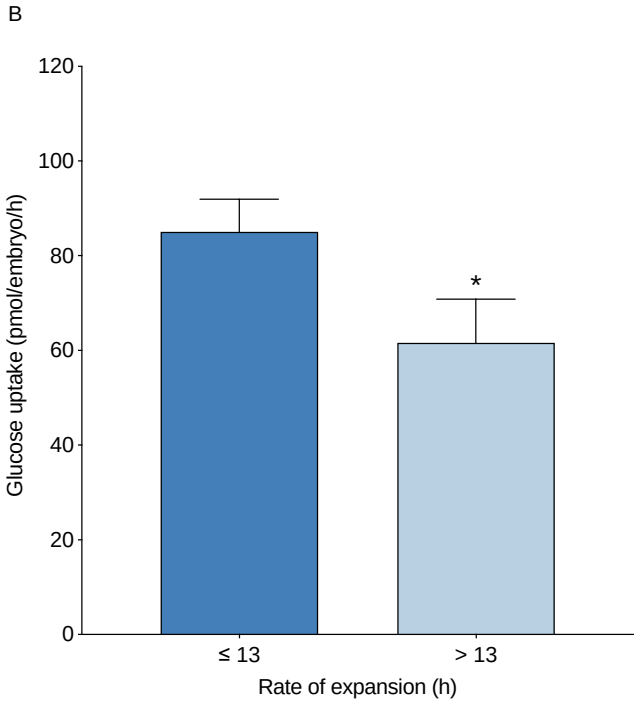
B
Glucose uptake (pmol/embryo/h)
120
100
80
60
40
20
0
*
≤ 13
> 13
Rate of expansion (h)

Figure 3.5 Blastocyst glucose uptake and the relationship to morphokinetic development of preimplantation embryos.

(A) Morphokinetic development of embryos that consumed high or low levels of glucose. Dark, circular data points represent the timing of each key morphokinetic event of embryos that consumed greater than the median amount of glucose (61 pmol/embryo/h). Light, square data points represent the timing of each key morphokinetic event of embryos that consumed less than the median amount of glucose. Morphokinetic parameters calculated as h post-insemination: 2-cell cleavage, t2 (n = 209); 3-cell cleavage, t3 (n = 209); 4-cell cleavage, t4 (n = 209); 5-cell cleavage t5 (n = 209); 6-cell cleavage, t6 (n = 205); 7-cell cleavage, t7 (n = 200); 8-cell cleavage, t8 (n = 200); morulae, tM (n = 204); start of blastulation, tSB (n = 209); blastocyst, tB (n = 209); expanded blastocyst, tEB (n = 201); and hatching blastocyst, tH (n = 137). (B) Glucose uptake of embryos that fully expanded (tEB-tSB) in ≤ 13 hours (n = 116) compared to embryos that fully expanded in >13 hours (n = 84). Data is expressed as mean ± SEM, significance is measured utilizing Mann-Whitney U test and asterisks denote significance, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Table 3.1 Morphokinetic development of embryos and their correlation to blastocyst consumption of individual amino acids.

		Timing of specific morphokinetic events (h)			
Consumption of amino acids (pmol/embryo/h)		tM	tSB	tB	tEB
Arginine	> median	86.1 ± 1.1	98.3 ± 0.7*	106.4 ± 0.9	111.1 ± 0.9
	≤ median	88.3 ± 1.1	101.0 ± 1.1	108.9 ± 1.1	113.7 ± 1.1
Asparagine	> median	86.6 ± 0.7	98.5 ± 0.7	106.3 ± 0.9*	111.0 ± 0.8*
	≤ median	88.7 ± 1.0	100.9 ± 1.0	109.0 ± 1.1	113.9 ± 1.1
Aspartate	> median	86.4 ± 0.7*	98.3 ± 0.7*	106.4 ± 0.8	111.0 ± 0.8
	≤ median	89.0 ± 1.0	101.1 ± 1.1	108.9 ± 1.1	113.8 ± 1.1
Isoleucine	> median	85.8 ± 1.1	98.5 ± 0.7	106.2 ± 0.8*	110.9 ± 0.8*
	≤ median	88.6 ± 1.0	100.9 ± 1.0	109.1 ± 1.1	114.0 ± 1.1
Lysine	> median	86.0 ± 1.1	98.5 ± 0.7	106.3 ± 0.8*	111.0 ± 0.8*
	≤ median	88.5 ± 1.0	100.8 ± 1.1	109.9 ± 1.2	113.9 ± 1.2
Taurine	> median	86.9 ± 0.7	98.5 ± 0.8	106.0 ± 0.8*	111.2 ± 0.9
	≤ median	88.4 ± 1.0	100.9 ± 1.0	109.3 ± 1.1	113.7 ± 1.1
Valine	> median	87.0 ± 0.8	98.0 ± 0.7	106.1 ± 0.8*	110.8 ± 0.9
	≤ median	88.3 ± 1.0	101.4 ± 1.0	109.2 ± 1.1	114.0 ± 1.1

Median consumption (pmol/embryo/h): arginine 5.10; asparagine 0.37; aspartate 1.40; isoleucine 6.10; lysine 0.15; taurine 0.20; valine 5.40. The timing of specific morphological events (time to morulae (tM), n = 204; start of blastulation (tSB), n = 209; blastocyst (tB), n = 209; and expanded blastocyst (tEB), n =201; expressed as mean ± SEM (hours post-insemination)). Grey boxes indicate significantly different levels of amino acid consumption respective to morphokinetic event. Significance is measured utilizing Student's t-test and asterisks denotes level of significance, *P <0.05.

Table 3.2 Morphokinetic development of embryos and their correlation to blastocyst production of individual amino acids.

		Timing of specific morphokinetic events (h)			
Production of amino acids (pmol/embryo/h)		tM	tSB	tB	tEB
Alanine	> median	88.3 ± 1.0	101.4 ± 1.0	109.2 ± 1.1	114.0 ± 1.1
	≤ median	87.0 ± 0.8	98.0 ± 0.7**	106.1 ± 0.8*	110.8 ± 0.9*
Glutamate	> median	89.1 ± 1.0	101.5 ± 1.1	109.8 ± 1.1	114.2 ± 1.1
	≤ median	86.2 ± 0.7*	97.9 ± 0.7***	105.5 ± 0.8**	110.7 ± 0.8*

Median production (pmol/embryo/h): alanine 8.95; glutamate 6.70. The timing of specific morphological events (time to morulae (tM), n = 204; start of blastulation (tSB), n = 209; blastocyst (tB), n = 209; and expanded blastocyst (tEB), n = 201; expressed as mean ± SEM (hours post-insemination)).Grey boxes indicate significantly different levels of amino acid consumption respective to morphokinetic event. Significance is measured utilizing Student's t-test and asterisks denotes level of significance, *P <0.05, **P <0.01 and ***P <0.001

Discussion

In this study blastocyst metabolism was shown to positively correlate with existing methods of embryo selection, Gardner grade, D5 KIDScore algorithm as well as the AI EmbryoScore. Of paramount significance, glucose consumption was found to be over 40% higher in blastocysts with a higher developmental and implantation potential. Indeed, when related to clinical pregnancy, glucose consumption was doubled in blastocysts that led to a successful pregnancy. Furthermore, blastocysts with a higher developmental potential and probability to succeed post-transfer also exhibited distinct amino acid profiles than blastocysts of lower viability, irrespective of which grading system was used.

Glucose is the predominant energy source utilized during blastocyst formation, supporting formation of the blastocoel, development of the first epithelium, and exponential cellular growth; processes which are energetically demanding (Biggers, et al., 1988, Watson, et al., 1992, Watson, et al., 1990). High glucose consumption ensures carbon flux through the pentose phosphate pathway required to produce biosynthetic precursors for DNA, RNA and lipid synthesis (Gardner, 1998, Morgan, et al., 1981, Reitzer, et al., 1980). Furthermore, the blastocyst has the capacity to convert glucose to lactate, even in the presence of sufficient oxygen, through aerobic glycolysis (Gardner, 1998). Lactate, long perceived as a by-product or regulator of metabolism (Lane, et al., 1996, Lane, et al., 2000), has more recently been implicated as a key signaling molecule and immunomodulator, thereby regulating initial events of implantation (Gardner, 2015). Additionally, lactate may directly affect embryonic epigenetic state and gene expression through lactylation of histone proteins (Zhang, et al., 2019), an example of a

metaboloepigenetic interaction, further highlighting the significance of blastocyst glucose metabolism in assessing embryonic physiology and health. Consistent with previous reports that embryos consuming higher levels of glucose are associated with a higher developmental potential (Gardner, et al., 2001, Gardner, et al., 1987, Gardner, et al., 2011, Lee, et al., 2015, Renard, et al., 1980), we show that blastocysts with higher viability consume more glucose during day 5 of development than less viable blastocysts. Furthermore, embryos that experienced a delay of 2-4 h in development to the blastocyst stage consumed lower levels of glucose on day 5 of the preimplantation period. This is consistent with data on the mouse embryo which determined that slow cleaving embryos give rise to blastocysts with reduced glucose uptake (Lee, et al., 2015). Together, these results support the hypothesis that slower developing embryos, or embryos of lower viability, have a diminished ability to maintain the energy and biosynthetic requirements to support viable blastocyst formation, and subsequently viability post-transfer (Edwards, et al., 1984, Lee, et al., 2015, Meseguer, et al., 2011, Wong, et al., 2010).

Blastocyst size has been linked to metabolic activity and viability due to the high energy demands of blastocyst expansion (Gardner, et al., 2016, Niederberger, et al., 2019). Blastocyst diameter, as an accurate measure of blastocyst size, is positively correlated to clinical pregnancy rates where larger embryos are more likely to establish a clinical pregnancy (Shapiro, et al., 2008). Similarly, this study found a positive relationship between blastocyst diameter and glucose consumption. Consistently, blastocysts with a faster expansion rate (tEB-tSB) consumed more glucose than slower expanding blastocysts, plausibly due to these embryos having a higher capacity to maintain energy requirements, subsequently supporting high developmental potential. However, it has

been established that even when blastocysts do have the same diameter (Gardner, et al., 2001, Lane, et al., 1996), there is still a large metabolic variation between embryos, inferring that although the size of a blastocyst is related to metabolism, it is not the sole factor dictating the overall metabolic activity of the embryo.

Of further interest, Desai and colleagues found embryos that have a faster expansion rate were associated with a lower rate of aneuploidy (Desai, et al., 2018). Changes to karyotypes associated with aneuploidy leads to alterations in gene expression, the proteome and metabolism a phenomenon termed aneuploid stress (Zhu, et al., 2018), suggesting embryonic metabolism may be directly related to the genetic composition of embryos in addition to embryo viability and health. Ongoing investigations are currently underway to determine whether metabolic biomarkers can provide a non-invasive measure of embryonic genetic composition.

Retrospective analysis of clinical pregnancy rates confirm that glucose consumption is significantly higher in blastocysts that successfully implant and result in a clinical pregnancy compared to blastocysts that fail to development post-transfer. These data are consistent with Gardner and colleagues who determined that glucose consumption of human blastocysts cultured at 5% oxygen was higher in blastocysts with positive transfer outcomes (Gardner, et al., 2011). It was further noted in this study that while data was not significantly different, plausibly due to low sample numbers, blastocysts with a top Gardner grade (AA) that failed to lead to a successful pregnancy had a lower glucose uptake than lower graded blastocysts which were successful post-transfer. This supports the findings of Gardner and colleagues who concluded glucose metabolism was

a stronger predictor of pregnancy success than morphological grade (Gardner, et al., 2011).

Higher developmental potential and probability of forming a viable pregnancy was also related to amino acid profiles which may provide an additional means of measuring embryo physiology and health. Specifically, alanine, arginine, aspartate, isoleucine, leucine, lysine, glutamate, glutamine, and threonine were utilized differently by blastocysts with high viability, regardless of the embryo selection method used. Of significance, arginine and leucine are both implicated in regulating the mammalian target of rapamycin (mTOR) pathway (González, et al., 2012). mTOR is responsible for maintaining cellular homeostasis by responding to nutrient, energy and/or oxygen availability and regulates translation machinery and metabolic processes accordingly. A significant increase in arginine and leucine consumption by blastocysts with higher viability suggests less viable blastocysts are unable to maintain cellular homeostasis as effectively thereby impacting embryo developmental potential. Additionally, arginine and leucine, together and individually, also promote TE motility, which assists TE invasion and is a requirement for penetration of the endometrium during implantation (Gangloff, et al., 2004, González, et al., 2012, Guertin, et al., 2006, Martin, et al., 2003). Of further significance, blastocysts that consumed high levels of glucose also consumed significantly more aspartate than less viable embryos. Aspartate is the rate-limiting factor in the malate-aspartate shuttle (MAS) that facilitates the regeneration of cytosolic nicotinamide adenine dinucleotide (NAD^+) required for glucose metabolism (Gardner, 1998, Lane, et al., 2005, Mitchell, et al., 2009). More recently NAD^+ has been recognized as a metaboloepigenetic signaling molecule via activation of sirtuins, a class of NAD^+-

dependent histone deacetylases (Haigis, et al., 2010, Tatone, et al., 2018). Hence, the relative availability of aspartate and subsequently NAD^+ may also influence embryonic gene regulation and subsequent health (Donohoe, et al., 2012, Ferrick, et al., 2019, Gardner, et al., 2015, Harvey, et al., 2016, Houtkooper, et al., 2010, Lees, et al., 2020). Given the wide array of metabolic and regulatory pathways amino acids are involved in, it is evident that between blastocysts of high and low viability, individual amino acid profiles represent distinct physiologies and provide a means of assessing important regulators of cellular homeostasis, cell-signaling, implantation and embryonic health. Of note, the amino acid analysis performed in this study assessed amino acid profiles over a 24 h period during blastocyst formation and is therefore limited in its ability to identify differences in flux and interactions between amino acid pathways. Targeted amino acid assays, designed to quantitate the utilization of an individual amino acids with high accuracy will plausibly provide further insight into amino utilization, and further studies are required for the development and validation of such assays.

The results of this study demonstrate that a blastocyst with a high probability of developing into viable and healthy pregnancy is one that consumes high levels of glucose and specific amino acids. Furthermore, this study shows no significant difference in total amino acid turnover, despite the distinct patterns of amino acid consumption and production between blastocysts with different levels of viability. Consequently, these data do not align with the Quiet Hypothesis, which states that the most viable preimplantation embryo has the lowest overall metabolism and the lowest amino acid turnover (Leese, 2002). Of note, studies that lay the foundation for this hypothesis utilized 20% oxygen for culture or metabolic analysis (Bowman, et al., 1970, Houghton,

et al., 2002, Leese, 1991, Turner, et al., 1994). Given that 20% oxygen directly perturbs both carbohydrate and amino acid metabolism leading to an altered physiology of the preimplantation embryo, it is plausible that the differences observed in this study and those which formed the basis of the Quiet Hypothesis are due to the concentration of oxygen utilized (Gardner, 2016, Gardner, et al., 2017, Guerin, et al., 2001, Kelley, et al., 2016, Kelley, et al., 2017, Kelley, et al., 2019, Umaoka, et al., 1992, Wale, et al., 2012). It was proposed by Gardner and Wale (2013) that viable embryos metabolize an optimal range of metabolites, i.e. one that is not suppressed nor unduly elevated (Gardner, et al., 2013). This concept has gained traction and has been coined the 'Goldilocks Principle' (Leese, et al., 2016). Our data have shown that within this optimal range the most viable blastocyst is a blastocyst that is characterized by an 'active' metabolism and utilizes metabolites in a distinct manner. This metabolic profile represents the embryos ability to maintain sufficient energy to support both cellular homeostasis and biosynthesis required to support embryo development, viability and health of the resulting offspring.

Due to the retrospective nature of this study and low numbers of transferred embryos it was not feasible to establish the cumulative predictive power of metabolism combined with either morphological grades, KIDScore or EmbryoScore. However, as it was shown by Gardner and colleagues that metabolism is a stronger predictor of transfer success than morphological grades (Gardner, et al., 2011), it is tempting to speculate that the assessment of blastocyst metabolism (and indirectly the metaboloepigenome) will be complimentary to any system used to assess developmental potential based on morphological parameters such as the KIDScore and EmbryoScore. For example, of the 64 blastocysts with both a high KIDScore and an EmbryoScore, inferring high viability,

over a third had reduced glucose uptake indicating an impaired physiology which may therefore prevent such top scoring embryos from resulting in a successful pregnancy. We therefore propose that a combination of metabolic activity together with morphological analysis will culminate in a single algorithm that provides greater power in predicting success post-transfer as well as subsequent fetal health.

This study is the first of its kind to incorporate analysis of both glucose and amino acid metabolism of individual human preimplantation embryos under physiological (5%) oxygen concentrations. Of significance, while UMF is highly sensitive technology and LC-MS is a great discovery tool as it allows analysis all amino acids in a single sample, neither are translatable to a clinical setting. Bench top assay technologies however have previously been used to measure mouse embryo carbohydrates metabolism (Guerif, et al., 2013, Kelley, et al., 2019) and amino acids (Lee and Gardner, unpublished) and hence provide a means of assessing metabolic biomarkers clinically within an hour, making assessment of metabolic activity prior to a fresh transfer feasible. Consequently, we are now evaluating the predictive power of metabolic biomarkers to create an “embryo health” selection algorithm incorporating morphology, morphokinetics (optimized through AI) and metabolism in order to reduce the time to pregnancy and facilitate the birth of healthy children.

Chapter 4. Morphology, morphokinetics, AI and metabolism are reflective of aneuploid stress in human preimplantation embryos

Abstract

Study question: Are morphological grades, morphokinetic parameters, artificial intelligence (AI) and the metabolic profile of blastocysts related to the chromosomal status of embryos?

Summary answer: Aneuploid embryos exhibit aberrations in embryo developmental kinetics and subsequently lower KIDScores and AI EmbryoScores compared to euploid embryos. Further metabolic profiles of embryos with single chromosome aneuploidy are significantly different to embryos with multiple chromosome aneuploidy which reflects distinct physiologies.

What is already known: Morphokinetic based algorithms such as D5 KIDScore algorithm and AI EmbryoScores, can predict implantation potential and development post-transfer, respectively. Further, glucose and amino acid metabolism are related to embryo development and implantation potential.

Study design, size, duration: A retrospective cohort pilot study including 64 blastocysts from 25 patients undergoing an ICSI cycle with PGT-A between August 2019 and February 2020.

Participants/materials, setting, methods: Blastocysts were biopsied for genetic testing and corresponding spent blastocyst culture media were analysed for metabolite concentrations. Time-lapse images were captured and analysed for embryo morphology, blastocyst diameter, developmental timings, KIDScore and AI EmbryoScore. Genetic testing results were analysed and compared to morphology, morphokinetics, KIDScores, AI EmbryoScores and metabolic profile of blastocysts.

Main results and the role of chance: On average, aneuploid blastocysts had a lower KIDScore (P <0.05) and AI EmbryoScore (P <0.01) compared to euploid blastocysts,

however there was no significant difference in morphological grading between aneuploid and euploid blastocysts. Aneuploid blastocysts expanded significantly slower (tEB-tSB; 16.4 ± 1.0 versus 13.6 ± 0.5 h, *P* <0.01) and at 115 h post-insemination were significantly smaller in diameter (148.4 ± 4.1 versus 164.5 ± 3.9 µm, *P* <0.05) compared to euploid blastocysts. Additionally, aneuploid embryos experienced a significant delay in the time to hatching (8 h; *P* <0.05). Although euploid and aneuploid embryos metabolic profiles were not significantly different, aneuploid embryos with multiple chromosome aneuploidies consumed significantly higher levels of asparagine, lysine phenylalanine, serine, tryptophan and tyrosine than embryos with a single chromosome deletion or duplication (*P* <0.05).

Limitations: This is a pilot study of 64 blastocysts from 25 patients.

Wider implications of findings: Aberrations in embryo development and physiology were observed in aneuploid embryos. While morphokinetic or metabolic parameters and algorithms cannot replace PGT-A in selecting euploid embryos for transfer, they do provide a measure of aneuploid associated stress and thereby information regarding which embryos are more likely to be aneuploid and may benefit from PGT-A.

Key words: Artificial intelligence, glucose, amino acids, blastocyst, PGT-A

Introduction

Current embryo selection methods following IVF include morphological (Gardner, et al., 2000) and morphokinetic (Basile, et al., 2015, Liu, et al., 2016, Milewski, et al., 2015, Petersen, et al., 2016, Reignier, et al., 2019, Rubio, et al., 2014) based algorithms assessing the development of preimplantation embryos to assist in reducing time to pregnancy, and preimplantation genetic testing for aneuploidy (PGT-A) to facilitate the transfer of a single euploid embryo. Aneuploidy in embryos is the leading cause of pregnancy loss and a major contributor to birth defects (Hassold, et al., 2001). Selecting embryos without aneuploidy subsequently reduces both the time to pregnancy and miscarriage rates (Grifo, et al., 2013, Scott Jr, et al., 2013, Simon, et al., 2018, Yang, et al., 2012).

Aneuploidy rates in embryos are known to increase with maternal age (Munné, et al., 1995), consequently when PGT-A has been used in younger patients, mixed results in transfer outcomes have been observed (Forman, et al., 2013, Kang, et al., 2016, Ozgur, et al., 2019, Scott Jr, et al., 2013, Yang, et al., 2012). The Single embryo TrAnsfeR (STAR) trial demonstrated PGT-A did not improve ongoing pregnancy rates for all patients, but rather for a subset of patients aged 35-40 years (Munné, et al., 2019). Likewise, Murphy and colleagues observed a significant improvement in live birth rates in older patients, while younger patients (≤37) on their first IVF cycle showed a small improvement in live birth rates and a reduction in cumulative pregnancy rates (Murphy, et al., 2019), possibly partly due to the manipulation of embryos during biopsy.

Trophectoderm biopsy involves manipulation of the zona pellucida and removal of a small number of trophectoderm (TE) cells for testing (Capalbo, et al., 2014, McArthur, et al., 2005, Schoolcraft, et al., 2010). Although, Scott and colleagues reported that trophectoderm biopsy has no impact on the viability of the embryo (Scott Jr, et al., 2013), other studies have demonstrated that different methods of biopsy (Rubino, et al., 2020) and the numbers of cells biopsied (Guzman, et al., 2019) can impact pregnancy outcomes. Such results demonstrate that blastocyst biopsy can have an impact on embryo viability in some circumstances and should not be considered completely benign. Hence, despite the widespread use of PGT-A to provide a measure of embryo ploidy, there remains discussion of whether PGT-A should be used for all patients, or whether PGT-A should be targeted to women with embryos at high risk of aneuploidy (Maxwell, et al., 2018, Mochizuki, et al., 2020, Paulson, 2020). Indeed, many clinics refer patients for PGT-A if they are of advanced maternal age, have experienced recurrent implantation failure or recurrent miscarriages. As patients with unexplained implantation failure are not diagnosed until they have had multiple embryo transfers, identifying additional characteristics associated with aneuploidy in embryos would assist in identifying which embryos and/or patients would benefit most from PGT-A early on.

Chromosomal imbalance associated with aneuploidy leads to perturbations in gene expression profiles, which in turn will alter the proteome, culminating in altered patterns of metabolism, a phenomenon in somatic cells referred to as aneuploid stress (Zhu, et al., 2018). Typically, aberrations in embryo metabolism, a regulator of embryo development, are manifested as altered morphological and morphokinetic development (Gardner, 1998, Lane, et al., 1998, Mitchell, et al., 2009, Wale, et al., 2012). In support of

aneuploid associated stress in embryos, both morphological parameters (Alfarawati, et al., 2011, Capalbo, et al., 2014, Tilia, et al., 2016, Wang, et al., 2018) and morphokinetic parameters (Campbell, et al., 2013, Campbell, et al., 2014, Chavez, et al., 2012, Chawla, et al., 2015, Desai, et al., 2018, Huang, et al., 2019, Kaing, et al., 2018, Nogales, et al., 2017, Patel, et al., 2016, Vega, et al., 2014) have been reported to be related to the ploidy status of embryos. Additionally, the amino acid utilization of euploid and aneuploid cleavage stage embryos appear to differ (Picton, et al., 2010) and utilization of Raman spectroscopy identified that chromosomal abnormalities impact the metabolic footprint of preimplantation embryos (Liang, et al., 2019).

Subsequently, morphological and morphokinetic based algorithms widely used to select blastocysts for transfer, including artificial intelligence (AI) selection methods (Tran, et al., 2019), and the metabolic profile of embryos which has been related to viability of embryos post-transfer (Gardner, et al., 2001, Gardner, et al., 2011, Houghton, et al., 2002, Kelley, et al., 2019, Lee, et al., 2015), may also provide non-invasive tools to quantitate aneuploid stress in embryos. A more holistic analysis of embryo development may therefore assist in identifying embryos at high risk of aneuploidy and subsequently which patients would benefit most from PGT-A regardless of age and cycle number. Consequently, this pilot study aimed to establish whether blastocyst morphology, morphokinetic parameters and viability scores (KIDScore and AI EmbryoScore) in parallel with blastocyst metabolism, can provide a means of assessing the level of aneuploid stress.

Materials and methods

Patient selection and stimulation protocol

Sixty-four blastocysts were included in this study from 25 patients who were undergoing an ICSI cycle between August 2019 and February 2020 (Fig. 4.1). These patients were 35-43 years, an average age of 39 years, at the time of treatment. Ovarian stimulation was performed utilising a standard gonadotropin-releasing hormone (GnRH) antagonist protocol incorporating recombinant follicle stimulating hormone (FSH), GnRH antagonist and human chorionic gonadotrophin (hCG) injections (Gonal F, Cetrotidel, Ovidrel; Merck, Australia). Collection of oocytes was performed 36 h post hCG injection.

Embryo culture

Oocytes were denuded of surrounding cumulus cells 2 h post oocyte collection and fertilization conducted 2 h later. Post-insemination, embryos were individually cultured at 5% O_2, 6% CO_2, 89% N_2, 37°C within an EmbryoScope® time-lapse incubator (Vitrolife AB, Sweden). Each well in an EmbryoSlide® culture dish contained 25 µl G-TL medium overlaid with 1.4 ml Ovoil (Vitrolife). On day 4 of culture (~90 h post-insemination) embryos were washed in culture medium and transferred to a new pre-equilibrated EmbryoSlide® culture dish containing 15 µl G-TL medium in each well overlaid with 1.4 ml Ovoil. Embryos were cultured for a further 24, 48 or 72 h until embryos reached blastocyst stage and biopsy could be performed (day 5-7). Spent media from these culture dishes were collected and stored at -20°C until collection for metabolic analysis.

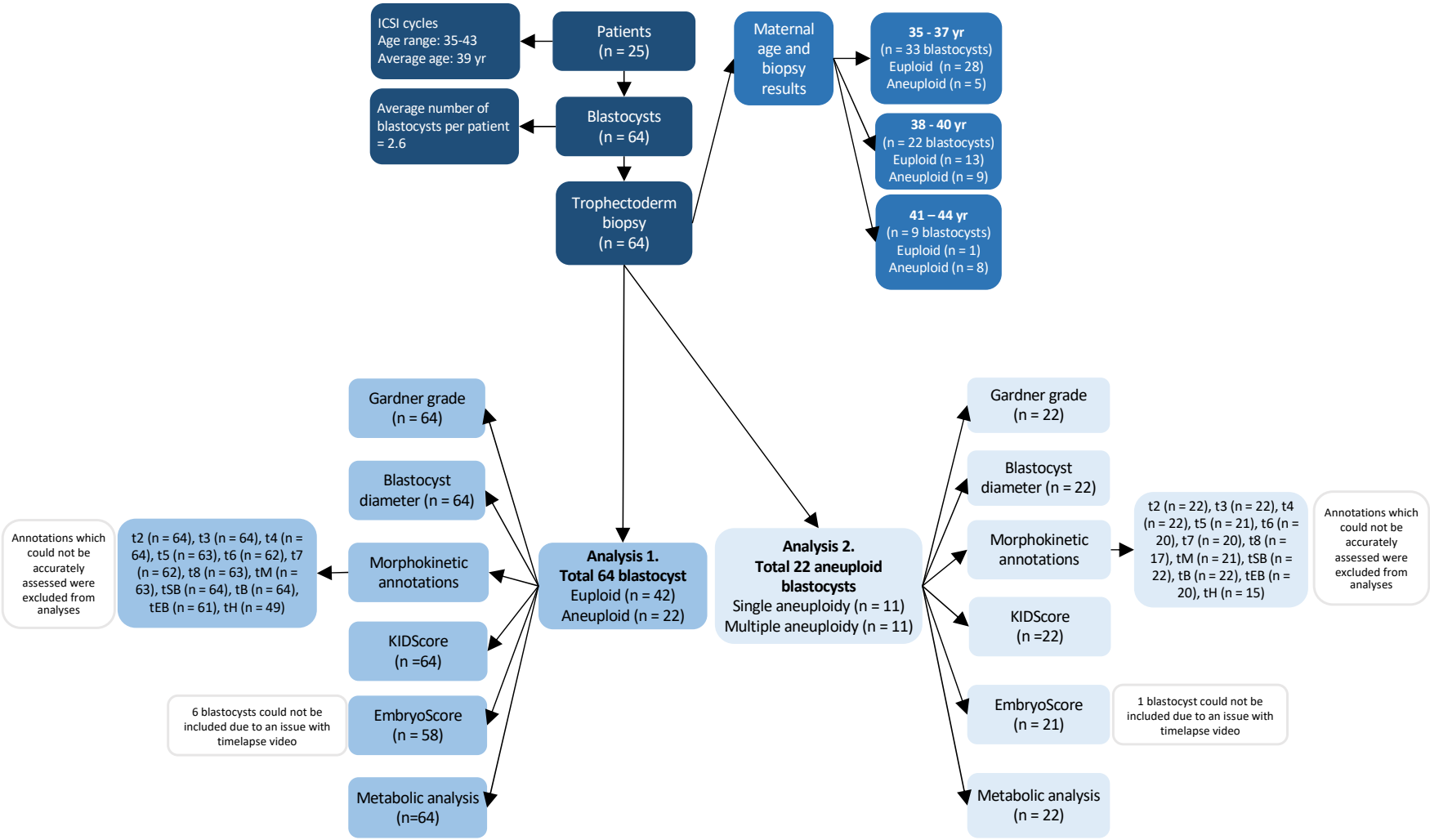
ICSI cycles
Age range: 35-43
Average age: 39 yr
Patients
(n = 25)
Average number of blastocysts per patient = 2.6
Blastocysts
(n = 64)
Trophectoderm biopsy
(n = 64)
Maternal age and biopsy results
35 - 37 yr
(n = 33 blastocysts)
Euploid (n = 28)
Aneuploid (n = 5)
38 - 40 yr
(n = 22 blastocysts)
Euploid (n = 13)
Aneuploid (n = 9)
41 – 44 yr
(n = 9 blastocysts)
Euploid (n = 1)
Aneuploid (n = 8)
Gardner grade
(n = 64)
Blastocyst diameter (n = 64)
Morphokinetic annotations
KIDScore
(n =64)
EmbryoScore
(n = 58)
Metabolic analysis
(n=64)
Annotations which could not be accurately assessed were excluded from analyses
t2 (n = 64), t3 (n = 64), t4 (n = 64), t5 (n = 63), t6 (n = 62), t7 (n = 62), t8 (n = 63), tM (n = 63), tSB (n = 64), tB (n = 64), tEB (n = 61), tH (n = 49)
6 blastocysts could not be included due to an issue with timelapse video
Analysis 1.
Total 64 blastocyst
Euploid (n = 42)
Aneuploid (n = 22)
Analysis 2.
Total 22 aneuploid blastocysts
Single aneuploidy (n = 11)
Multiple aneuploidy (n = 11)
Gardner grade
(n = 22)
Blastocyst diameter (n = 22)
Morphokinetic annotations
KIDScore
(n =22)
EmbryoScore
(n = 21)
Metabolic analysis
(n = 22)
t2 (n = 22), t3 (n = 22), t4 (n = 22), t5 (n = 21), t6 (n = 20), t7 (n = 20), t8 (n = 17), tM (n = 21), tSB (n = 22), tB (n = 22), tEB (n = 20), tH (n = 15)
Annotations which could not be accurately assessed were excluded from analyses
1 blastocyst could not be included due to an issue with timelapse video

Figure 4.1 Study flow chart, detailing analyses and sample sizes.

Blastocysts were considered euploid (no abnormality detected) or aneuploidy according to biopsy results. Morphokinetic parameters t2, t3, t4, t5, t6, t7, t8, tM, tSB, tB, tEB, and tH represent 2-8 cell cleavage, morulae, start of blastulation, blastocyst, expanded blastocyst tEB and hatching blastocyst respectively.

Preimplantation genetic testing

Trophectoderm biopsy was performed on suitable blastocysts on day 5-7 of culture and embryos were vitrified post-biopsy. The trophectoderm biopsy was amplified using SurePLEX (Illumina, USA). Library of all successfully amplified samples was constructed using VeriSeq PGS Library Prep Kit following manufacturer's instructions. Sequencing was performed by MiSeq Reagent Kit v3-PGS on Miseq system (Illumina). Analysis and copy number variant calling were performed using BlueFuse Multi software (Illumina). Only embryos considered euploid, due to no abnormality detected (NAD), or embryos with uniform chromosome abnormalities were included in this study. No mosaic embryos were detected. Aneuploid rates were calculated as the number of aneuploid embryo divided by the total number of embryos biopsied and the number of chromosomal deletions and/or duplications was also recorded for all aneuploid embryos.

Retrospective analysis of embryo viability

All embryos were retrospectively analysed for blastocyst diameter, morphokinetic parameters, and assigned a morphological grade, KIDScore and AI EmbryoScore. The Gardner grading system was used to assess embryo morphology at 115 h as previous described (Gardner, et al., 2000). Blastocyst diameter was measured, at 115 h, from the outside of the trophectoderm (TE) using EmbryoViewer® software diameter tool (version 7.5.201.20844, Vitrolife) (Almagor, et al., 2016). The timings of the morphokinetic parameters including early cleavage divisions (2-cell (t2), 3-cell (t3), 4-cell (t4), 5-cell (t5), 6-cell (t6), 7-cell (t7), 8-cell (t8)), morulae (tM), start of blastulation (tSB), blastocyst (tB), expanded blastocyst (tEB) and hatching blastocyst (tH) were recorded for embryos which could be accurately assessed followed by the assessment of rate of expansion (tEB-tSB)

as previously described (Desai, et al., 2018). Additionally, implantation potential score (KIDScore), was calculated with EmbryoViewer® software utilizing D5 KIDScore algorithm (version 2) incorporating TE quality and annotations for t2, t3, t4, t5 and tB (Petersen, et al., 2016, Reignier, et al., 2019). Finally, each embryo was assigned an EmbryoScore (%) generated by an AI system (IVY, version 1.0.0, Harrison AI, Australia). This score represents the relative ranking that an embryo will develop a fetal heartbeat (Tran, et al., 2019). Of the 64 blastocysts, 6 could not be included in EmbryoScore analysis due to an interference in the time-lapse video, which can impact overall EmbryoScore.

Analysis of glucose metabolism

Glucose metabolism of all individual embryos was measured using ultramicrofluorescence as previously described (Gardner, et al., 2001, Leese, et al., 1984). Metabolite concentrations of embryo spent media samples (test) were subtracted from metabolite concentration of no-embryo spent media samples (control) to determine overall metabolite consumption/production.

Analysis of amino acid metabolism

Amine analysis was conducted on 1 µl of spent media from each embryo using a liquid chromatography-mass spectrometry (LC-MS) analysis utilizing an Agilent 6490 Triple Quadrupole LC-MS with iFunnel Technology coupled with an Agilent 1290 liquid chromatography system (Agilent, USA). Both embryo and control spent media were analysed and Agilent MassHunter Quantitative Analysis software (B.08.0, Agilent) was used to calculate relative abundances as previously described (Lee, et al., 2015, Wale, et al., 2012).

Statistical analysis

All analyses were conducted on two groups: (1) euploid versus aneuploid embryos, and (2) aneuploid embryos with single chromosome deletion or duplication versus aneuploid embryos with multiple chromosome deletions and/or duplications. Normality was assessed using Shapiro-Wilk test and analyses were performed using the parametric Student's t-test or nonparametric Mann-Whitney U test using Prism 8 (version 8.3.0, GraphPad, LLC), with the exception of the relationship between ploidy status and morphological grade which was analysed using Fisher's exact test, and the correlation between morphokinetics, viability scores and metabolism with maternal age was calculated using Person's correlation coefficient. Data was expressed as mean ± SEM, and *P* value threshold was set to 0.05.

Ethical approval

Consent from patients to join this study was obtained during clinical consultation and ethical approval was provided by Melbourne IVF Human Research Ethics Committee (53/117-MIVF).

Results

Morphology, KIDScores and EmbryoScores of aneuploid and euploid blastocysts

Of the 40 blastocysts that were graded a Gardner grade of greater than BB, 27.5% were aneuploid. In contrast, of the 24 blastocyst that were graded BB or less, 45.8% were aneuploid, however this difference was not significant. Additionally, euploid blastocysts had a significantly higher average KIDScore (7.2 versus 6.4, $P < 0.05$) and EmbryoScore compared to aneuploid blastocysts (34.8 versus 16.2%, $P < 0.001$) (Fig. 4.2).

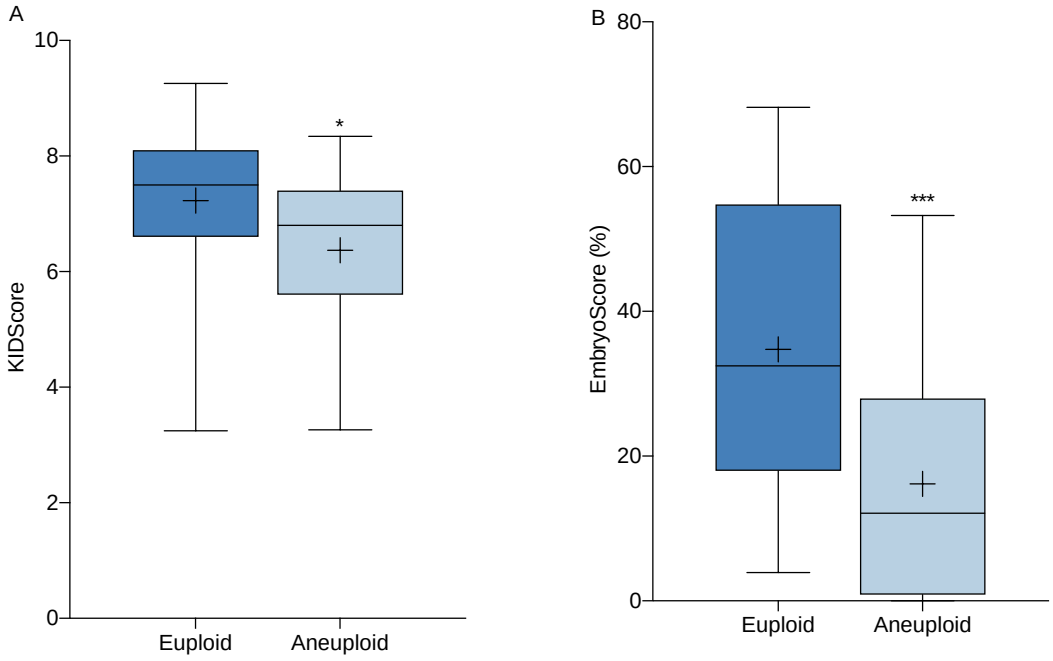

Figure 4.2 Viability scores of euploid and aneuploid blastocysts.

(A) KIDScore of euploid (n = 42) and aneuploid (n = 22) embryos, (B) EmbryoScore of euploid (n = 37) and aneuploid embryos (n = 21). Boxes represent 25th to 75th percentile, '+' represents mean, whiskers represent 5th to 95th percentile, and median represented by horizontal line. Asterisks denote the level of significance, *P <0.05, ***P <0.001.

Euploid blastocysts developed faster and are larger than aneuploid blastocysts

Analysis of morphokinetic parameters demonstrated that the time between tSB and tEB, i.e. rate of blastocyst expansion, was significantly different due to embryo ploidy status. Aneuploid embryos had a significant delay in expansion rate of more than 2 h when compared to euploid blastocysts (16.4 ± 1.0 versus 13.6 ± 0.5 h, P <0.01) (Fig. 3A). This was reflected in blastocyst size, whereby aneuploid blastocysts had a significantly smaller diameter than euploid blastocysts (148.4 ± 4.1 versus 164.5 ± 3.9 µm, P <0.05) (Fig. 3B). Additionally, aneuploid embryos experienced a significant delay of greater than 8 h to the time of hatching (P <0.001) (Table 4.1).

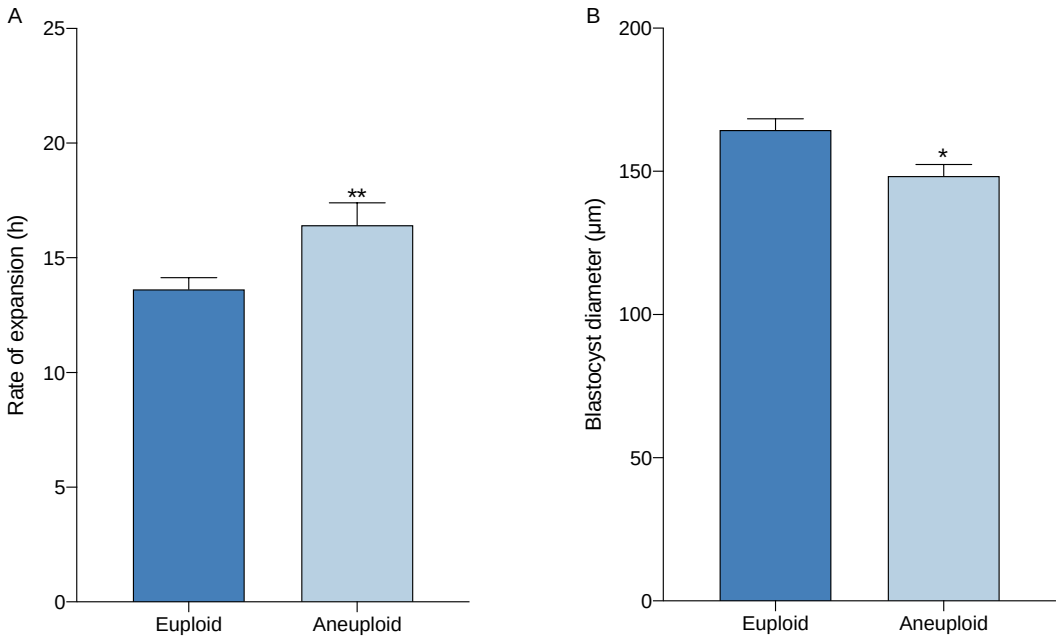

Figure 4.3 Blastocyst size and rate of expansion compared to preimplantation genetic test for aneuploid results.

A) Blastocyst diameter (µm) of euploid (n = 42) and aneuploid embryos (n = 22). (B) Rate of expansion (tEB-tSB) of euploid (n = 37) and aneuploid (n = 21) blastocysts. All data are expressed as mean ± SEM. Significance denoted by asterisks, *P <0.05, **P <0.01.

Table 4.1 Morphokinetic development of euploid and aneuploid embryos.

Morphokinetic parameters	Euploid embryos	Aneuploid embryos
t2	24.7 ± 0.5	24.8 ± 0.6
t3	35.2 ± 0.6	35.7 ± 0.9
t4	37.5 ± 0.8	36.9 ± 0.8
t5	48.0 ± 1.0	49.7 ± 1.7
t6	50.0 ± 1.0	51.2 ± 1.6
t7	52.6 ± 1.1	54.5 ± 1.6
t8	55.1 ± 1.3	57.8 ± 1.8
tM	85.4 ± 1.2	84.8 ± 2.0
tSB	95.9 ± 1.0	97.1 ± 1.5
tB	104.4 ± 1.1	106.4 ± 1.8
tEB	109.5 ± 1.1	113.1 ± 1.8
tH	117.3 ± 1.8	125.6 ± 2.1**

Morphokinetic parameters (2-cell cleavage, t2 (n = 64);3-cell cleavage, t3 (n = 64); 4-cell cleavage, t4 (n = 64); 5-cell cleavage, t5 (n = 63); 6-cell cleavage, t6 (n = 62); 7-cell cleavage, t7 (n = 62); 8-cell cleavage, t8 (n = 56); morulae, tM (n = 63); start of blastulation, tSB (n = 63); blastocyst tB (n = 64); expanded blastocyst tEB (n = 61); and hatching blastocyst, tH (n = 49)) calculated as h post-insemination (h) and data expressed as mean ± SEM. Asterisks denotes the significance between euploid embryos and aneuploid embryos, $^{**}P < 0.01$.

Metabolic profile of aneuploid and euploid embryos

Although not statistically significant, the metabolic profile of euploid and aneuploid embryos appeared to have altered trends. Euploid blastocysts consumed 32% more glucose than aneuploid blastocysts (50.7 ± 6.2 versus 38.3 ± 5.2 pmol/embryo/h) (Fig. 4.4A) and appeared to consume more amino acids (Fig. 4.4B).

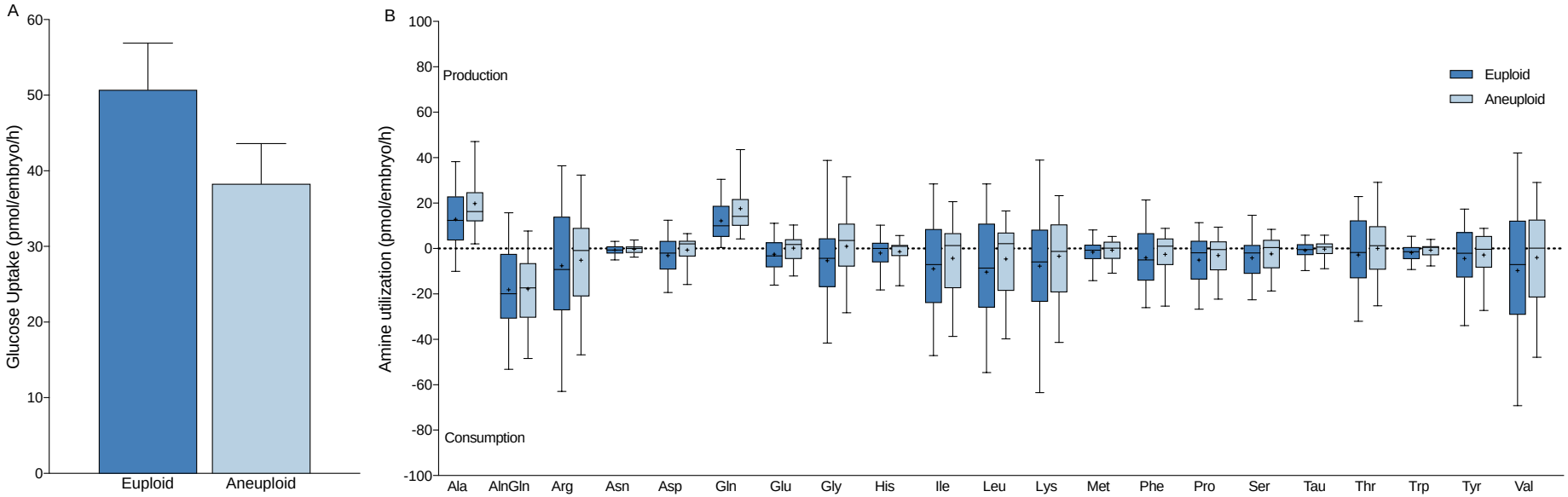

Figure 4.4 Metabolic profiles of euploid and aneuploid blastocysts.

(A) Glucose consumption (pmol/embryo/h) of euploid (n = 42) and aneuploid blastocysts (n = 22). Data are expressed as mean ± SEM. (B) Individual amino acid profiles of euploid (n = 42) and aneuploid (n = 22) blastocysts. Consumption of amino acids represented by negative values and production of amino acids represented by positive values. Boxes represent 25th to 75th percentile, '+' represents mean, whiskers represent 5th to 95th percentile, and median represented by horizontal line. No statistical significance was observed.

Biomarkers of embryo viability and maternal age

Aneuploidy rates were found to increase with maternal age (37-37 yr 15.2%, 38-40 yr 40.9%, and 41-43 yr, 88.9%). To determine if patient age was a confounding variable, analysis of maternal age and rate of blastocyst expansion, KIDScore, EmbryoScore and glucose and amino acid metabolism was undertaken. A positive correlation between maternal age and rate of expansion was observed whereby embryos from older patients were associated with longer blastocyst expansion rates (P <0.05) (Fig. 4.5A). Additionally, maternal age was inversely related to EmbryoScores (P <0.05) (Fig. 4.5B). No correlation between KIDScore or glucose uptake was found with maternal age (Fig. 4.5C-D). Furthermore, alanine, alanyl-glutamine, arginine, aspartate, glutamine, glycine histidine, isoleucine, leucine, phenylalanine, proline, tryptophan and valine utilization were correlated with maternal age (P <0.05), while asparagine, glutamate, lysine, methionine, serine, taurine, threonine and tyrosine were not.

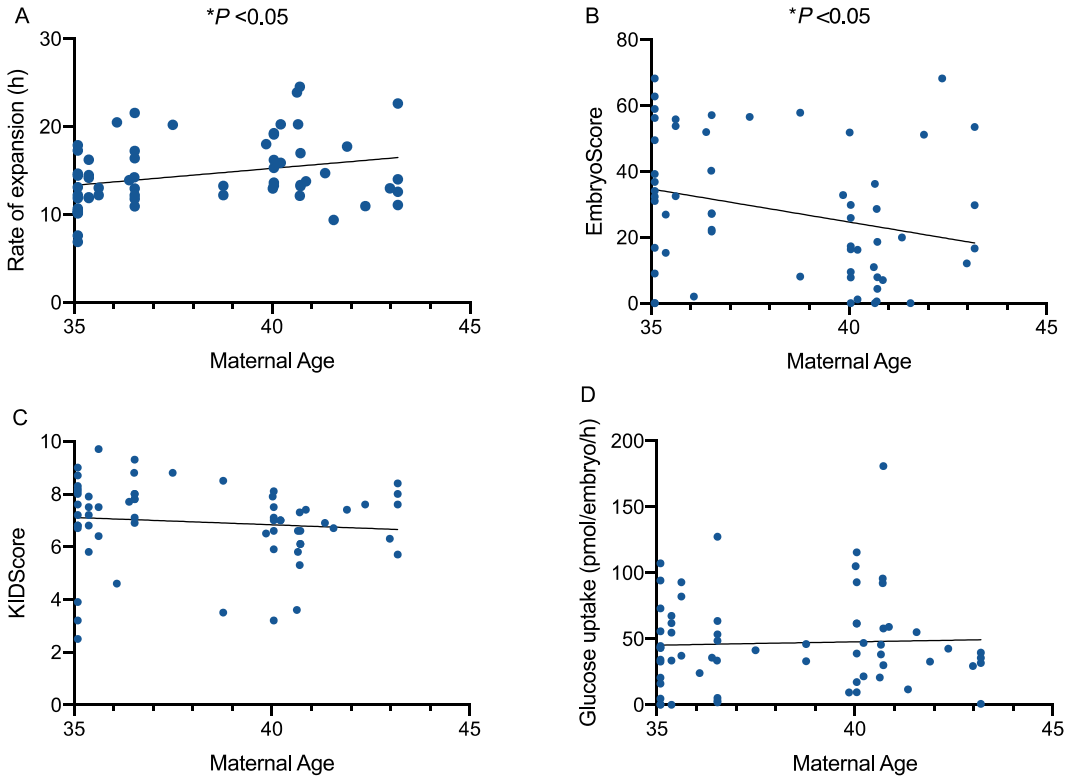

Figure 4.5 Linear correlation between blastocyst morphokinetics, viability scores, metabolism and maternal age.

(A) Blastocyst rate of expansion (h) and maternal age (n = 64); (B) Blastocyst EmbryoScores and maternal age (n = 58); (C) Blastocyst KIDScores and maternal age (n = 64); (D) Blastocyst glucose uptake (pmol/embryo/h) and maternal age (n = 64). Significance denoted by asterisks, *P <0.05.

The development, viability scores and metabolic profile of single and multiple aneuploidy embryos

Analysis of aneuploid embryos (n = 22) with either single or multiple aneuploidy, revealed no significant differences in morphological grades, morphokinetic parameters, viability scores (both KIDScore and EmbryoScore) or glucose uptake (Table 4.2). However, a significant difference in the utilisation of asparagine, lysine phenylalanine, serine, tryptophan and tyrosine were observed. Aneuploid embryos with multiple aneuploidy consumed significantly higher levels of each of these amino acids than aneuploid embryos with a single aneuploidy (Table 4.2).

Table 4.2 Morphology, morphokinetic development, viability scores and metabolic profiles of aneuploid embryos with single or multiple chromosomal abnormalities.

	Single chromosome aneuploidy	Multiple chromosome aneuploidy
Embryo morphology		
>BB	63.4%	36.4%
Blastocyst diameter	150.6 ± 5.6	146.1 ± 6.1
Embryo morphokinetics		
t2	24.5 ± 0.7	25.2 ± 0.9
t3	34.5 ± 1.5	36.9 ± 1.1
t4	36.0 ± 1.0	37.9 ± 1.3
t5	47.2 ± 2.7	52.0 ± 1.8
t6	50.4 ± 2.2	53.6 ± 2.1
t7	53.3 ± 2.0	55.5 ± 2.3
t8	55.8 ± 2.7	59.6 ± 2.4
tM	84.6 ± 2.7	85.0 ± 3.0
tSB	97.6 ± 1.9	96.6 ± 2.3
tB	105.5 ± 2.1	107.3 ± 3.0
tEB	113.1 ± 2.6	113.1 ± 2.8
tH	126.8 ± 2.6	124.3 ± 3.4
tEB-tSB	15.2 ± 1.2	17.8 ± 1.7
Embryo viability scores		
KIDScore	6.4 ± 0.3	6.2 ± 0.5
EmbryoScore	21.3 ± 5.8	10.5 ± 3.5
Metabolic profile		
Glucose uptake	37.0 ± 7.0	39.6 ± 8.3
Alanine utilization	23.4 ± 4.8	16.3 ± 3.2
Alanyl-Glutamine utilization	-13.0 ± 5.0	-22.7 ± 4.4
Arginine utilization	2.5 ± 6.4	-12.73 ± 6.1

Asparagine utilization	0.6 ± 0.5	-1.3 ± 0.5*
Aspartate utilization	-1.1 ± 2.0	-2.3 ± 1.8
Glutamine utilization	20.2 ± 4.2	15.0 ± 2.3
Glutamate utilization	2.0 ± 1.7	-1.6 ± 1.6
Glycine utilization	7.2. ± 4.4	-5.3 ± 4.3
Histidine utilization	0.1 ± 1.5	-2.9 ± 1.8
Isoleucine utilization	1.7 ± 5.0	-10.4 ± 4.9
Leucine utilization	1.2 ± 5.2	-10.4 ± 5.1
Lysine utilization	3.5 ± 5.7	-10.3 ± 4.6*
Methionine utilization	1.1 ± 1.2	-2.5 ± 1.4
Phenylalanine utilization	0.9 ± 2.8	-6.0 ± 2.9*
Proline utilization	-0.2 ± 2.6	-5.8 ± 2.8
Serine utilization	0.7 ± 2.1	-5.3 ± 2.2*
Taurine utilization	0.8 ± 1.1	-1.3 ± 1.1
Threonine utilization	4.8 ± 4.1	-4.7 ± 3.5
Tryptophan utilization	0.3 ± 0.9	-1.7 ± 0.8*
Tyrosine utilization	0.8 ± 3.0	-6.5 ± 3.0*
Valine utilization	3.8 ± 7.4	-11.9 ± 6.0

Gardner grade (% of embryos graded >BB), n = 22; Blastocyst diameter (µm), n = 22; KIDScore (0-9.9) (n = 22), EmbryoScore (%) (n = 21), and glucose metabolism and individual amino acid metabolism (pmol/embryo/h) (n = 22). Morphokinetic parameters calculated as h post-insemination: 2-cell cleavage, t2 (n = 22); 3-cell cleavage, t3 (n = 22); 4-cell cleavage, t4 (n = 22); 5-cell cleavage t5 (n = 21); 6-cell cleavage, t6 (n = 20); 7-cell cleavage, t7 (n = 20); 8-cell cleavage, t8 (n = 17); morulae, tM (n = 21); start of blastulation, tSB (n = 22); blastocyst, tB (n = 22); expanded blastocyst, tEB (n = 20); hatching blastocyst, tH (n = 15); and rate of expansion, tEB-tSB (n = 19). All data expressed as mean ± SEM. Grey boxes indicates significance and the level of significance is denoted by asterisks, * P <0.05.

Discussion

Data presented demonstrates that aneuploid embryos exhibit aberrations in embryo developmental kinetics when compared to euploid embryos. Subsequently, morphokinetic based algorithms and AI generated scores, which are currently used to assess embryo potential, appear to reflect the presence of aneuploid stress in embryos. Of further significance, embryos with multiple chromosomal aneuploidy appear to have distinct metabolic profiles when compared to aneuploid embryos with single aneuploidy.

The majority of morphological grading systems, such as the Gardner grade, include an assessment of inner cell mass (ICM) and TE quality as an indicator of embryo viability (Gardner, et al., 2000). In contrast to this study, which did not show a significant correlation between blastocyst ICM and TE grades and ploidy status, Capalbo and colleagues found embryos with top morphological grades for both ICM and TE (AA) had a euploid rate of 56.4% compared to 25.5% in poor quality blastocysts (Capalbo, et al., 2014). Consistently, Minasi and colleagues found euploid embryos had a higher incidence of top morphological grades, although they concluded that morphological grades cannot replace genetic testing as an assessment of embryonic ploidy status (Minasi, et al., 2016). This conclusion aligns with an earlier study by Alfarawati and colleagues who reported a weak association between blastocyst morphology and aneuploidy rates (Alfarawati, et al., 2011). These studies and the results presented in the current study reiterate that morphology alone is not an absolute means of measuring ploidy status however, it is plausible that in a larger cohort study a significant correlation between morphological grade and ploidy status will be observed and subsequently align with previous reports.

The D5 KIDScore algorithm facilitates the identification of blastocysts with a high implantation potential (Reignier, et al., 2019). This study demonstrates that aneuploid embryos had significantly lower KIDScores than euploid embryos. Similarly, Gazzo and colleagues showed blastocysts with a higher KIDScore had a higher probability of being euploid, in addition to KIDScore improving implantation rates of single euploid blastocyst transfers compared to morphological grading (Gazzo, et al., 2020). Of note, regardless of whether embryo selection is assessed using morphological grades or morphokinetic based algorithm, a level of intra- and inter-observer subjectivity exists and can impact the success of embryo selection (Storr, et al., 2017). In contrast, AI has the potential to both automate and standardize embryo selection, eliminating such observer subjectivity. The generation of an AI-based EmbryoScore is derived from the analysis of the entire preimplantation period facilitated by time-lapse systems (Tran, et al., 2019) and its clinical benefits are currently under investigation in a prospective randomised trial. Similar to their KIDScores, aneuploid blastocysts scored lower EmbryoScores than euploid blastocysts. Of significance, AI systems utilize deep-learning technology to analyse time-lapse videos and does so with no pre-set parameters. Consequently, it is plausible that such AI systems are able to recognize additional characteristics associated with embryo viability and ploidy status which are yet to be identified. This, in part, may help to explain why the relationship between EmbryoScore and aneuploidy rate is greater than the relationship between aneuploidy rate and KIDScore or morphological grade. Consequently, such an approach may assist in identifying embryos at risk of aneuploidy and hence those that will benefit from PGT-A.

Specific morphokinetic parameters have previously been investigated for an association with embryonic ploidy status. Indeed, a relationship between early preimplantation development and morphology, and the ploidy status of embryos has been observed (Balakier, et al., 2016, Basile, et al., 2014, Chavez, et al., 2012, Chawla, et al., 2015, Kaing, et al., 2018, Patel, et al., 2016, Tilia, et al., 2020, Vera-Rodriguez, et al., 2015). Of significant interest, in addition to showing that the duration of mitotic phase was associated with embryo ploidy status, Vera-Rodriguez and colleagues showed aneuploid embryos differentially expressed a subset of genes during the first 30 h of the preimplantation period (Vera-Rodriguez, et al., 2015). These data provide an example of the impact of aneuploid stress on embryonic gene expression profiles and how this can consequently result in aberrations in the developmental kinetics of preimplantation embryos.

In this study a significant delay in development to hatching was observed in aneuploid embryos, consistent with previous reports (Minasi, et al., 2016). Additionally, analysis of the rate of blastocyst expansion revealed aneuploid blastocysts experienced a significant delay in expansion. This aligns with the work of Desai and colleagues who demonstrated that embryos with a slower rate of expansion (> 13 h) had a lower euploid rate (Desai, et al., 2018). Plausibly, given the role of the TE in forming the blastocoel during blastocyst formation, the observed delay to hatching and slower expansion rates could be related to impaired TE function as a result of aneuploid stress (Huang, et al., 2019).

In cancer cells, aneuploid stress has been recognized to lead to an increase in reactive oxygen species (ROS) and a loss of cellular homeostasis resulting in cell death (Andriani,

et al., 2016, Limoli, et al., 2003, Newman, et al., 2019, Roh, et al., 2012). Of note, exposure to high levels of ROS and oxidative stress in preimplantation embryos causes aberrations in embryo development and physiology (Burton, et al., 2003, Guerin, et al., 2001, Kelley, et al., 2017, Lane, et al., 2005, Rinaudo, et al., 2006, Takahashi, et al., 2000, Wale, et al., 2012, Yang, et al., 1998) and homeostatic pathways such as the mammalian target of rapamycin (mTOR), which are essential for viable development during the preimplantation period (Gangloff, et al., 2004, González, et al., 2012, Krisher, et al., 2012). Such cellular aberrations as a result of aneuploidy may help to explain the impact observed on embryo development. Furthermore, fully understanding the impact of aneuploid stress at a cellular level may in turn help to identify additional developmental and physiological aberrations in preimplantation embryos that would assist in developing algorithms to measure aneuploid stress.

While a significant delay in development was not observed between aneuploid embryos with single or multiple aneuploidies, Nogales and colleagues revealed the rate of morphokinetic development is positively related to the complexity of chromosome disorders (Nogales, et al., 2017). These results provide further support for the presence of aneuploid stress in human preimplantation embryos and how it manifests as aberrations in specific morphological and morphokinetic developmental parameters. Subsequently, such parameters could be used to develop an algorithm to assess aneuploid stress. Campbell and colleagues reported a significant difference in time to the start of blastulation and to the blastocyst stage based on the ploidy status and established a morphokinetic-based aneuploidy risk classification model (Campbell, et al., 2013). Evaluating the effectiveness of this model, they concluded that it could be used to help

prioritize embryos for further genetic screening, however, they also suggested that due to intrinsic and extrinsic factors it may not be transferable between clinics and to a wide spectrum of patient populations (Campbell, et al., 2013). Indeed, Kramer and colleagues were not able to repeat these results using the same aneuploidy risk classification model in a different clinic (Kramer, et al., 2014). It is plausible that AI systems may represent a means of standardizing such algorithms to predict embryonic ploidy status and aneuploid stress. Further, as aneuploidy increases with maternal age and both rate of expansion and EmbryoScores were correlated with maternal age, different thresholds could be considered for specific patient populations including specific age groups.

Consistent with the rate of expansion, the relationship between blastocyst diameter and ploidy status revealed smaller blastocysts are more likely to be aneuploid. We have previously shown larger and faster developing blastocysts have a more active metabolism and a higher implantation potential than slower and smaller blastocysts (Ferrick, et al., 2020). Specifically, glucose and amino acids regulate the formation of the blastocyst and have been recognized as biomarkers of embryo viability (Gardner, et al., 2001, Gardner, et al., 2011, Houghton, et al., 2002, Kelley, et al., 2019, Lee, et al., 2015, Wale, et al., 2012). As aneuploid stress can result in changes to the metabolome, it is plausible that measuring blastocyst metabolism provides an indication of viability post-transfer and could also provide a means of measuring the impact of aneuploid stress. In this study, glucose and amino acids were analysed in relation to the ploidy status of blastocysts. Results were not significant, plausibly due to the low sample size, however euploid blastocysts did demonstrate a trend towards consuming more glucose and amino acids than aneuploid blastocysts.

Of interest, blastocysts with multiple chromosome aneuploidy exhibited a higher level of amino acid consumption than single aneuploidy blastocysts, reflecting distinct physiologies. These results are consistent with previous reports that aneuploidy can impact the metabolic footprint of preimplantation embryos, and the amino acid utilization of cleavage-stage embryos (Liang, et al., 2019, Picton, et al., 2010). However, based on previous reports that higher quality blastocysts exhibit higher amino acid consumption (Ferrick, et al., 2020), one may have predicted embryos with multiple aneuploidy would consume less amino acids than embryos with single aneuploidy. Based on the results of this pilot study and the possibility that specific aneuploidies may impair genes that result in specific metabolic phenotypes, further research on aneuploid stress and metabolism in a larger cohort study is clearly warranted.

The analysis of embryonic chromosomal status utilizing PGT-A technologies is recognized to carry potential risks associated with the invasive nature of embryo biopsy and are additionally costly and time-consuming. The recent discovery of cell-free DNA in both embryo culture medium and blastocoel fluid has raised the possibility of non-invasive PGT (niPGT) (Palini, et al., 2013, Stigliani, et al., 2013) which would eliminate any potential risk associated with embryo biopsy. However, the concordance of niPGT results with PGT-A results from embryo biopsy remains variable (Ho, et al., 2018, Huang, et al., 2019, Leaver, et al., 2020, Liu, et al., 2017, Rubio, et al., 2019, Vera-Rodriguez, et al., 2018, Xu, et al., 2016). The preliminary data presented indicate that aneuploidy is associated with aberrations in embryo development and physiology and such data are now being followed up in a larger cohort study. It is proposed that incorporation of viability scores such as the KIDScore and EmbryoScore, and metabolic biomarkers could assist in

identifying embryos at greatest risk of being aneuploid and will benefit from further genetic testing. Furthermore, in clinics which do not have access to PGT-A technologies, it is possible that the creation of a novel algorithm which incorporates markers of aneuploid stress will assist in ranking embryos for transfer.

Chapter 5. Carbohydrate uptake of vitrified blastocysts post-warm and its relationship to re-expansion and implantation potential

Abstract

Study question: Is metabolism post-warm a biomarker of mouse and human blastocyst re-expansion and implantation potential?

Summary answer: Post-warm, mouse and human blastocyst metabolism was not significantly different between viable and non-viable blastocysts; however, a significant association was observed between human blastocyst re-expansion grades and live birth rates.

What is already known: Blastocyst metabolism is related to embryo development and implantation potential. The re-expansion of warmed or thawed blastocysts is a measure of transfer success.

Study design, size, duration: One hundred and five vitrified mouse blastocysts and 100 vitrified human blastocysts were warmed and incubated in EmbryoGlue media prior to an outgrowth assay and frozen embryo transfer, respectively. Retrospective analysis of carbohydrate uptake post-warm, re-expansion grades and implantation potential was conducted.

Participants/materials, setting, methods: Pyruvate and glucose uptake were analyzed in spent modified EmbryoGlue media samples from mouse blastocysts and spent EmbryoGlue media from human blastocysts were analyzed for pyruvate uptake. Outgrowth area was measured as an indicator of implantation potential in mouse embryos and live birth rates were recorded for human embryos. Blastocyst morphology was assessed immediately after warming (initial re-expansion grade) and prior to outgrowth assay or transfer (final re-expansion grade).

Main results and the role of chance: Mouse blastocyst pyruvate and glucose uptake did not differ significantly based on degree of re-expansion or implantation potential.

Additionally, pyruvate uptake by human blastocysts was not related to blastocyst re-expansion post-warm or live birth rates. Mouse re-expansion grades were also not significantly associated with implantation potential; however, human blastocyst final re-expansion grades were positivity correlated with live birth rates ($P < 0.01$).

Limitations: Glucose consumption could not be measured in human spent media samples due to high glucose concentration present in commercial EmbryoGlue media (Vitrolife).

Wider implications of findings: Data supports the clinical use of re-expansion grades as a useful tool to reassess viability of blastocysts prior to frozen embryo transfers.

Keywords: glucose, pyruvate, viability, live birth rates

Introduction

The first reported pregnancies and live births from cryopreserved embryos were in the late 70's and early 80's and utilized slow freezing protocols (Mukerji S., et al., 1978, Trounson, et al., 1983, Zeilmaker, et al., 1984). Over the past 20 years, such protocols have been widely replaced by vitrification, an ultra-rapid cooling method that eliminates ice-crystal formation and has resulted in higher survival rates in human embryos (Balaban, et al., 2008, Rezazadeh Valojerdi, et al., 2009, Son, et al., 2009, Stehlik, et al., 2005) and higher live birth rates (LBR) following transfer (Debrock, et al., 2015). As a result of improvements in embryo culture systems, extended culture to the blastocyst stage has supported a shift to single blastocyst transfers (SBT) (Gardner, et al., 2000, Harbottle, et al., 2015, Penzias, et al., 2017). Subsequently, vitrification is routinely utilized to cryopreserve supernumerary blastocysts for subsequent frozen embryo transfers (FET), which thereby contributes to cumulative pregnancy rates (Tiitinen, et al., 2004). Additionally, vitrification facilitates preimplantation genetic testing (PGT) and the incidence of PGT has significantly increased in recent years to assist in the transfer of a single euploid blastocyst (Grifo, et al., 2013). Further, more clinics are utilizing freeze-all cycles (Wong, et al., 2017), where all viable blastocysts within a cohort are vitrified, allowing blastocysts to be transferred in a subsequent natural cycle. Freeze-all cycles are beneficial for patients who are at risk of ovarian hyper stimulation syndrome (OHSS), and patients undergoing an agonist gonadotrophin releasing hormone (GnRH) protocol to trigger ovulation (Nagy, et al., 2020). GnRH protocols can alter the receptivity of the endometrium (Dosouto, et al., 2017) and transferring blastocysts in a FET cycle may increase implantation and clinical pregnancy (Özgür, et al., 2015).

Morphological grading systems (Cummins, et al., 1986, Gardner, et al., 2000) and, in those clinics with time-lapse incubators, morphokinetic based algorithms (Basile, et al., 2015, Milewski, et al., 2015, Petersen, et al., 2016, Reignier, et al., 2019, Rubio, et al., 2014) are routinely utilized to assess blastocyst viability and assist in ranking embryos for fresh SBT or warming for a FET. However, vitrification and warming exposes blastocysts to high concentrations of chemical cryoprotectants, with the embryo undergoing a series of morphological changes as it shrinks and subsequently re-expands (Nagy, et al., 2020), hence an assessment of blastocyst re-expansion in the time prior to transfer provides an opportunity to reassess embryo morphology and viability (Coello, et al., 2017). Indeed, the re-expansion of blastocysts post-warm has been positively correlated with implantation rates (Coello, et al., 2017), clinical pregnancy (Yin, et al., 2016) and LBR (Du, et al., 2016). Furthermore, as vitrification can impair cellular function, it is plausible that an assessment of blastocyst physiology post-warm may provide an additional biomarker of embryo viability. Therefore, a reassessment of blastocyst viability may help to decide whether or not, due to low quality, a subsequent blastocyst should be warmed for transfer.

Blastocoel expansion or re-expansion requires the movement of sodium ions across the basolateral membrane of trophectoderm cells (Watson, et al., 1988) as well as the movement of water via aquaporins (Barcroft, et al., 2003). The activity of the Na^+,K^+-ATPases increases subsequent energy demands and is supported by an increase in oxidative metabolism as observed by an increase in oxygen consumption (Houghton, et al., 1996). Pyruvate is an important energy substrate facilitating mitochondrial oxidative metabolism and at the blastocysts stage, pyruvate is predominantly converted from

exogenous glucose. However, exogenous pyruvate can be transported via monocarboxylate transporters (MCT) across the plasma membrane (Gardner, et al., 1988, Hérubel, et al., 2002). Additionally, around 50% of glucose consumed by the blastocyst is converted to lactate, a phenomenon known as the Warburg effect and was first reported in cancer cells (Gardner, 1998, Warburg, 1956). Lactate is released into the microenvironment, where it is has been hypothesized to have a signaling role in mediating endometrium receptivity (Gardner, 2015). Aberrations in embryo carbohydrate metabolism during the preimplantation period have been directly related to poor development (Gardner, 1998, Gardner, et al., 1993, Kelley, et al., 2019, Lee, et al., 2015, Wale, et al., 2012), and blastocyst carbohydrate metabolism has been reported as a biomarker of blastocyst viability and health (Ferrick, et al., 2019, Ferrick, et al., 2020, Gardner, et al., 2001, Gardner, et al., 2011). Therefore, it is plausible an assessment of blastocyst carbohydrate metabolism post-warm may provide an additional biomarker which could be used to reassess embryo viability and health prior to FET.

An analysis of slow-frozen bovine blastocyst carbohydrate metabolism in the 5 h following thaw revealed significantly higher glucose and pyruvate uptake, and higher lactate production in blastocysts that were capable of re-expanding (Gardner, et al., 1996). Therefore, indicating the carbohydrate metabolic profile may provide a measure of cryopreserved blastocyst viability post-thaw. A comparison between non-cryopreserved, slow-frozen and vitrified mouse embryos found blastocysts vitrified in liquid nitrogen exhibited a glucose uptake post-warm similar to non-cryopreserved embryos (Walker, et al., 2005). In contrast, slow-frozen blastocyst glucose uptake was significantly lower post-thaw than control blastocysts indicating slow-freezing may

induce higher levels of metabolic perturbation in blastocysts. Therefore, as an assessment of vitrified blastocyst carbohydrate metabolism post-warm has yet to be investigated as a biomarker of blastocyst viability, the objective of this study was to analyze carbohydrate utilization of mouse and human blastocyst post-warm and relate this to blastocyst re-expansion and implantation potential, which subsequently could be used to help reassess embryo viability post-warm, and clinically could identify cycles in which a second blastocyst requires warming.

Materials and methods

Mouse embryo culture

Female F1 (C57 x CBA) mice, 3 to 4 weeks old, were injected with 5 IU pregnant mare's serum gonadotrophin (PMSG; Folligon, Intervet, UK) to stimulate follicle growth and 48 h later were injected with 5 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet) and mated with males of the same strain. The presence of a vaginal plug was recorded the following morning as an indicator of mating and females were sacrificed via cervical dislocation (Gardner, et al., 2014). Embryos were collected in 500 µl of G-MOPS PLUS handling medium (Vitrolife, AB, Sweden) and denuded of cumulus cells by adding 500 µl pre-warmed hyaluronidase solution (300 IU/ml) (bovine testes type IV, Sigma Aldrich, Australia) (Gardner, et al., 2019). Denuded embryos were washed three times in G-MOPS PLUS before being transferred to the culture dish. Embryos were cultured individually in 10 µl of G-TL culture medium under Ovoil (Vitrolife) at 37°C, 6% CO_2, 5% O_2, 89% N_2 and left undisturbed for 74 h. On day 4 of culture, blastocysts were graded and vitrified. A total of 105 mouse blastocysts were included in this study and ethical approval was granted by the Faculty of Science Animal Ethics Committee, University of Melbourne.

Human embryo culture

One hundred human embryos from 100 patients from IVF or ICSI cycles were included in this study. Embryos were cultured in G-series medium (Vitrolife) at 37°C, 6% CO_2, 5% O_2, 89% N_2. These embryos were vitrified on either day 5 or 6 of development once they had reached the blastocyst stage and warmed for a SBT between February and December 2018. At the time of transfer patients were ≤ 38 years of age and had less than 5 previous

transfers. Ethical approval was obtained from Melbourne IVF Human Research Ethics Committee (53/117-MIVF).

Vitrification and warming of mouse and human blastocysts

Mouse blastocysts were vitrified and warmed using RapidVit™ Blast kits (Vitrolife) and RapidWarm™ Blast kits (Vitrolife) as per manufacturer's instructions and with a cryo-loop as the cryo-device (Lane, et al., 1999). Human blastocysts were vitrified and warmed using the same commercial kits and a Rapid-i Vitrification System (Vitrolife) (Desai, et al., 2013). Immediately after warming, mouse blastocysts were washed in 20 µl modified EmbryoGlue media (mEG; 0.5 mM glucose, prepared in-house) and transferred to 0.5 µl mEG overlaid with Ovoil for 4 h prior to outgrowth assay and EmbryoGlue dishes were stored at -80°C until metabolic analysis. Human blastocysts were immediately washed twice with G-TL medium (Vitrolife) and once with EmbryoGlue medium (Vitrolife) before being incubated in 10 µl EmbryoGlue medium under 2 ml Ovoil for 1-5 h awaiting transfer. Once the embryo was transferred, the EmbryoGlue dishes were stored at -20°C until collection for metabolic analysis.

Analysis of embryo re-expansion

Mouse and human blastocyst re-expansion were assessed immediately after warming (initial re-expansion grade). Each embryo was assigned an alphabetical grade and represented the proportion of the blastocyst which the blastocoel cavity occupied (A: 75-100%, B: 50-75%, C: <50% and D: collapsed). Using the same alphabetical grading system, mouse and human blastocysts were assessed again prior to outgrowth assay and FET, respectively (final re-expansion grade).

Analysis of embryo carbohydrate utilization

EmbryoGlue and mEG media samples were analyzed for carbohydrate utilization by ultramicrofluorescence (UMF) as previously described (Gardner, et al., 1986, Leese, et al., 1984). Mouse embryo spent media and no-embryo control media were analyzed for both pyruvate and glucose uptake. Human embryo spent media and no-embryo control media were analyzed for pyruvate uptake only. High glucose concentrations in commercial EmbryoGlue media (3.15 mM) meant human spent media samples could not be accurately measured for glucose uptake in the time prior to FET due to limitations in UMF sensitivity. Overall metabolite uptake was calculated as the difference between no embryo control and each spent medium sample.

Analysis of embryo implantation potential

Outgrowth assay

An outgrowth assay was conducted on mouse blastocysts as a measure of implantation potential (Binder, et al., 2015). Microwell plates (Nunc MicroWell MiniTrays, Thermo Fisher Scientific, Australia) were prepared by rinsing with sterile phosphate-buffered saline (PBS), incubated with 10µg/ml fibronectin solution (Corning, USA) for 1 h at room temperature and washing again with sterile PBS (Hannan, et al., 2011). Wells were then incubated in 2.5 µl of bovine serum albumin solution (BSA; 4mg/mL, MP Biomedicals, Australia) and washed thoroughly with sterile PBS. For outgrowth assay, 5 µl G2 medium with 5% fetal calf serum (FCS; Invitrogen, Thermo Fisher Scientific) was added to wells followed by 5 µl Ovoil and preequilibrated to 37°C, 6% CO_2, 5% O_2 and 89% N_2 for 4 h. Embryos were cultured individually in microwells and imaged at 48, 60, 72, and 84 h post-

culture using NIS Elements BR 3.00, SP7 Laboratory Imaging software. Area of outgrowth was calculated using FIJI ImageJ software (ImageJ 2.0.0).

Pregnancy assessment in the human

Ongoing pregnancies were confirmed at a 6 week ultrasound with the presence of a positive fetal heartbeat and gestational sac. LBR and gender were recorded at the time of birth.

Statistical analysis

All data were tested for normality prior to statistical tests using Shapiro-Wilk test. The comparisons between two groups were conducted using the Student's *t*-test and for analyses with groups >2 data were subject to a one-way analysis of variance (ANOVA) and Bonferroni's Multiple Comparison test for comparison within groups. To analyze the relationship between mouse blastocyst metabolism and outgrowth area, embryos were grouped based on the mean pyruvate and glucose uptake (1.9 pmol/embryo/h and 7.7 pmol/embryo/h, respectively). Chi square test was used to analyze the correlation between LBR and the expansion grade of human blastocysts. All analyses were conducted using Prism 8 (version 8.3.0, GraphPad, LLC) and significance threshold was set as $P < 0.05$.

Results

Mouse blastocyst pyruvate and glucose uptake post-warm and the relationship to re-expansion grades and implantation potential

No significant differences were found between initial or final re-expansion grades and mouse blastocyst pyruvate or glucose uptake (Table 5.1-2). Fifty-one percent of mouse blastocysts successfully attached and grew in the outgrowth assay (n = 54). A comparison between pyruvate uptake and outgrowth area of these blastocysts revealed mouse blastocyst outgrowth area did not significantly differ based on the mean cohort pyruvate uptake (1.9 pmol/embryo/h). Similarly, outgrowth area did not significantly differ based on the mean cohort glucose uptake (7.7 pmol/embryo/h) (Fig. 5.1).

Table 5.1 Relationship between mouse blastocyst carbohydrate uptake and initial re-expansion grade.

	Initial re-expansion grade			
	A	B	C	D
Pyruvate uptake	1.52 ± 1.00	3.02 ± 0.50	1.74 ± 0.44	1.51 ± 0.20
Glucose uptake	8.95 ± 1.60	11.36 ± 1.49	9.27 ± 1.00	6.98 ± 0.67

Pyruvate and glucose uptake (pmol/embryo/h) of embryos graded A (n = 7), B (n = 12), C (n = 22) and D (n = 64). Initial re-expansion grade represents the proportion of the blastocyst which the blastocoel occupied (A: 75-100%, B: 50-75%, C: <50% and D: collapsed) immediately after warming. Data expressed as mean ± SEM. No significant differences were observed.

Table 5.2 Relationship between mouse blastocyst carbohydrate uptake and final re-expansion grade.

	Final re-expansion grade			
	A	B	C	D
Pyruvate uptake	2.37 ± 0.57	1.94 ± 0.34	1.52 ± 0.34	1.46 ± 0.26
Glucose uptake	9.68 ± 1.15	8.26 ± 1.00	7.30 ± 0.91	7.79 ± 0.96

Pyruvate and glucose uptake (pmol/embryo/h) of embryos graded A (n = 18), B (n = 21), C (n = 25) and D (n = 41). Final re-expansion grade represents the proportion of the blastocyst which the blastocoel occupied (A: 75-100%, B: 50-75%, C: <50% and D: collapsed) prior to outgrowth assay. Data expressed as mean ± SEM. No significant differences were observed.

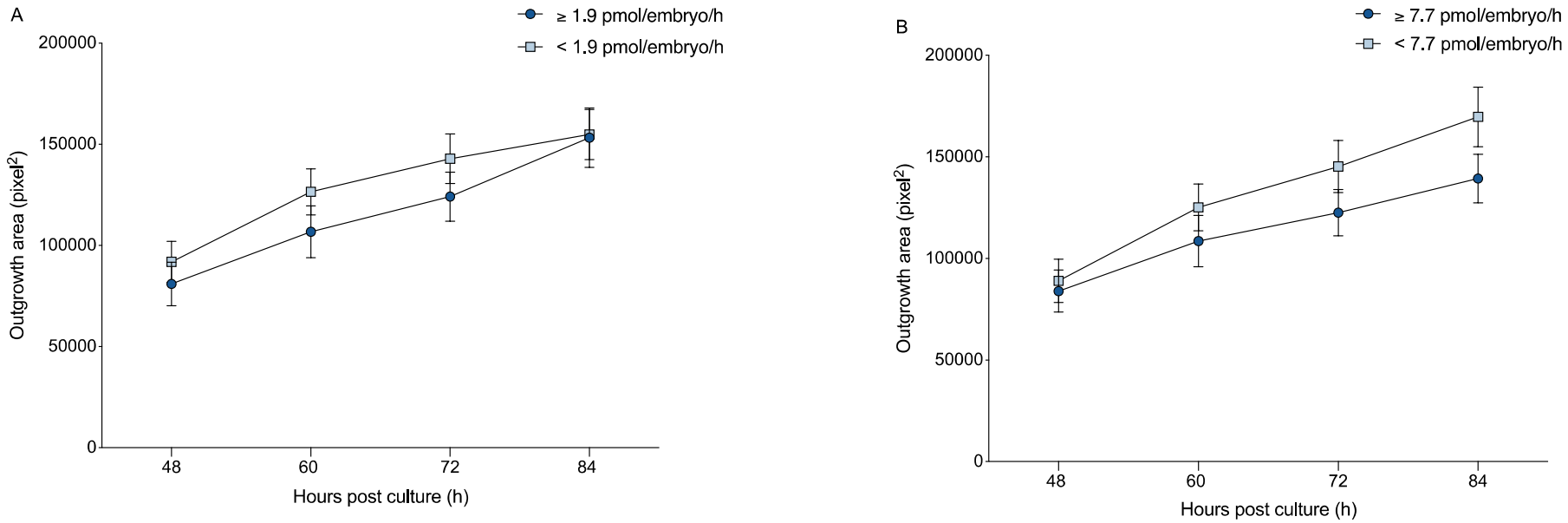

Figure 5.1 Relationship between mouse blastocyst pyruvate and glucose uptake and implantation potential.

(A) Pyruvate uptake compared to embryo outgrowth area (pixel^2) measured at 48, 60, 72 and 84 h. Dark data points represent blastocysts that consumed ≥ mean cohort pyruvate uptake (pmol/embryo/h), n = 27, light data points represent embryos that consumed < cohort mean pyruvate uptake (pmol/embryo/h), n = 27. (B) Glucose uptake compared to embryo outgrowth area (pixel^2), measured at 48, 60, 72 and 84 h. Dark data points represent blastocysts that consumed ≥ mean cohort glucose uptake (pmol/embryo/h), n = 28, light data points represent embryos that consumed < cohort mean glucose uptake (pmol/embryo/h), n = 26. All data expressed as mean ± SEM, no significant differences were observed.

Mouse blastocyst re-expansion post-warm and implantation potential

Both initial and final re-expansion grades did not significantly differ between embryos which failed to attach (n = 51) and those that outgrew (n = 54). Of the blastocysts that attached and grew in an outgrowth assay, there was no significant difference in outgrowth area based on both initial and final re-expansion grades (Fig. 5.2).

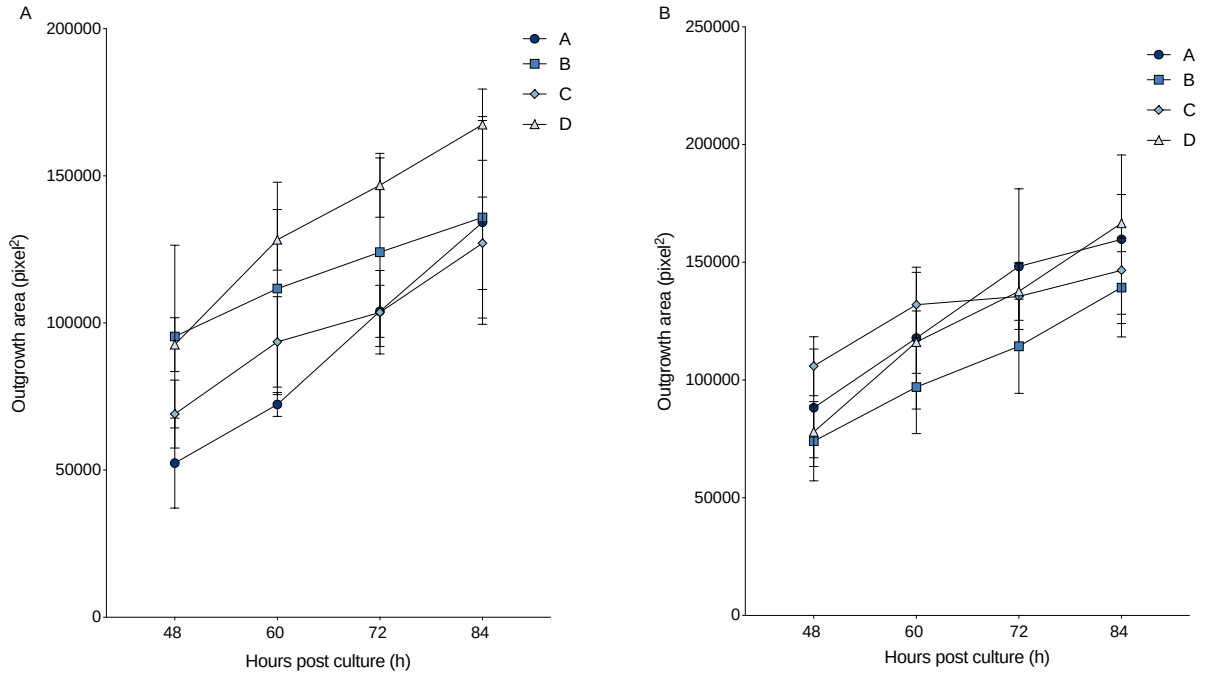

Figure 5.2 Relationship between mouse blastocyst implantation potential and re-expansion grades post-warm.

(A) Implantation potential and initial re-expansion grade, A (n = 3), B (n = 7), C (n = 10) and D (n = 34), whereby grade represents the proportion of the blastocyst which the blastocoel occupied (A: 75-100%, B: 50-75%, C: <50% and D: collapsed) and were assigned immediately post-warm. (B) Implantation potential and final re-expansion grade A (n = 8), B (n = 12), C (n = 15) and D (n = 19), whereby grades were assigned prior to outgrowth assay. Data expressed as mean ± SEM, no significant differences were observed.

The relationship between human blastocyst pyruvate uptake, re-expansion post-warm and live birth rates

No significant differences were observed between the final re-expansion grade of human blastocysts and pyruvate uptake (Table 5.3). Of note, an analysis of initial re-expansion grade for human blastocysts revealed that majority of embryos (92%) were collapsed and graded 'D', hence a comparison with pyruvate uptake was not conducted. Of the 100 human embryos transferred in an FET cycle, 26 led to a live birth resulting in a LBR of 26%. No significant difference in pyruvate uptake was observed between blastocysts that resulted in a live birth compared to those that failed to establish a pregnancy (Fig. 5.3). To ensure there was no gender bias, pyruvate uptake of both male and female blastocysts was analyzed, and no significant differences were observed (Table 5.4).

Table 5.3 Relationship between human blastocyst pyruvate uptake and final re-expansion grade.

	Final re-expansion grade			
	A	B	C	D
Pyruvate uptake	56.69 ± 24.91	84.65 ± 28.41	13.22 ± 14.45	77.04 ± 31.19

Pyruvate uptake(pmol/embryo/h) of embryos graded A (n = 46), B (n = 17), C (n = 5) and D (n = 32). Final re-expansion grade represents the proportion of the embryo which the blastocoel occupied (A: 75-100%, B: 50-75%, C: <50% and D: collapsed) and were assigned prior to frozen embryo transfer. Data expressed as mean ± SEM. No significant differences were observed.

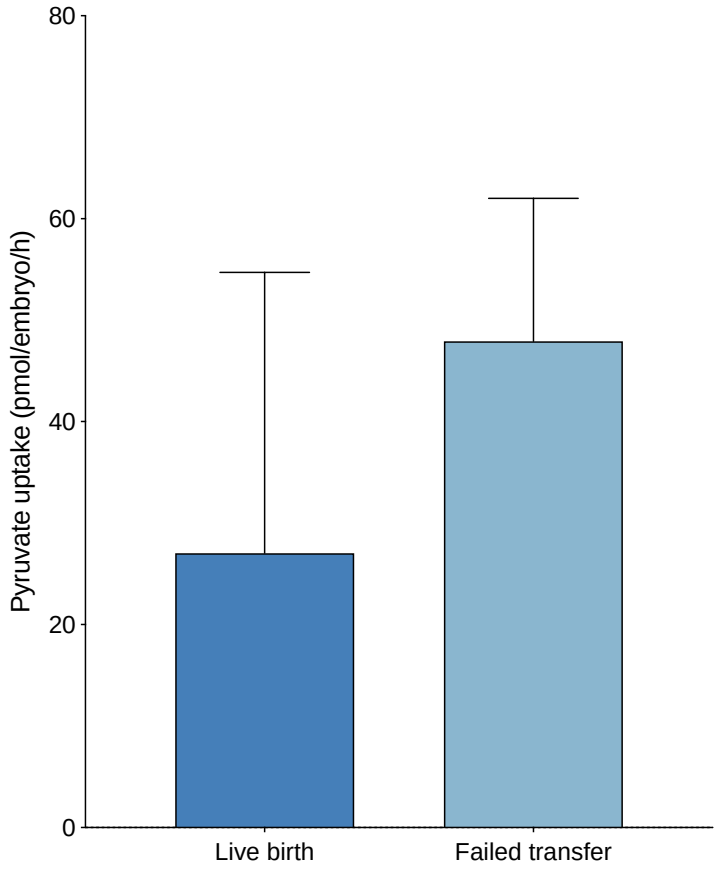

Figure 5.3 Relationship between pyruvate uptake of human blastocysts and live birth rates.

Data are expressed as mean ± SEM (pmol/embryo/h), live births n = 26 and failed transfers n = 74. No significant difference was observed.

Table 5.4 Pyruvate uptake of human male and female blastocysts post-warm.

	Female	**Male**
Pyruvate uptake	36.56 ± 38.17	9.11 ± 36.52

Pyruvate uptake (pmol/embryo/h) of female (n = 17) and male (n = 9). Data expressed as mean ± SEM. No significant difference was observed.

Human blastocyst re-expansion post-warm is correlated to live birth rates

Live birth rates were found to be significantly associated with final re-expansion grades ($P < 0.01$) (Fig. 5.4). Thirty-nine percent of human blastocysts with a top final re-expansion grade (A) resulted in a live birth, compared to 9% of human blastocysts with the lowest final re-expansion grade (D). Furthermore, 48% of blastocysts were vitrified on day 5 of development and had a LBR of 27%. The remaining 52% of blastocysts were vitrified on day 6 of development and had a LBR of 25%.

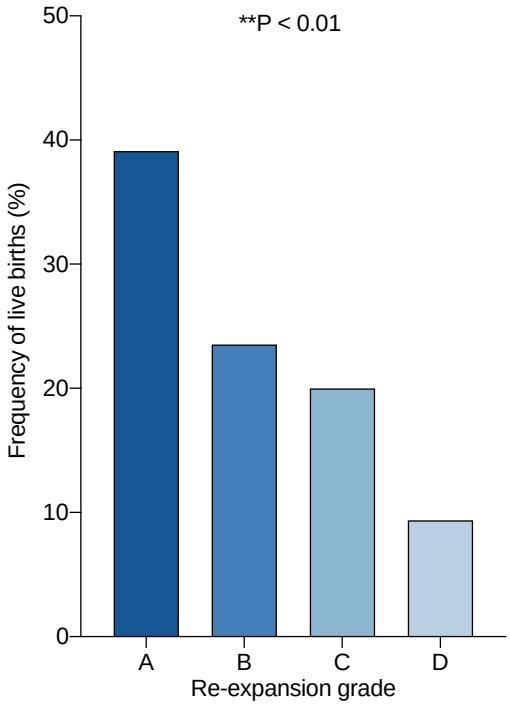

Figure 5.4 Correlation between transfer outcome of human single frozen embryo transfers and re-expansion grades post-warm.

Blastocysts final re-expansion grades, A (n = 46), B (n = 17), C (n = 5) and D (n = 32), representing the proportion of the blastocyst which the blastocoel occupied (A: 75-100%, B: 50-75%, C: <50% and D: collapsed), and the frequency of a live birth. Significant correlation between live birth rates and re-expansion grades denoted by asterisks and *P* value.

Discussion

It was determined that human blastocyst re-expansion grades post-warm were positively correlated to LBR, confirming that blastocyst morphology post-warm is a valuable biomarker of embryo viability and success of FET. In contrast, no significant differences were observed in human blastocyst pyruvate uptake post-warm or mouse blastocyst pyruvate and glucose uptake when compared to the degree of blastocyst re-expansion or implantation potential. Therefore, data do not support the use of metabolic biomarkers post-warm in predicting transfer success following FET, however, optimization of a glucose UMF assay to accurately measure human glucose uptake post-warm may provide further insight into blastocyst metabolism.

Mouse and human blastocysts were found to consume pyruvate post-warm, however, a large variation between blastocysts with different degrees of re-expansion or implantation potential, indicates pyruvate uptake could not provide a measure of blastocyst viability. In contrast, Gardner and colleagues found pyruvate utilization was significantly higher in viable bovine blastocysts or blastocysts that were capable of re-expanding post-thaw, compared to non-viable blastocysts or blastocysts that remained collapsed (Gardner, et al., 1996). However, an analysis of the variation of pyruvate uptake did not reveal two distinct populations suggesting pyruvate uptake post-thaw is limited in predicting embryo viability alone. Of significance, Gardner and colleagues utilized slow-freezing cryopreservation protocols, whereas vitrification was used in the present study and the level of cryo-damage associated with each cryopreservation technique may explain the conflicting results. Indeed, slow-freezing protocols have been shown to induce higher levels of metabolic trauma than vitrification in human cleavage stage

embryos (Balaban, et al., 2008). Furthermore, while gene expression analysis revealed the gene expression profile of vitrified blastocysts did not significantly differ when compared to non-cryopreserved blastocysts (Larman, et al., 2011), slow freezing was found to significantly impact the expression of 115 genes involved in protein metabolism, transcription, cell organization, signal transduction, intracellular transport, macromolecule biosynthesis and development (Larman, et al., 2011). Therefore, indicating slow-freezing induces a higher level of cryo-damage than vitrification leading to perturbations in blastocyst physiology and metabolism.

The repair of cryo-damage post-thaw can induce an increase in reactive oxygen species (ROS) (Tatone, et al., 2010), which, if not removed through antioxidants mechanisms, can further compromise embryo development and viability. Of significance, pyruvate, as an α-keto carboxylic acid, can react with hydrogen peroxide (H_2O_2) to produce acetate, carbon dioxide and water (Bunton, 1949) and has been shown to attenuate the toxic effects of H_2O_2 in mouse and bovine embryos (Morales, et al., 1999, O'Fallon, et al., 1995). Therefore, due to antioxidant properties it is plausible an increase in pyruvate utilization by viable thawed bovine blastocysts may represent the reduction in ROS accumulated during cryo-damage repair post-thaw. Together, these data suggest, while pyruvate uptake may provide a biomarker of embryo viability post-thaw, it does not hold the same predictive power in assessing viability of vitrified blastocysts post-warm which are exposed to lower levels of cryo-damage.

Mouse blastocyst glucose uptake post-warm was not significantly different when compared to degree of re-expansion or implantation potential, suggesting glucose may

not be a valuable biomarker of embryo viability post-warm. Of note, the average glucose uptake of mouse blastocyst post-warm was 7.7 pmol/embryo/h and while metabolism prior to vitrification was not measured, these values are consistent with those measured in non-cryopreserved mouse blastocysts cultured in the same conditions (Kelley, et al., 2019). Subsequently, unlike slow-frozen bovine blastocysts which experienced a significant drop in metabolic activity post-thaw (Gardner, et al., 1996), these data further support the hypothesis that vitrification causes less metabolic trauma to embryos. However, while 80-90% of fresh mouse blastocysts transferred to an outgrowth assay have been shown attach (Kelley, et al., 2019), in the present study 49% of mouse blastocysts failed to attach and grow in an outgrowth assay following warming and therefore suggests vitrified blastocysts are not fully resistant to cryo-damage. Indeed, vitrification of mouse pronucleate oocytes has been shown to alter the mitochondrial distribution and the mitochondrial membrane potential compared to fresh pronucleate oocytes and this cryo-damage was shown to persist in embryo development and resulted in lower blastocyst development rates (Zhao, et al., 2009). Additionally, an overall increase in ROS and a reduction in mitochondrial activity in vitrified mouse blastocysts (Martino, et al., 2013), suggests cryo-damage is evident in vitrified mouse blastocysts and the analysis of the oxidative capacity of blastocysts via carbohydrate metabolism post-warm may be limited in its ability to discriminate based on embryo viability. Furthermore, a study on sheep oocytes revealed mitochondrial function in vitrified oocytes took 4-6 h post-warm to recover from vitrification induced cryo-damage (Succu, et al., 2018), therefore suggesting the current time prior to transfer (1-5 h) may also impact the ability to assess the true oxidative capacity of blastocysts.

The average glucose uptake in mouse blastocysts was higher (7.7 pmol/embryo/h) than pyruvate uptake (1.9 pmol/embryo/h) and hence consistent with mouse blastocysts preferentially consuming glucose to support re-expansion post-warm. In light of the predominant role of glucose metabolism in blastocyst expansion, is has been shown that prior to vitrification or transfer, human blastocysts that expanded at a significantly faster rate consumed more glucose and were associated with higher viability than slower expanding blastocysts (Ferrick, et al., 2020). Further, day 4 and 5 glucose consumption has previously been related to both human blastocyst quality and pregnancy rates following transfer (Gardner, et al., 2001, Gardner, et al., 2011). While pyruvate was shown to be higher on day 4 in embryos that development to the blastocyst stage, pyruvate uptake did not differ between blastocyst of different qualities based on their morphological grade (Gardner, et al., 2001) and it is plausible that blastocyst expansion may rely on the conversion of exogenous glucose to pyruvate to facilitate oxidative metabolism more so than the consumption of exogenous pyruvate. Furthermore, the significant production of lactate through aerobic glycolysis, which is exported out of the cell via MCTs to create a unique microenvironment characteristic of low pH and high lactate, may create competition for MCTs and impact the transport of exogenous pyruvate across the plasma membrane and into the embryo (Lane, et al., 2000).

The sensitivity of ultramicrofluorescent carbohydrate assays heavily relies on the incubation conditions such as, time, media volume, the concentrations of metabolites in media and also the metabolic activity of the embryos. Mouse blastocysts were cultured in 0.5 µl of mEG medium with 0.5 mM glucose, which represents the smallest culture volume logistically possible and an 84% reduction in glucose concentration compared to

commercial compositions. Over a 4 h period where, based on the average consumption of 7.7 pmol/embryo/h of glucose, this equates to a 1.2% change in the overall glucose concentration. Therefore, it is plausible that a lack of significance in glucose consumption observed post-warm of blastocysts with different degrees of re-expansion or implantation potential is due to a limitation in the assay's sensitivity utilizing these culture conditions. To fully elucidate whether glucose metabolism is a valid biomarker which could be used clinically to assess embryo viability post-warm, future studies could focus on analysis of glucose metabolism of vitrified human blastocysts and should consider the delicate balance in incubation time, media volume, media composition to ensure assay sensitivity is capable of detecting difference in individual blastocyst glucose metabolism.

Following FET, the LBR for human blastocysts was 26%, whereby 48% of blastocysts were vitrified on day 5 of development and had a LBR of 27%, and the remaining blastocysts were vitrified on day 6 representing a delay in blastocyst formation and had a LBR of 25%. Similarly, Ferreux and colleagues reported a 26.3% LBR of vitrified blastocysts transferred individually in an FET (Ferreux, et al., 2018). Furthermore, they found blastocysts vitrified on day 5 had a higher LBR than blastocysts vitrified on day 6 (29.6% versus 17.0%) (Ferreux, et al., 2018). Indeed, regardless of whether blastocysts are transferred in a fresh or frozen embryo transfer, day 5 blastocysts are reported to have greater success rates than day 6 blastocysts (Haas, et al., 2016, Shapiro, et al., 2001), suggesting a the delay in blastocyst formation is a sign of lower viability and future experiments should be mindful of which day blastocysts are vitrified when analyzing results. The average time between warming and FET ranged from 1-5 h with an average time of 2.3 h. As it has been shown an assessment of blastocyst morphology within 2 h immediately following warming can

predict pregnancy outcomes (Ebner, et al., 2009) these results suggests an assessment of blastocyst morphology post-warm and prior to transfer is clinically feasible. Of those human blastocysts that resulted in a live birth, 39% had expanded by 75-100% prior to transfer and just 9% were collapsed, therefore, suggests that the ability of blastocysts to re-expand is indeed a measure of embryo competency. These results aligns with previous reports that re-expansion of blastocysts post-warm is significantly associated with live birth rates following FET (Du, et al., 2016) and the speed of blastocyst re-expansion following vitrification is significantly associated with clinical pregnancy rates (Yin, et al., 2016). Of note, given 9% of collapsed blastocysts resulted in a live birth, this suggests re-expansion grades post-warm are not an absolute measure of embryo viability and perhaps a combination of biomarkers will provide a more accurate measure of embryo viability post-transfer (Ferrick, et al., 2020).

Data presented indicate pyruvate uptake post-warm has limited value as a biomarker of human embryo viability; however, as the predominant carbohydrate consumed during blastocyst expansion, glucose may be capable of predicting success post-FET for human vitrified blastocysts and a follow-up study, plausibly using reduced glucose concentrations, is required for validation. Morphological parameters of re-expansion post-warm, however, were significantly associated with LBR and therefore can be used to reassess embryo viability following vitrification and warming. Viability grades post-warm can provide fundamental information that could assist in the decision-making process of whether a FET should go ahead, or perhaps due to low viability, whether a subsequent embryo should be warmed. Additionally, having a better understanding of

the potential an embryo will succeed post-transfer also provides an essential for tool for patient counseling and the management of patient expectations.

Chapter 6. Addition of antioxidants to embryo culture medium reduces reactive oxygen species production and alters metabolic activity in mouse preimplantation embryos

Abstract

Study question: Does acetyl-*L*-carnitine (ALC), *N*-acetyl-*L*-cysteine (NAC) and α-lipoic acid (ALA) individually, and in combination (A3) impact mitochondrial and intracellular reactive oxygen species (ROS) levels and affect mouse blastocyst carbohydrate metabolism?

Summary answer: The addition of ALC, NAC, ALA and A3 to embryo culture media reduced ROS levels, regulated blastocyst metabolism and subsequently improved blastocyst development. The impact on blastocyst metabolism was dependent on whether antioxidants were added to embryo culture media individually or in combination.

What is already known: Culturing mouse embryos in the presence of A3 improves IVF, embryo quality and transfer outcome. Additionally, patients aged 35-40 years have higher implantation and pregnancy rates when oocytes and embryos are cultured in the presence of A3.

Study design, size, duration: This study was conducted on mouse pronucleate oocytes cultured to day 5 in the presence or absence of 10 µM ALC, 10 µM NAC and 5µM ALA individually or as a group (A3).

Participants/materials, setting, methods: Blastocyst total cell numbers were analyzed by nuclear staining as a measure of embryo development. Glucose and lactate metabolism were quantitated using ultramicrofluorescence and glycolytic rate was calculated. Confocal microscopy was employed to measure glutathione levels, mitochondrial membrane potential and mitochondrial superoxide. Additionally, intracellular hydrogen peroxide levels were measured, and the redox state of embryos assessed by NADH autofluorescence.

Main results and the role of chance: In the presence of individual antioxidants and A3, blastocysts had an increase in cell number and a reduction in ROS, with ROS reduction being greatest in the presence of A3. While ALC did not affect blastocyst metabolism, NAC and ALA increased glucose consumption but did not alter lactate production. In contrast, the presence of A3 did not impact glucose uptake, but increased lactate production, and glycolytic rate. The changes in blastocyst metabolism were consistent with a reduction in NADH autofluorescence.

Limitations: Analyses were performed using mouse embryos only.

Wider implications of findings: The results of this study indicate a combination of antioxidants has a synergistic affect in reducing blastocyst ROS production associated with improved blastocyst development. Furthermore, while individual antioxidants are able to regulate carbohydrate metabolism to maintain mitochondrial function, a combination of antioxidants supported a more in vivo-like metabolism. These data highlight the importance of understanding the impact alterations to embryonic culture conditions have on embryo physiology.

Key words: metabolism, glucose, lactate, glycolytic rate, viability

Introduction

In vivo, preimplantation embryos are exposed to an oxygen gradient in which the oviduct oxygen concentration is 5-10% and the uterus is 1-5% (Fischer, et al., 1993, Mastroianna, et al., 1965). As early as 1969, the benefits of low oxygen concentrations in in vitro culture were reported in mouse preimplantation embryos (Whitten, 1969) and subsequent studies on other mammalian species reported improved preimplantation embryo development in the sheep (Thompson, et al., 1990), cow (Thompson, et al., 1990), goat (Batt, et al., 1991), pig (Berthelot, et al., 1996) and mouse (Gardner, et al., 1996) when exposed to low (5-7%) oxygen compared to atmospheric (20%) oxygen during culture. Indeed, exposing embryos to 20% oxygen can induce aberrations in embryonic gene expression (Gardner, et al., 2005, Harvey, et al., 2004, Rinaudo, et al., 2006), epigenome (Ghosh, et al., 2017), proteome (Katz-Jaffe, et al., 2005) and metabolome (Khurana, et al., 1989, Wale, et al., 2012) plausibly due to oxidative stress which increases with oxygen concentration (Thompson, et al., 1996).

Despite these substantial data demonstrating the negative effects of 20% oxygen to the developing embryo, 34% of clinics worldwide use a combination of 5% and 20% oxygen and a further 38% of clinics exclusively use 20% oxygen culture (Christianson, et al., 2014). It has been documented that culturing human preimplantation embryos at 20% oxygen delayed cleavage rates (Kirkegaard, et al., 2013) and reduced blastocyst formation (Dumoulin, et al., 1999, Kirkegaard, et al., 2013, Waldenström, et al., 2009). Conversely, culturing human embryos at 5% oxygen increased the number and quality of blastocysts, therefore the number of embryos cryopreserved per cycle (Van Montfoort, et al., 2020, Waldenström, et al., 2009) and subsequently increased cumulative pregnancy rates (Van

Montfoort, et al., 2020). Furthermore, live birth rates were significantly higher when preimplantation embryos are cultured at 5% oxygen (Bontekoe, et al., 2012, Meintjes, et al., 2009).

Even when embryos are cultured in the presence of 5% oxygen, they are exposed to 20% oxygen during IVF procedures including gamete collection, denudation, insemination, media change overs, biopsy for preimplantation genetic testing, vitrification and transfer (Agarwal, et al., 2014, Gupta, et al., 2009, Truong, et al., 2020, Truong, et al., 2016). Crucially, it has been identified that exposure to 20% oxygen for as little as 1 to 4 h can negatively impact the developing embryo (Gardner, et al., 1996, Pabon Jr, et al., 1989, Truong, et al., 2017). Furthermore, oxygen is an important metabolite consumed by the preimplantation embryo (Houghton, et al., 1996, Magnusson, et al., 1986, Thompson, et al., 1996) and oxidative metabolism, via the mitochondria, results in the production of reactive oxygen species (ROS) (Murphy, 2009), a further source of oxidative stress. Subsequently, in vivo and in vitro derived embryos are unavoidably exposed to oxidative stress.

In addition to low oxygen concentrations present within the maternal reproductive tract, in vivo preimplantation embryos are exposed to a comprehensive antioxidant system. Antioxidants can be either enzymatic such as superoxide dismutase, or non-enzymatic such glutathione (GSH), cysteine (Agarwal, et al., 2012, Gupta, et al., 2009) and pyruvate (Morales, et al., 1999, O'Fallon, et al., 1995). A balance between pro-oxidant and antioxidants prevents the accumulation of ROS during the preimplantation period protecting against oxidative damage while maintaining ROS signaling capacity which is

important for normal cellular function (Diebold, et al., 2016, Hamanaka, et al., 2010, Harvey, 2007, Lees, et al., 2017). To date, antioxidants are typically absent from commercial embryo culture media.

Carnitine (and its acetylated derivative), acetyl-cysteine and lipoic acid have been shown to have beneficial effects in reducing oxidative stress and improving mammalian oocyte and embryo development. Carnitine is capable of scavenging free radicals (Gülcin, 2006) and is involved in the regulation of fatty acid transport into the mitochondria for β-oxidation and ATP production (Somfai, et al., 2011). The inclusion of l-carnitine to embryo culture media has been reported to decrease DNA damage and improve mouse blastocyst development (Abdelrazik, et al., 2009). Cysteine is the rate-limiting factor in GSH production, a potent antioxidant (Anderson, et al., 1983, De Matos, et al., 2000, De Matos, et al., 1995) and the inclusion of *N*-acetyl-*L*-cysteine (NAC) to pig IVM media significantly reduced DNA fragmentation in oocytes and led to an increase in blastocyst development rates (Whitaker, et al., 2012). Lipoic acid can reduce other antioxidants, recycling their antioxidant function and is hence referred to as the antioxidant of antioxidants (Bilska, et al., 2005, Packer, et al., 1995). Further, lipoic acid is a coenzyme of pyruvate dehydrogenase (PDH) complex necessary for mitochondrial ATP production (Palaniappan, et al., 2007) and the inclusion of α-lipoic acid (ALA) to mouse embryo culture media significantly increased blastocyst cell number and reduced the levels of extracellular ROS in culture medium (Linck, et al., 2007).

Due to their individual ability to reduce ROS, the addition of acetyl-*L*-carnitine (ALC), NAC and ALA in combination (A3) to mouse embryo culture medium has been investigated.

Culturing embryos in the presence of A3 revealed faster cleavage rates, maintenance of intracellular GSH levels and subsequently improved embryo and fetal development (Truong, et al., 2016). Further, Truong and colleagues utilized a defined culture matrix to investigate the impact of A3 when embryos were exposed to different culture conditions and subsequent stresses. Specifically, the culture matrix consisted of 5% or 20% oxygen and individual or group culture, creating four culture conditions with varying levels of stress (group, 5% oxygen; group, 20% oxygen; individual, 5% oxygen; individual, 20% oxygen). Embryos cultured individually at 20% oxygen, a high oxidative stress environment, which exhibited the lowest overall blastocyst cell number exhibited the largest increase in cell numbers when in the presence of A3 (Truong, et al., 2016). These results were consistent with previous reports showing that a combination of both 20% oxygen and individual culture were of further detriment to the developing embryo when compared to 20% oxygen or individual culture alone (Kelley, et al., 2016). Therefore, indicating exposure of embryos to a single stress increases the susceptibility to any additional stresses (Kelley, et al., 2016). Furthermore, A3 still remained beneficial to embryos in groups at 5% oxygen possibly due to a reduction in the accumulation of endogenous ROS generated during mitochondrial metabolism (Truong, et al., 2016).

The beneficial effects of A3 in embryo culture media, prompted investigating in mouse IVF. A3 resulted in improved blastocyst development and significantly reduced hydrogen peroxide (H_2O_2) levels (Truong, et al., 2017). Additionally, the inclusion of A3 to vitrification media increased mouse blastocyst viability and fetal development (Truong, et al., 2020). Furthermore, benefits have been observed in human embryos. A human prospective randomized multicenter comparison was performed on 1563 embryos, from

133 patients cultured in the presence or absence of A3 at 5% oxygen, and exposure of oocytes and embryos to A3 resulted in a significant increase in day 3 embryo quality. However, an increase in both implantation and pregnancy rates following blastocyst transfer was restricted to patients aged 35-40 years (Gardner, et al., 2020). Therefore, indicating A3 may have a role in reducing oxidative stress associated with reproductive aging (Igarashi, et al., 2015). Together, data from both mouse and human studies illustrate the beneficial effects on embryos cultured in the presence of a combination of antioxidants.

Alterations to embryo culture conditions and medium composition have been shown to directly impact embryonic metabolic activity (Gardner, et al., 2017, Gardner, et al., 1993, Kelley, et al., 2016, Kelley, et al., 2019, Lane, et al., 1998, Lane, et al., 2005, Wale, et al., 2012). For example, the addition of a combination of amino acids, vitamins, insulin, epidermal growth factor and transferrin (AVIET) to mouse embryo culture media significantly altered both embryonic glucose uptake and lactate production compared to embryos cultured in the absence of AVIET (Gardner, et al., 1993). Additionally, the inclusion of vitamins and amino acids to mouse embryo culture media was found to regulate the relative activity of both oxidative and glycolytic metabolism (Lane, et al., 1998), which supported improved fetal development, similar to in vivo derived embryos, compared to embryos cultured in the absence of vitamins and amino acids. Furthermore, utilizing the same culture matrix as Truong and colleagues, blastocyst glucose uptake was found to be significantly reduced when embryos were cultured individually at 20% oxygen compared to 5% oxygen or group culture (Kelley, et al., 2019) indicating a metabolism response to oxidative and culture stresses. While, the addition of A3 to embryo culture

media has shown to improve embryo development and viability post-transfer, the direct impact of these antioxidants on embryo metabolism, when added to embryo culture medium both individually and in combination, has not been determined.

The relative activities of metabolic pathways have recently been shown to affect epigenetic landscape through their impact on the levels of key metabolites and co-factors involved in epigenetic regulation, a phenomenon referred to as metaboloepigenetics (Donohoe, et al., 2012, Gardner, et al., 2015, Harvey, et al., 2016). As such, metaboloepigenetics represents a mechanism linking embryo culture to Developmental Origins of Health and Disease (DOHaD) (Fleming, et al., 2015). Consequently, embryo metabolism is not only an important biomarker of embryonic viability (Ferrick, et al., 2020, Gardner, et al., 2011, Renard, et al., 1980) but also indirectly a biomarker of embryonic health and its analysis can help to elucidate changes in embryo development and transfer success (Ferrick, et al., 2019). Subsequently, the aim of this study was to investigate whether the benefits of ALC, NAC, ALA and A3 on embryo development is driven by a reduction in ROS and/or a direct regulation of blastocyst metabolism and therefore, to establish the importance of understanding how changes to embryo culture media may influence embryonic health.

Materials and methods

Mouse embryo collection

Embryos were obtained from 3 to 4 week old F1 (C57 x CBA) mice, housed in a 12 h light/dark photoperiod with food and water *ad libitum*. Females were superovulated via injection with 5 IU pregnant mare's serum gonadotrophin (PMSG; Folligon, Intervet, UK) followed by an injection of 5 IU human chorionic gonadotrophin (hCG; Chorulon, Intervet) 48 h later and mated with males of the same strain. The following morning presence of a vaginal plug was used as an indicator of mating and cervical dislocation is used to sacrifice females (Gardner, et al., 2014). Five to six mice were required for each biological replicate performed. All mouse experimentation was approved by the Faculty of Science Animal Ethics Committee, University of Melbourne (1914891.1).

Pronucleate oocytes were collected 20 h post-hCG injection in 500 µl handling medium (G-MOPS PLUS; Vitrolife AB, Sweden) pre-warmed to 37°C (Gardner, et al., 2014). Surrounding cumulus cells were denuded from oocytes following the addition of 500 µl pre-warmed hyaluronidase solution (300 IU/ml) (Bovine testes type IV, Sigma Aldrich, Australia). Denuded embryos were washed of hyaluronidase and debris three times in G-MOPS PLUS and once in G1 medium with HSA (5mg/ml) before culture (Gardner, et al., 2014, Gardner, et al., 2019). Embryos were pooled before allocation to account for any animal variation.

Mouse embryo culture

Mouse embryos were cultured individually in 2 µl of culture medium or in groups of 10 in 20 µl culture media in 60 mm petri dishes (Falcon, BD Biosciences, Australia) overlay with Ovoil (Vitrolife) in a humidified incubator (Sanyo MCOK-5M[RC], Japan) at 37°C, 6% CO_2, at 5% or 20% O_2. The first 48 h embryos were cultured in G1 medium with HSA (5mg/ml), followed by G2 medium with HSA (5mg/ml) for a further 48 h (Gardner, et al., 2014, Gardner, et al., 2019). Media was made in-house using formulas replicating commercial media. Treatment culture media (G1 and G2) were supplemented with 10 µM acetyl-*L*-carnitine (ALC), 10 µM *N*-acetyl-*L*-cysteine (NAC), 5µM α-lipoic acid (ALA), or A3 (10 µM ALC, 10 µM NAC, and 5µM ALA) (Truong, et al., 2016).

Experimental design

Impact of antioxidants in a high oxidative stress environment

Embryos were cultured individually in 2 µl of culture medium at 20% O_2. Treatment G1/G2 culture media was supplemented with individual antioxidants or A3.

Impact A3 in a defined culture matrix

Analysis of blastocyst total cell number and carbohydrate metabolism in the presence or absence of A3, was additionally conducted using a culture matrix. Embryos were cultured either individually in 2 µl culture medium or as groups of 10 in 20 µl culture medium under Ovoil with or without A3 and were exposed to either 5% or 20% oxygen creating four culture conditions (group, 5% oxygen; group, 20% oxygen; individual, 5% oxygen; individual, 20% oxygen).

Analysis of blastocyst cell number

Following carbohydrate analysis blastocysts were washed in G-MOPS PLUS handling media and incubated in G-MOPS PLUS with 0.1 mg/ml bisbenzimide (Hoechst 33258, Sigma Aldrich) and 10% ethanol for 20 mins at 37°C for nuclear staining. Stained blastocysts were individually mounted in 100% glycerol on microscope slides (SuperFrost Plus, Thermo Scientific, Germany) and imaged using a fluorescence microscopy (Nikon Eclipse TS100). Cell numbers were determined using FIJI ImageJ software (ImageJ 2.0.0, www.imagej.net).

Confocal microscopy

To quantify embryonic mitochondrial membrane potential (MMP), mitochondrial superoxide concentrations and GSH levels in blastocysts, blastocysts were stained with different fluorescent probes and analyzed using confocal microscopy. Following 96 h of culture embryos were washed in G2-MOPS handling medium and incubated in G2-MOPS supplemented with 1.5 µM JC-1 (MP30168, Invitrogen, USA) to determine embryonic MMP, 5 µM MitoSOX™ (M36008, Invitrogen) to determine mitochondrial superoxide concentrations, or 20 µM ThioTracker™ (T10095, Invitrogen) to measure GSH levels, for 15 mins at 37°C. Embryos were incubated for a further 15 mins with the same fluorescent probe combined with 10 µM DRAQ5 (62251, Thermo Scientific, Illinois, USA) nuclear stain. Embryos were then washed three times in G2-MOPS and transferred to 0.2 µl of G2-MOPS in 60 mm coverslip bottom dish (FluroDish, World Precision Instruments Ltd, USA) under Ovoil. Individual embryos were imaged using a confocal microscope (Nikon A1R, Nikon Instruments Inc., USA) and NIS Element software version 4.6. Stain intensities were quantified using Imaris software 8.4.1 (Oxford instruments, UK).

Analysis of intracellular hydrogen peroxide levels

Analysis of intracellular levels of H_2O_2 in blastocysts was achieved using peroxyfluor1 (PF1) (Centre for Nanoscale BioPhotonics, Australia). Embryos were cultured with or without antioxidant treatment for 96 h and were then incubated with 0.01 µM PF1 for 1 h under the same culture conditions (Truong, et al., 2017). Following PF1 incubation, embryos were transferred to 0.2 µl of G2-MOPS in 60 mm coverslip bottom imaging dish under Ovoil (Vitrolife). Fluorescent intensities were captured using inverted microscope with heated stage (Ti-U eclipse; Nikon Instruments) with a CoolSNAP HQ camera (Photometrics, USA) and NIS Elements BR 3.00, SP7 Laboratory Imaging software (Nikon Instruments) and quantified using FIJI ImageJ software (ImageJ 2.0.0).

Analysis of carbohydrate metabolism

Blastocysts cultured individually in 2 µl at 20% in the presence of ALC, NAC and ALA individually and blastocysts in the presence or absence of A3 as part of a culture matrix were transferred to 1 µl modified G2 culture media (mG2) (0.5 mM glucose, 0 mM lactate) in groups of four to five for a further 4 h following 96 h culture to quantitate blastocyst carbohydrate metabolism. Spent media samples (test) and no-embryo (control) media were collected and assayed using ultramicrofluoresence (Gardner, et al., 1986, Leese, et al., 1984) to determine glucose and lactate levels from which the glycolytic rate was calculated as previously described (Gardner, et al., 1990, Lane, et al., 1996).

Quantification of NADH Autofluorescence

NADH autofluorescence was quantified as a measure of the redox state of blastocysts cultured with or without antioxidant treatments after 96 h culture (Lane, et al., 2005). Individual blastocysts were transferred to 0.2 µl of G-MOPS in 60 mm coverslip bottom dish under Ovoil and imaging was achieved using inverted microscope with a heated stage (Ti-U eclipse; Nikon Instruments) with a CoolSNAP HQ camera (Photometrics) and NIS Elements BR 3.00 (Nikon Instruments). Each treatment group was transferred to imaging dish and imaged separately to reduce the level of light exposure before imaging. NADH fluorescent intensities were quantified for individual embryos using FIJI ImageJ software (ImageJ 2.0.0).

Statistical analysis

The mean total cell numbers, glucose consumption, lactate production and glycolytic rate for control and A3 were compared using Student's *t*-test. All other groups were subject to a one-way analysis of variance (ANOVA). All data is expressed as mean ± SEM and tested for normality prior to analysis using the Shapiro-Wilk test. Differences were considered statistically significant when *P* value was less than 0.05. All analyses were conducted using Prism 8 (version 8.3.0, GraphPad, LLC).

Results

Antioxidants increased blastocyst total cell number

An increase in blastocyst total cell number was observed in embryos cultured individually at 20% oxygen, in the presence of individual antioxidants, ALC (P <0.05), NAC (P <0.001) and ALA (P <0.01) (Table 6.1) and A3 (P <0.01) (Table 6.2). Additionally, embryos exhibited an increase in total blastocyst cell number when cultured at 20% oxygen in group culture (P <0.0001), and individually at 5% oxygen when exposed to A3 throughout culture (P <0.0001) (Table 6.3).

Table 6.1 Total cell numbers of blastocysts cultured with and without individual antioxidants.

	Control	Acetyl-*L*-carnitine (ALC)	*N*-acetyl-*L*-cysteine (NAC)	α-lipoic acid (ALA)
Total cell numbers	88.8 ± 3.2	99.7 ± 2.7*	106.2 ± 3.0***	102.5 ± 3.0**

Total cell number of blastocysts cultured individually at 20% oxygen. Control (n = 73), ALC (n = 73), NAC (n = 68) and ALA (n = 64) data expressed as mean ± SEM. Asterisks denotes level of significance, *P <0.05, **P <0.01, and ***P <0.001.

Table 6.2 Total cell numbers of blastocysts cultured with and without A3 in a defined culture matrix.

		Group culture, 5% oxygen	Group culture, 20% oxygen	Individual culture, 5% oxygen	Individual culture, 20% oxygen
Total cell numbers	*Control*	133 ± 3.3	84.4 ± 2.0	83.1 ± 2.4	80.0 ± 2.1
	A3	130 ± 3.1	98.4 ± 1.9****	100.8 ± 2.4****	90.1 ± 2.4**

Total cell number of embryos cultured at 5% oxygen in groups (n = 130) or individually (n = 179), and of embryos cultured at 20% oxygen in groups (n = 146) or individually (n = 161). Data expressed as mean ± SEM. Asterisks denotes level of significance between control and A3 groups, ***P* <0.01, and *****P* <0.0001.

Antioxidants reduce ROS when embryos are cultured in a high oxidative stress environment

To create an environment of high oxidative stress embryos were cultured individually at 20% oxygen and an analysis of mitochondrial superoxide found concentrations were significantly reduced in blastocysts cultured in the presence of ALC (P <0.05), NAC (P <0.0001) and A3 (P <0.0001) when compared to control (Fig 6.1A). However, no significant differences were observed when blastocysts were cultured in the presence of ALA. Additionally, when exposed to A3, mitochondrial superoxide levels were reduced by the greatest amount, 74%, compared to control (P <0.0001) (Fig 6.1A).

Hydrogen peroxide levels were significantly reduced when blastocysts, cultured individually at 20% oxygen, were cultured in the presence of ALA (P <0.01) and A3 (P <0.01) compared to control, and no impact was observed when blastocysts were cultured in the presence of ALC or NAC (Fig 6.1B). Furthermore, no statistical difference was found in GSH levels (arbitrary units) between blastocysts cultured in the presence of antioxidants, both individually (ALC, 21021 ± 2153, n = 43; NAC, 24186 ± 2384, n = 43; ALA, 20378 ± 2422, n = 42) and in combination (A3, 15295 ± 1687 , n = 42), when compared to control embryos (20811 ± 2056, n = 44).

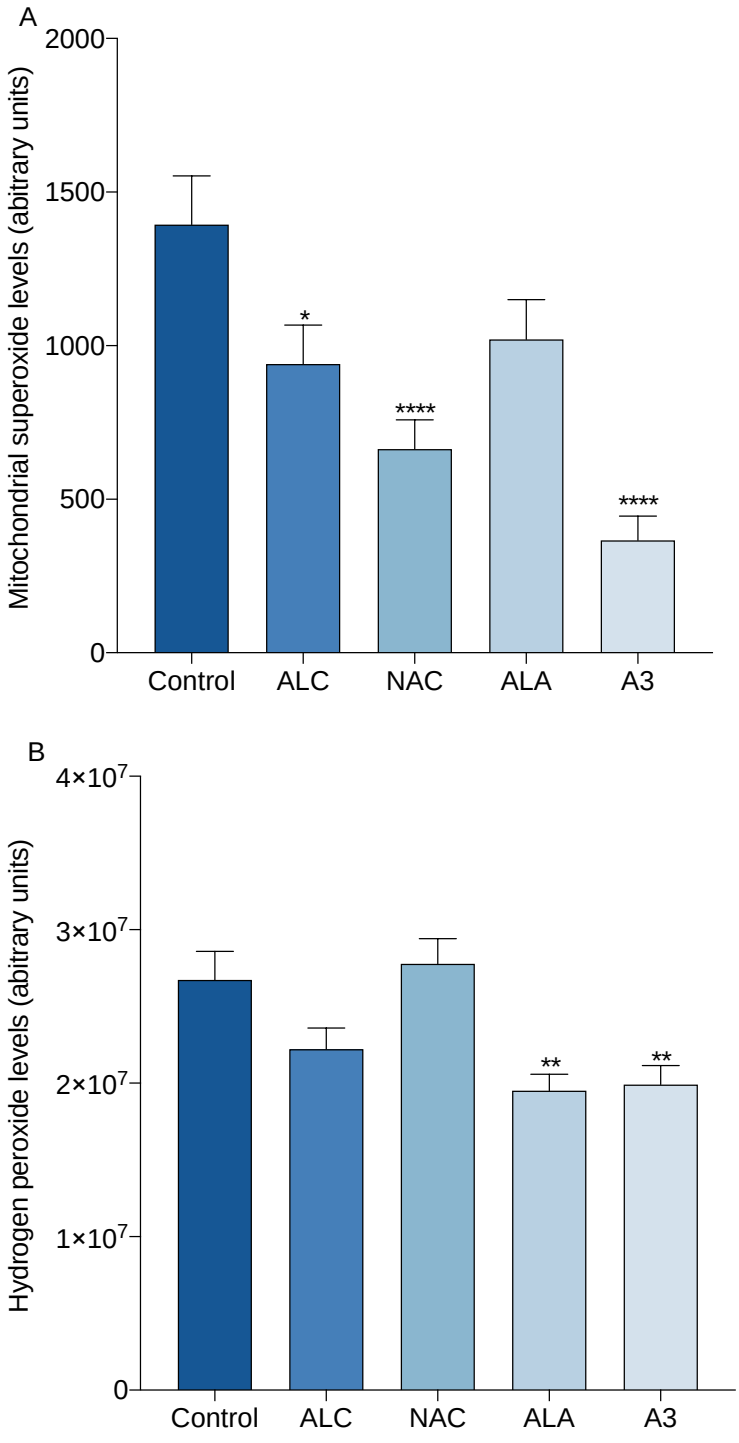
A
Mitochondrial superoxide levels (abitrary units)
2000
1500
1000
500
0
*
****
****
Control
ALC
NAC
ALA
A3
B
Hydrogen peroxide levels (abitrary units)
4×10^7
3×10^7
2×10^7
1×10^7
0
**
**
Control
ALC
NAC
ALA
A3

Figure 6.1 Addition of antioxidants to embryo culture medium and the impact on ROS levels in individual blastocysts.

A) Mitochondrial superoxide levels in embryos cultured in the absence of antioxidants (control, n = 37), or in the presence of 10 µM acetyl-*L*-carnitine (ALC) (n = 30), 10µM *N*-acetyl-*L*-cysteine (NAC) (n = 37), 5µM α-lipoic acid (ALA) (n = 29), or a combination of antioxidants (A3, n = 24). B) Hydrogen peroxide levels of embryos in the absence of antioxidants (control, n = 58), or in the presence of 10 µM acetyl-*L*-carnitine (n = 57), 10µM *N*-acetyl-*L*-cysteine (n = 58), 5µM α-lipoic acid (n = 53), or a combination of antioxidants (A3, n = 54). Blastocysts were cultured individually at 20% oxygen, data are expressed as mean ± SEM and asterisks denote significance from control, $*P < 0.05$, $**P < 0.01$, $****P < 0.0001$.

The addition of antioxidants to embryo culture medium affects blastocyst metabolism

Glucose consumption and lactate production were measured in blastocysts cultured in the presence of ALC, NAC, ALA and A3. A significant increase in glucose consumption compared to control embryos was observed in blastocysts cultured individually at 20% oxygen in the presence of NAC (P <0.05) and ALA (P <0.0001), however there was no impact when embryos were cultured in the presence of ALC (Fig 6.2A). Lactate production and glycolytic rate remained unaffected by the addition of individual antioxidants (Fig 6.2A, B). In contrast, the presence of A3 had no effect on glucose metabolism, however lactate production and glycolytic rate was significantly increased compared to control regardless of the culture conditions (Table 6.3). The greatest impact on carbohydrate metabolism was observed when embryos were cultured individually at 20% oxygen (P <0.001).

Mitochondrial membrane potential, as measured by JC-1 red:green fluorescence ratio, was not statistically different in embryos cultured individually at 20% in the presence of individual antioxidants (ALC, 0.7 ± 0.1, n = 17; NAC, 0.7 ± 0.1, n = 22; ALA, 0.8 ± 0.1, n = 13) or A3 (0.6 ± 0.1, n = 16) compared to control embryos (0.8 ± 0.1, n = 18).

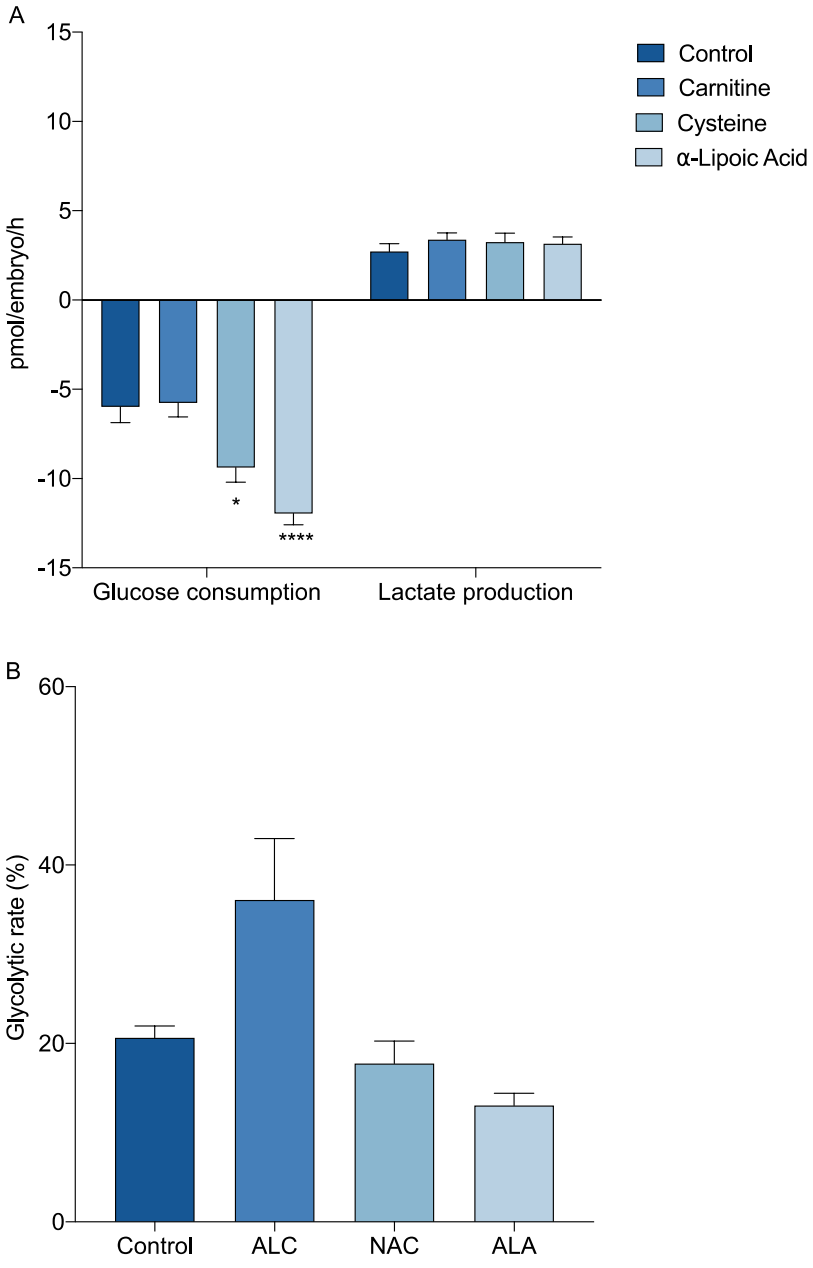
A
15
10
5
0
-5
-10
-15
pmol/embryo/h
Control
Carnitine
Cysteine
α-Lipoic Acid
*
****
Glucose consumption
Lactate production
B
60
40
20
0
Glycolytic rate (%)
Control
ALC
NAC
ALA

Figure 6.2 Addition of individual antioxidants to embryo culture medium and the impact on blastocyst carbohydrate metabolism.

A) Glucose consumption and lactate production of blastocysts cultured in the absence of antioxidants (control, n = 12) or in the presence of individual antioxidants (10 μM acetyl-*L*-carnitine (ALC), n = 17; 10μM *N*-acetyl-*L*-cysteine (NAC), n = 15; or 5μM α-lipoic acid (ALA), n = 17). B) Glycolytic rate of blastocysts cultured in the absence of antioxidants (control, n = 9) or in the presence of individual antioxidants (10 μM acetyl-*L*-carnitine (ALC), n = 15; 10μM *N*-acetyl-*L*-cysteine (NAC), n = 15; or 5μM α-lipoic acid (ALA), n = 17). n value represents the number of metabolic measurements. Blastocysts were cultured individually at 20% oxygen, data are expressed as mean ± SEM and asterisks denote significance from control, **P* <0.05, *****P* <0.0001.

Table 6.3 Carbohydrate metabolism of blastocysts cultured with and without A3 in a defined culture matrix.

		Group culture, 5% oxygen	Group culture, 20% oxygen	Individual culture, 5% oxygen	Individual culture, 20% oxygen
Glucose Uptake (pmol/embryo/h)	*Control*	15.2 ± 0.6	10.3 ± 0.9	14.7 ± 0.7	7.9 ± 0.4
	A3	14.7 ± 0.7	11.4 ± 0.6	14.3 ± 0.7	7.7 ± 0.5
Lactate Production (pmol/embryo/h)	*Control*	12.5 ± 1.7	6.8 ± 0.7	7.0 ± 0.9	4.0 ± 0.8
	A3	17.7 ± 1.1*	9.0 ± 0.5*	10.6 ± 1.2*	8.0 ± 0.7***
Glycolytic rate (%)	*Control*	41.1 ± 4.9	32.8 ± 3.2	23.5 ± 3.2	23.2 ± 5.1
	A3	62.7 ± 4.6**	42.7 ± 2.9*	40.2 ± 5.7*	51.6 ± 4.5***

Carbohydrate metabolism of embryos cultured at 5% oxygen in groups (n = 50) or individually (n = 50), and of embryos cultured at 20% oxygen in groups (n = 58) or individually (n = 52). n value represents the number of metabolic measurements. Data expressed as mean ± SEM. Asterisks denotes level of significance between control and A3 groups, *P <0.05, **P <0.01, and ***P <0.001.

The addition of A3 to culture medium alters the redox state of blastocysts

NADH autofluorescence did not significantly differ between blastocysts cultured individually at 20% oxygen in the presence of individual antioxidants (ALC, NAC or ALA). However, when cultured in the presence of A3, blastocysts exhibited a significant reduction in NADH levels compared to blastocysts cultured in the absence of antioxidants (P <0.01) (Fig 6.3).

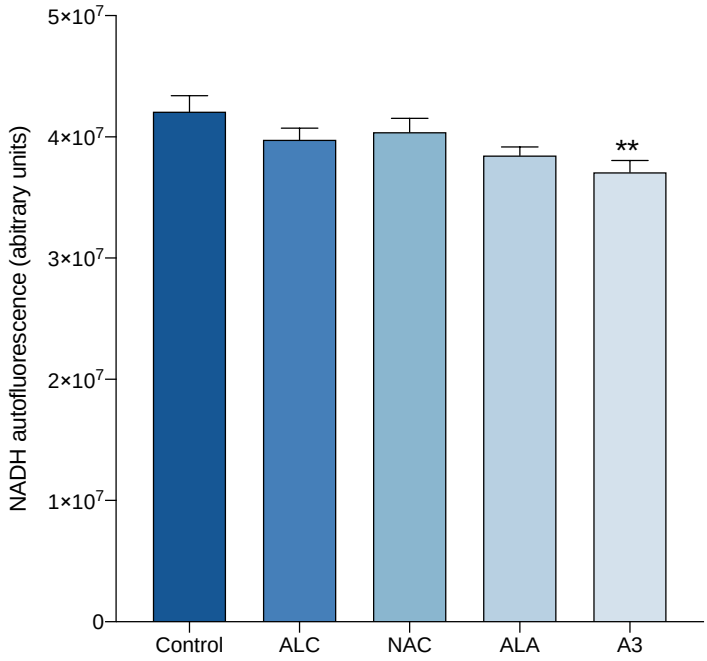

Figure 6.3 Addition of antioxidants to embryo culture medium and the impact on the redox status of individual blastocysts.

NADH autofluorescence of blastocysts cultured in the absence of antioxidants (control, n = 57), or in the presence of 10 µM acetyl-*L*-carnitine (n = 58), 10µM *N*-acetyl-*L*-cysteine (n = 59), 5µM α-lipoic acid (n = 50), or a combination of antioxidants (A3, n = 55). Blastocysts were cultured individually at 20% oxygen, data are expressed as mean ± SEM and asterisks denote significance from control, ***P* <0.01.

Discussion

The addition of ALC, NAC and ALA to embryo culture medium in addition to reducing ROS, regulates the metabolic profile of blastocysts, subsequently resulting in improved blastocyst development. Of note, the impact on blastocyst metabolic profile was dependent on whether antioxidants were added to embryo culture media individually or in combination. Individually, NAC and ALA increased glucose consumption and in combination, A3 significantly increased lactate production and subsequent glycolytic rate.

A significant increase in blastocyst total cell number was observed in embryos when cultured in the presence of ALC, NAC, ALA and A3. Using a culture matrix, A3 was found to significantly improve blastocyst development under all culture conditions, with the exception of embryos cultured in groups at 5% oxygen. Similarly, Truong and colleagues reported an increase in both trophectoderm (TE) and inner cell mass (ICM) cell numbers of mouse blastocysts when cultured in the presence of A3 (Truong, et al., 2016). However, while the impact of A3 was lowest when embryos were cultured in groups at 5% oxygen, an increase in blastocyst cell number was observed (Truong, et al., 2016). The difference between these studies is plausibly due to the timing of nuclear staining. Truong and colleagues conducted blastocyst nuclear staining in the morning of day 5 whereas in the present study this was performed 6-8 h later which is adequate time for control blastocysts to catch up (Ciemerych, et al., 2005). Together these data indicate A3 is able to improve the development of mouse embryos, in particular, those that are exposed to high levels of oxidative and culture stress.

Truong and colleagues demonstrated a significant reduction in H_2O_2 levels in pronucleate mouse embryos when exposed to A3 for 4 h (Truong, et al., 2017) suggesting the improvements in embryo development may be a result of reduction in oxidative stress. However, they did not analyze oxidative stress at the blastocyst stage. Supplementation of ALC, NAC, and ALA individually to culture media were found to reduce the levels of mitochondrial superoxide or H_2O_2, at the blastocyst stage when embryos cultured in a high oxidative stress environment. Individually, both ALC and NAC reduced the levels of mitochondrial superoxide suggesting they are capable of alleviating mitochondrial dysfunction through the reduction of oxidative stress. Indeed, the addition of NAC to human hepatocytes, treated with D-galactosamine to induced ROS, was found to significantly reduce ROS, support the recovery of mitochondrial complexes activities and subsequently increase cellular ATP production (González, et al., 2009). An increase in glucose uptake was observed in blastocysts that were cultured in the presence of NAC and aligns with an increase in ATP production as the conversion of glucose, the predominantly carbohydrate at the blastocyst stage, to endogenous pyruvate facilitates mitochondrial metabolism. These results therefore suggest NAC, through the reduction of ROS, may indirectly support mitochondrial function.

In align with the role of cysteine in GSH production, the addition of cysteine to culture media has been shown to increase intracellular GSH production and prevent a decrease during proliferation in lymphocytes (Zmuda, et al., 1983). Conversely, no significant difference in GSH levels were observed in mouse embryos cultured in the presence of NAC or any other antioxidant. However, GSH analysis measured the reduced state of GSH and did not account for oxidized GSH and therefore the total GSH present. Through the

activity of glutathione peroxidase (GXP), GSH can sequester H_2O_2 (Anderson, et al., 1983, De Matos, et al., 1995). However, Pigeolet and colleagues demonstrated GXP activity is sensitive to the concentrations of its own substrates, where an increase in H_2O_2 can inactivate GXP (Pigeolet, et al., 1990). As embryos were cultured in a high oxidative stress environment (20% individual culture), it is plausible GXP activity may have been affected by elevated H_2O_2 and may help explain why NAC had no impact on H_2O_2 concentrations.

The reduction in mitochondrial superoxide in the presence of ALC aligns with previous reports that supplementation of l-carnitine to aged bovine oocyte IVM media significantly reduced intracellular ROS levels (Jiang, et al., 2020), although, Jiang and colleagues did not delineate between mitochondrial and intracellular ROS. Here the data presented suggest that ALC is specifically targeted to mitochondrial oxidative damage. In support of this, mitochondria function was significantly improved in 3T3-L1 adipocytes when cultured in the presence of a combination of ALC and ALA (Shen, et al., 2008) and suggests through the reduction of mitochondrial ROS, ALC is capable of indirectly improving mitochondrial function. In contrast, ALA did not significantly reduce mitochondrial superoxide, however, as mitochondrial metabolism can result in the production of superoxide (Murphy, 2009) these results may indicate an increase in oxidative phosphorylation (OXPHOS). Indeed, ALA is a cofactor of the PDH complex which facilitate the decarboxylation of pyruvate to acetyl-CoA for oxidative metabolism. An increase in glucose consumption and no change in lactate production observed in the presence of ALA may suggest an increase in the conversion of endogenous pyruvate and further supports an increase in oxidative metabolism. In support of this, previous reports have shown mice deficient in lipoic acid synthetase led to impairment of glucose oxidative

metabolism via the mitochondria (Yi, et al., 2005). Therefore, inferring ALA is capable of directly increasing mitochondrial activity while concomitantly reducing intracellular H_2O_2 levels.

Together these data suggest an improvement in embryo developmental competence in the presence of ALC, NAC or ALA individually may be due to their antioxidant defense mechanism thereby reducing ROS and supporting mitochondrial function, and/or directly regulating oxidative metabolism. Of note, a significant difference in MMP was not observed despite evidence suggesting antioxidants regulated mitochondrial activity and function. As analysis of MMP provides a qualitative assessment of mitochondrial activity, a quantitative assessment of oxygen consumption (Hashimoto, et al., 2017, Yamanaka, et al., 2011) of blastocysts cultured in the presence of ALC, NAC and ALA, at both 5% and 20% oxygen would further elucidate the relationship between antioxidants and mitochondrial function.

The addition of A3 to embryo culture media significantly reduced both mitochondrial superoxide and H_2O_2, subsequently providing evidence for the synergistic effects of antioxidants. These results support the inclusion of A3 to embryo culture medium to protect against the damaging effects of ROS accumulation associated with in vitro culture. Of significance, culturing human oocytes and resultant embryos in the presence of A3 was found to significantly increase the quality of day 3 blastocysts, however, an increase in implantation and pregnancy rates were only observed in patients aged 35-40 years (Gardner, et al., 2020). Aging in oocytes is largely due to mitochondrial dysfunction and high levels of oxidative stress. Aged oocytes exhibit an elevation in ROS (Igarashi, et

al., 2015, Sasaki, et al., 2019) and further a loss in antioxidant defense mechanisms (Tatone, et al., 2008). It is the exposure to such oxidative stress that may be responsible for aberrations in oocyte chromosome segregation and genetic stability (Desler, et al., 2007, Eichenlaub-Ritter, et al., 2004, Schon, et al., 2000) resulting in high levels of aneuploidy (Franasiak, et al., 2014) and subsequent low implantation rates (Bartmann, et al., 2004) in older patients. Subsequently, the inclusion of A3 to embryo culture media may assist in alleviating endogenous oxidative stress associated with aging and an analysis of the beneficial effects of antioxidants based on maternal age warrants further investigation.

While A3 observed the greatest reduction in ROS overall, these levels were not eliminated. In various cell types, physiological levels of ROS has been recognized to have important signaling roles involved in regulating the activity of kinases and phosphatases, transcription and cellular proliferation (Diebold, et al., 2016, Hamanaka, et al., 2010, Harvey, 2007, Lees, et al., 2017). For example, H_2O_2 is capable of targeting cysteine residues on proteins, modifying protein activity which alters signal transduction (D'Autréaux, et al., 2007). Therefore, the addition of A3 to embryo culture media in addition to reducing the accumulation of ROS, plausibly also maintains ROS signaling capacity.

Metabolic analyses revealed, unlike NAC and ALA, A3 did not alter the rate of glucose consumption, rather increased lactate production which led to an increase in the overall glycolytic rate. As proposed by the 'Rate and Fate' hypothesis, not only is the amount of nutrient utilization (rate) important, but also the metabolic pathways and fate of the

metabolite (Gardner, et al., 2013). Subsequently, the increase in glycolytic rate and further, the reduction in mitochondrial superoxide levels by A3 indicating low oxidative metabolism, suggests an alteration in the fate of glucose in the presence of A3. The glycolytic rate of in vivo-derived blastocysts is ~50% where there is a balance between OXPHOS, supporting exponential cellular growth and maintenance of the blastocoel, and lactate production to support development during implantation (Gardner, et al., 1990). The glycolytic rate of control blastocysts in the present study was 20-40% and this increased in the presence of A3 to 40-60%, depending on the oxygen concentration and embryo density and therefore, suggests an increase in glycolytic rate supports higher viability. Subsequently, the data presented in this study suggest that while individual antioxidants were able to reduce ROS and subsequently improve mitochondrial function, A3 had the greatest impact in reducing ROS and also supports a more in vivo-like metabolism. Furthermore, while the impact of A3 on carbohydrate metabolism was greatest in the presence of both 20% oxygen and individual culture, A3 also significantly increased the glycolytic rate of embryos cultured at 5% oxygen and/or in groups. Therefore, these results support the addition of A3 to embryo culture regardless of the culture conditions.

The glycolytic rate can influence the redox state of cells i.e. the equilibrium between NADH and NAD^+ (Gardner, et al., 2000, Harvey, et al., 2002), and a decrease in NADH levels is consistent with an increase in glycolytic rate. Indeed, a reduction in NADH autofluorescence was observed when blastocysts were cultured in the presence of A3 and further supports that ability of A3 to alter the fate of glucose consumed by the blastocyst. Concomitantly, the inclusion of A3 to embryo culture media has the potential

to influence NAD^+ availability. Not only is NAD^+ a key regulator of several metabolic pathways including, glycolysis, TCA cycle, oxidative phosphorylation and malate-aspartate shuttle (Gardner, et al., 2015, Lane, et al., 2005), but NAD^+ is a cofactor of sirtuins, an important epigenetic modulator which can subsequently alter gene expression (Donohoe, et al., 2012, Gardner, et al., 2015, Harvey, et al., 2016). Sirtuins are NAD^+-dependent deacetylases which through the remodeling of histones can subsequently influence the gene expression profile of developing embryos (Tatone, et al., 2018). A previous analysis of the acetylation levels of vitrified blastocysts revealed the addition of A3 to mouse vitrification media influenced embryonic H3K9ac levels at the blastocyst stage, indicating a metaboloepigenetic response to A3 (Truong, et al., 2020). Additionally, lactate has recently been recognized to have implications in epigenetic remodeling through lactylation which is a modification of histone lysine residues that stimulates gene expression (Zhang, et al., 2019) and subsequently, provides an additional metaboloepigenetic interaction which A3 has the potential to regulate.

In summary, the inclusion of ALC, NAC and ALA individually to embryo culture media alleviated ROS accumulation associated with in vitro culture. These data suggest these antioxidants may directly or indirectly regulate mitochondrial function and support improved embryonic developmental competence. Further, these antioxidants in combination reduced the accumulation of ROS by the greatest magnitude while plausibly maintaining ROS signaling capacity and supported a more in vivo-like metabolism. However, to fully elucidate the impact that A3 on the preimplantation embryonic health, gene expression analyses and longitudinal studies analyzing the long-term health of offspring is warranted, prior to these antioxidants being adopted in routine clinical

embryo culture systems. Of further significance, these data highlight the importance of considering the physiological changes that may occur to the developing embryo when altering embryo culture conditions in particular media compositions. Finally, it is not amiss that a physiological combination of an array of other antioxidants in addition to A3, such as the inclusion of melatonin (Manjunatha, et al., 2009, Rodriguez-Osorio, et al., 2007), ascorbate (Lane, et al., 2002), β-mercaptoethanol (Feugang, et al., 2004) and coenzyme Q10 (Akarsu, et al., 2017, Liang, et al., 2017), may provide a means of further improving clinical success rates.

Chapter 7. General Discussion

As single blastocyst transfer has become routine practice in human IVF clinics worldwide, the success of an IVF cycle and the ability to reduce time to pregnancy is dependent on the selection of the most competent embryo for transfer. Traditional morphological grading systems are central tools in assessing embryo viability, as well as morphokinetic algorithms, however, they do not provide a direct measure of embryo physiology. This is important as embryo physiology is a known regulator of embryo development and subsequent embryonic health. The studies presented in this thesis confirm that human blastocyst carbohydrate and amino acid metabolism are valuable biomarkers of embryo viability, and potentially the health of the child conceived. Together with current embryo selection tools, these biomarkers have the potential to improve embryo selection for both fresh and frozen transfers, identify blastocysts at risk of aneuploidy and assist in validating advances in embryo culture media.

The metabolism of human blastocysts cultured at physiological (5%) oxygen was analyzed (chapter 3), with the aim to determine the relationship between blastocyst glucose and amino acid utilization. Blastocysts with high viability, as determined by Gardner grade, KIDScore and EmbryoScore, were found to consume significantly more glucose than lower quality blastocysts, indicating the dynamic development of the blastocyst relies on high levels of glucose uptake. These results are consistent with previous work in human blastocysts (Gardner, et al., 2001), whereby a higher glucose uptake was predictive of blastocysts of high quality. Subsequently, as the predominant carbohydrate consumed during blastocyst development (Gardner, et al., 1988), an increase in glucose uptake may indicate a higher capacity to support the energy and biosynthetic requirements associated with blastocyst formation and expansion.

Implantation is a highly coordinated and complex series of events which includes the timely expansion of blastocysts, an energetically demanding process, that facilitates thinning of the zona pellucida (ZP) (Cole, 1967, Leonavicius, et al., 2018) and subsequent hatching of the trophectoderm (TE) cells in preparation for invasion of the endometrium. Additionally, lactate produced by the blastocyst through aerobic glycolysis facilitates the establishment of unique microenvironment which may mediate the maternal-fetal dialogue essential for successful implantation (Gardner, 2015). Of note, both blastocyst expansion and lactate production are regulated by glucose consumption and in support of this, results from chapter 3 revealed human blastocysts which expanded in 13 h or less were found to consume more glucose than slower expanding blastocysts. Further, human blastocysts that were transferred in a fresh single blastocyst transfer (SBT) cycle and established a viable pregnancy were found to consume more glucose than blastocysts that failed to implant post-transfer and were consistent with previous findings in human blastocysts (Gardner, et al., 2011). Therefore, indicating that glucose uptake is important in regulating implantation and may provide a measure of the physiological capacity of blastocysts to succeed post-transfer.

In align with previous work in human blastocysts (Gardner, et al., 2001), results from chapter 3 also revealed top quality blastocysts, according to current methods of embryo selection, exhibited a range of glucose uptakes. These findings are of relevance to human IVF as the selection of a single blastocyst for transfer among a number of morphologically similar blastocysts is currently a challenging task and can significantly impact the ability to reduce the time to pregnancy. The combination of glucose uptake as a biomarker of

embryo viability and current embryo selection tools could help to distinguish between these top quality blastocysts.

Current scientific literature on human embryo amino acid utilization and embryo quality is limited. Utilizing donor human embryos of presumable low quality, a previous study found day 2-3 amino acid turnover (the sum of consumption and production) was predictive of blastocyst development (Houghton, et al., 2002). A follow up study identified day 1-2 amino acid turnover was predictive of development following fresh human cleavage stage embryo transfers (Brison, et al., 2004). While there is some evidence that suggests the human embryo is transcriptionally active from the 1-cell stage (Xue, et al., 2013), the first major wave of embryonic genome activation (EGA) begins at 4-8 cell stage and the second wave of transcription occurs around the time of compaction (Braude, et al., 1988, Xue, et al., 2013). Consequently, current scientific literature of human embryo amino acid utilization has largely focused on oocyte quality. In contrast, the analysis of individual amino acid utilization of human blastocysts was investigated in chapter 3 and provides a detailed analysis of embryonic quality.

Overall amino acid utilization profiles revealed high quality human blastocysts consumed high levels of amino acids, while lower quality blastocysts were characterized by a higher level of amino acid production. These results suggest, based on embryonic quality, blastocysts exhibit distinct physiologies. Due to experimental design the analyses conducted were not able to provide a real-time analysis of the kinetics and flux of amino acid metabolism, however, the net consumption or production of specific amino acids during the blastocyst stage were found to be associated with metabolic pathways that

are fundamental for viable blastocyst development. Of particular interest is aspartate, the rate limiting factor in the malate-aspartate shuttle (MAS), and hence a key regulator of both mitochondrial and cytosolic metabolism (Lane, et al., 2005). Higher quality blastocysts, and therefore those predicted to have greater success post-transfer, were found to consume more aspartate than lower quality blastocysts. These results are consistent with a study conducted in the mouse which found inhibition of MAS resulted in lower inner cell mass (ICM) and TE cell numbers, and consequently reduced both implantation rates and fetal development (Mitchell, et al., 2009). Therefore, these data infer that an increase in MAS activity, represented by an increase in aspartate consumption, provides an indication of the blastocysts ability to maintain the mitochondrial and cytosolic metabolic balance necessary to support blastocyst development and success post-transfer.

Blastocysts of high quality were also found to consume significantly higher levels of both arginine and leucine compared to lower quality blastocysts. Arginine and leucine have a role in mediating the mammalian target of rapamycin (mTOR) pathway (González, et al., 2012) and subsequently the maintenance of cellular homeostasis. Specifically, studies in the mouse found the activation of mTORC1 protein complex is essential for implantation (Gangloff, et al., 2004, Guertin, et al., 2006) and both arginine and leucine were subsequently shown to activate mTORC1 to promote TE motility, a requirement for TE invasion of the endometrium during implantation (González, et al., 2012, Martin, et al., 2003). Therefore, results indicate that the consumption of arginine and leucine may also provide a measure of blastocyst physiological capacity to succeed post-transfer. The data presented subsequently identified specific amino acid biomarkers that could be used in

conjunction with glucose uptake and other embryo selection tools to aid in the selection of the most competent embryo for transfer. These amino acid biomarkers were not limited to those discussed above, in addition, alanine, asparagine, glutamine, glutamate, histidine, isoleucine, lysine, methionine phenylalanine, proline, serine, taurine, threonine, tryptophan and tyrosine were also found to be utilized differently by higher quality blastocysts. These results provide a substantial contribution to the knowledge of human blastocyst amino acid utilization.

Currently, amino acid composition in embryo culture medium is largely based on Eagle's minimal essential media for mammalian cell culture (Eagle, 1959). Importantly, the concentration of metabolites in the surrounding environment can affect the kinetics of metabolite consumption. For example, it has been shown an increase in glucose concentration in embryo culture media increases its uptake by mouse blastocysts (Gardner, et al., 1988). The amino acid utilization analyses performed in this thesis represent a potential means of investigating and optimizing the amino acid composition in embryo culture media to promote viable physiology and therefore may improve embryo development in vitro. While the technologies utilized in this thesis to measure both carbohydrate and amino acid utilization are not feasibly translatable to a clinical setting, carbohydrate enzymatic assays and the development of specific amino acid assays could be optimized for bench top assay technologies (Kelley, et al., 2019, Leary, et al., 2020). These technologies have the potential to provide an analysis of blastocyst metabolism within a matter of minutes within the IVF lab and provide an opportunity for metabolic biomarkers to be readily introduced to a clinical setting to aid in the selection of the most competent blastocyst.

As proposed by the Rate and Fate Hypothesis, not only is the amount of metabolite consumed important (rate), but so is the fate, or how that metabolite is utilized within the cell (Gardner, et al., 2013). This is of particular importance given the recent establishment of a relationship between metabolites and the epigenome, known as metaboloepigenetics (Donohoe, et al., 2012, Gardner, et al., 2015), whereby metabolites and their cofactors, such as lactate and NAD^+, have the ability to modify the epigenetic landscape and subsequent developmental programming of the embryo. These alterations have been shown to have significant consequences for fetal development and the early onset of adult diseases (Feuer, et al., 2016). Consistent with the dynamic development during the preimplantation period, the preimplantation embryo exhibits a level of plasticity, whereby in response to exogenous and endogenous stresses the embryo is capable of fine-tuning metabolic processes to ensure viable development (Feuer, et al., 2012). Consequently, due to these alteration in embryo metabolism, epigenetic modifications can plausibly occur without compromising blastocyst development and therefore go undiagnosed by current embryo selection methods. Therefore, a combination of morphological, morphokinetic and metabolic analyses conducted prior to transfer and may not only provide a detailed analysis of embryo viability, but plausibly the potential health of the resulting offspring.

Given alterations to karyotypes as a result of aneuploidy can lead to perturbations in gene expression and subsequently compromise cellular physiology (Zhu, et al., 2018), the morphological, morphokinetic and metabolic biomarkers established in chapter 3 were investigated in chapter 4 to establish whether these biomarkers could be used to non-invasively identify perturbations in blastocysts caused by subsequent aneuploid stress,

prior to preimplantation genetic testing (PGT). Consistent with reports in human blastocysts (Campbell, et al., 2013, Campbell, et al., 2014, Desai, et al., 2018, Huang, et al., 2019, Kaing, et al., 2018, Nogales, et al., 2017, Patel, et al., 2016), aberrations in embryonic morphokinetics were observed in aneuploid blastocysts. Specifically, a delay in the time of hatching and a significantly slower rate of blastocyst expansion was observed in aneuploid blastocysts. Given blastocyst expansion facilitates hatching of the TE cells from the ZP (Cole, 1967, Leonavicius, et al., 2018), the delay in the time to hatching may have been a consequence of the delay in blastocyst expansion. Results from chapter 3 infer this delay may be due to aberrations in glucose metabolism and subsequently the energy requirements necessary for viable blastocyst development. However, due to low sample size, no significant difference in blastocyst metabolism was observed between aneuploid and euploid blastocysts. Subsequently, to fully understand whether these developmental delays are a direct result of perturbations in embryo metabolism, a follow up larger cohort study is warranted.

Aberrations in embryo development, as a result of aneuploidy, did however result in significantly lower KIDScores and EmbryoScores. Therefore, these current biomarkers of embryo viability might be used to identify blastocysts at risk of aneuploid prior to PGT and would be of considerable value to clinics which currently do not have access to PGT technologies. Of further significance, EmbryoScores generated via an artificial intelligence (AI) program appeared to be more closely associated with embryonic ploidy status and blastocyst metabolic profiles, compared to KIDScores and morphological grades. While morphological grading and KIDScores are based on preset parameters, the AI program was trained to predict embryo viability with no preset assumptions using deep

learning and neural networks. In future, there is potential for AI to be trained to identify characteristics associated with embryonic ploidy status and viability. Additionally, AI eliminates the level of subjectivity that existed between embryologists when annotating preset parameters (Storr, et al., 2017) and requires validation in robust randomized clinical trials. In conclusion, the data presented in this thesis supports the incorporation of AI to embryo selection methods in human IVF clinics.

Consistent with the concept of aneuploid stress, a previous report in human embryos found embryo cleavage stage kinetics were significantly delayed in complex aneuploidy embryos when compared to single aneuploidy (Nogales, et al., 2017). Further, euploid embryos were found to be two times more likely to undergo blastulation than aneuploid blastocysts and the blastulation rates declined in a linear manner as the number of aneuploidies increased (Vega, et al., 2014). Here it was determined that blastocysts with a single chromosome deletion or duplication were found to have altered amino acid metabolism in comparison to blastocysts with complex aneuploidies. Specifically, asparagine, lysine, phenylalanine, serine, tryptophan and tyrosine were utilized differently by blastocysts with complex aneuploidies. These results provide further evidence for the presence of aneuploid stress in human blastocysts and a possible mechanism responsible for perturbations in development of embryos with complex aneuploidy. An analysis of blastocyst metabolism in a larger study is required to confirm whether specific chromosomal errors and subsequent metabolic phenotypes are reflected in specific metabolic profiles.

While results from chapter 4 revealed an increase in aneuploidy rates with maternal age, blastocyst glucose metabolism did not appear to be correlated with maternal age. This is of particular importance as some younger patients undergoing IVF treatment can experience high levels of aneuploidy (Baart, et al., 2006). As PGT is both invasive and expensive, younger patients often experience recurrent pregnancy losses before a diagnosis is established and PGT is recommended. Results from both chapters 3 and 4 suggest a combination of morphological, morphokinetic and metabolic biomarkers has the potential to assess not only blastocyst viability, but also the level of aneuploid stress and embryonic health for women of all ages. Of note, the utilization of a number of specific amino acids appeared to be correlated with maternal age and should be confirmed in a larger cohort study. Furthermore, as female fertility significantly declines from 35 years (Baird, et al., 2005, van Noord-Zaadstra, et al., 1991) and perturbations in embryo development seem to be of higher frequency in older females (Janny, et al., 1996), specific biomarker thresholds according to maternal age may be worth investigating.

Consistent with previous analyses in vitrified human blastocysts (Coello, et al., 2017, Du, et al., 2016, Yin, et al., 2016), results from chapter 5 identified vitrified human blastocysts that re-expanded to a greater degree following warming were found to be associated with higher live birth rates. Consequently, morphological assessment of blastocysts, in addition to an analysis of embryo viability at the end of culture, could be utilized post-warm to assess whether or not the stresses associated with vitrification and warming have compromised embryo development and its potential success post-transfer. These results align with data presented in chapter 4 which suggest blastocysts that were slower

to expand post-warm may consequently experience a delay in hatching following transfer to the uterus. As implantation is a highly dynamic and coordinated event between both embryo and endometrium (Bhusane, et al., 2016, Salamonsen, et al., 2016), it is plausible that this delay in development may result in the blastocyst missing the window of endometrium receptivity compromising implantation (Lessey, 2000). However, as no significant difference was observed in the mouse blastocyst re-expansion grades and outgrowth area, this indicated the grading system was not effective as an assessment of mouse embryo implantation potential.

An analysis of pyruvate uptake in mouse and human blastocysts post-warm did not reveal a significant difference based on the degree of blastocyst re-expansion or live birth rates following frozen embryo transfers (FET). This was in contrast to slow-frozen bovine blastocysts, whereby blastocysts that re-expanded post-thaw consumed higher levels of pyruvate (Gardner, et al., 1996). It is possible that differences in results may be due to the additional stresses that are associated with slow-freezing (Nagy, et al., 2017). Furthermore, possibly due to limited sensitivity in ultramicrofluorescent assays, glucose uptake was not significantly different in vitrified mouse blastocysts based on re-expansion and viability. Therefore, together with pyruvate uptake indicates metabolic analyses of vitrified blastocysts post-warm does not have the same predictive value as analysis during blastocyst development. However, while glucose uptake could not be measured in human blastocyst due to high concentrations in commercial embryo culture medium, glucose is the predominant carbohydrate consumed at the blastocyst stage. Furthermore, given the clear correlation between glucose uptake and expansion in

human blastocysts observed in chapter 3, an analysis of glucose uptake of human blastocysts post-warm warrants further investigation.

An abundance of endogenous pyruvate and possible competition for transport into the cell may help to explain why exogenous pyruvate uptake did not significantly differ between vitrified blastocysts of varying qualities. It is plausible, post-warm the blastocyst may favor the conversion of exogenous glucose to pyruvate to support ATP production facilitating re-expansion. Additionally, the conversion of ~50% of glucose to lactate in preparation for implantation may affect the movement of exogenous pyruvate into the cell due to competition for monocarboxylate transporters (MCT) on the plasma membrane (Lane, et al., 2000). The data presented in chapter 5 suggests an analysis of blastocyst re-expansion and plausibly glucose uptake may provide a better understanding of the probability individual vitrified human blastocysts have at succeeding post-transfer. Therefore, could assist in the decision-making process of whether, due to low viability and health, a subsequent blastocyst should be warmed. Further a better understanding of the probability a blastocyst will succeed post-transfer would assist in patient counselling and the management of patient expectations.

The results from this thesis indicate, the metabolic profile characteristic of a viable, healthy blastocyst includes a high glucose uptake and high levels of specific amino acid consumption and consequently does not align with the Quiet hypothesis (Leese, 2002). The Quiet hypothesis states, the most viable preimplantation embryo has the lowest overall metabolism, as a high metabolic activity represents energy expenditure to repair cellular damage (Leese, et al., 2007). Of significant importance, previous analyses of

human amino acid metabolism (Brison, et al., 2004, Houghton, et al., 2002) and studies investigating carbohydrate metabolism in mammalian species (Bowman, et al., 1970, Lane, et al., 1996, Leese, 1991, Turner, et al., 1994), that lay the foundation for the Quiet Hypothesis, were conducted at 20% oxygen and this may explain the conflicting metabolic profiles of viable blastocysts. Arguably, the Quiet Hypothesis consequently describes the metabolic response of preimplantation embryos to a high level of oxidative stress. In contrast, the data presented in this thesis provides a comprehensive understanding of normal embryonic cellular function.

Extensive analyses have been performed using a mouse model investigating the inclusion of a combination of antioxidants (A3) (acetyl-*L*-carnitine (ALC), *N*-acetyl-*L*-cysteine (NAC) and α-lipoic acid (ALA)) to embryo culture, IVF and vitrification media to reduce oxidative stress associated with in vitro culture and has resulted in improve embryo and fetal development (Truong, et al., 2017, Truong, et al., 2020, Truong, et al., 2016). Further, exposing human oocytes and embryos to these antioxidants resulted in improved day 3 embryo quality and increase implantation and pregnancy rates for patients aged 35-40 years, but not patients younger than 35 (Gardner, et al., 2020). Using a mouse model, an analysis of reactive oxygen species (ROS) and blastocyst metabolism was conducted (chapter 6) to determine whether improvements in embryo development and implantation potential in the presence of A3 was due to a reduction in ROS and/or regulation of metabolism. A reduction in intracellular hydrogen peroxide was observed in the presence of ALA and A3, and a reduction in mitochondrial superoxide was found in the presence of ALC, NAC and A3. Further, ROS levels were reduced to the greatest extent by A3 when compared to individual antioxidants therefore demonstrating a synergistic

effect. These results suggest that the addition of antioxidants to embryo culture media plausibly improves embryo developmental competence as a result of a reduction in oxidative stress. Furthermore, A3 did not eliminate ROS, therefore suggesting its signaling capacity required for viable development may be maintained (Diebold, et al., 2016, Hamanaka, et al., 2010, Harvey, 2007, Lees, et al., 2017).

The reduction in mitochondrial superoxide may suggest an improvement in mitochondrial function which is of particular importance in human IVF for patients of advanced maternal age. Central to reproductive aging is mitochondrial dysfunction and high levels of oxidative stress which may result in high aneuploidy rates in embryos (Duncan, et al., 2012, Franasiak, et al., 2014) and possibly aberrations in metabolic function due to subsequent aneuploid stress as observed in chapter 4. Indeed, a previous study culturing oocytes and embryos from aged mice (13.5 months) in the presence of a combination of antioxidants (ALA, α-tocopherol, hypotaurine, NAC, and sirtuin) exhibited improved blastocyst development and also mitochondrial function (Silva, et al., 2015). Therefore, together with the data presented in this study, may help to explain why only patients aged 35-40 years observed a significant increase in both implantation and pregnancy rates as compared to younger patients (Gardner, et al., 2020).

An analysis of blastocyst carbohydrate metabolism revealed a dynamic response to the inclusion of antioxidants, whereby individually, NAC and ALA increased glucose uptake compared to embryos cultured in the absence of antioxidants and was consistent with improved viability. Conversely, A3 had no impact on the rate of blastocyst glucose uptake, however, altered the metabolic fate of the glucose. A3 increased lactate production in

blastocysts and subsequently resulting in an elevation of glycolytic rate to ~50%, which is similar to previous reports of in vivo derived blastocysts (Gardner, et al., 1990). A glycolytic rate of 50% presumably ensures a viable balance between oxidative phosphorylation maintaining energy requirements and appropriate lactate production necessary to facilitate implantation (Gardner, 2015). The data from chapter 6 subsequently provides an example of how alterations to embryo culture media can influence the metabolic profile of blastocysts and concomitantly how an analysis of blastocyst metabolic profiles can help validate advances in culture systems. These data suggest A3 has the capacity to reduce ROS and regulate blastocyst metabolism to support viable development. However, elevated glycolytic rates were also accompanied by a reduction of NADH, plausibly altering NAD^+ availability and may regulate embryonic epigenetic landscape, suggesting longitudinal studies analyzing the long-term health of offspring warrants further investigation.

Conclusions

The data presented in this thesis provides the largest, most comprehensive analysis of blastocyst metabolism, at physiological oxygen, to date and subsequently provides a valuable contribution to our understanding of blastocyst metabolism and the requirements for viable development and success post-transfer. These data lay the foundation for the development of an algorithm incorporating morphological, morphokinetic and metabolic biomarkers which could be used generate a health index score to predict the probability each individual blastocyst will result in the birth of a healthy child. The utilization of such an algorithm in human IVF could significantly improve embryo selection methods, reduce the time to pregnancy and subsequently,

minimize the psychological and financial burdens associated with assisted reproductive technologies. Of further significance, as metabolic profiles can be influenced by changes in embryo culture systems, the development of an embryonic health algorithm could also be used to facilitate quality control checks on culture systems within human IVF clinics and to mediate the introduction of advances to embryo culture conditions which could improve the quality of in vitro cultured embryos.

Appendices

Appendix A

Table A1. Stock for in-house media preparation

Stock	Component	Molecular weight	Weight (g) for stock	Final media conc. (mM)
Salts 100 mL x10 Expiry 3 months	NaCl	58.44	5.2643	90.08
	KCl	74.56	0.4108	5.5
	NaH_2PO_4	119.98	0.02995	0.25
	$MgSO_4.7H_20$	246.47	0.24647	1
Bicarbonate 20 mL x10 Expiry 1 month	$NaHCO_3$	84.01	0.42006	2
Calcium 5 mL x100 Expiry 1 month	$CaCl_2$	147.02	0.13232	1.8
G1 Carbohydrate 5 mL x10 Expiry 3 months	Glucose	180.16	0.04504	0.5
	Sodium-L-Lactate acid	112.06	0.5883	10.5
	Sodium pyruvate	110.04	0.01761	0.32
G2 Carbohydrate 5 mL x10 Expiry 3 months	Glucose	180.16	0.28375	3.15
	Sodium-L-Lactate acid	112.06	0.3289	5.87
	Sodium pyruvate	110.04	0.0055	0.1
mG2 Carbohydrate 5 mL x10 Expiry 3 months	Glucose	180.16	0.28375	0.5
EDTA 5 mL x100 Expiry 1 month	EDTA	372.24	0.0019	0.01
Aln-Gln 5 mL	Alanyl-glutamine	217.2	0.2172	0.5

x100 Expiry 1 month				
NEAA stock 20 mL x100 Expiry 3 months	L-Alanine	89.09	0.0178	0.1
	L-Aspartic acid	133.10	0.0266	0.1
	L-Asparagine-H_2O	150.14	0.03003	0.1
	L-Glutamic acid	169.10	0.03382	0.1
	Glycine	75.07	0.01501	0.1
	L-Proline	115.13	0.02301	0.1
	L-Serine	105.09	0.02102	0.1
Taurine 10 mL x100 Expiry 3 months	Taurine	125.14	0.0125	0.1
Vitamin stock 20 mL x100 Expiry 3 months	D-Ca Pantothenate	238.30	0.002	0.0042
	Pyridoxal-HCl	203.62	0.002	0.0049
	Thiamine-HCl	337.27	0.00202	0.0030
	Riboflavin	367.37	0.0002	0.0003
Hyaluronan stock 50 mL x25 Expiry 3 months	Hyaluronan		0.16076	0.125 mg/ml
Gentamicin 1 mL x40000	Gentamicin		0.04	0.01 mg/ml
MOPS 100 mL x10 Expiry 3 months	MOPS	209.3	4.8140	23

Table A2. Preparation of in-house media solutions from stock solution

Stock	G1 (mL)	G2 (mL)	mG2 (mL)	G2MOPS (mL)	mEmbryoGlue (mL)
H_2O	6.5	6.4	6.4	7.02	6
Salts	1	1	1	1	1
Bicarbonate	1	1	1	0.08	1
Calcium	0.1	0.1	0.1	0.1	0.1
Carbs (G1)	0.1	0	0	0	0
Carbs (G2)	0	0.1	0	0.1	0
Carbs (mG2)	0	0	0.1	0	0
EDTA	0.1	0	0	0	0.1
Aln-Gln	0.1	0.2	0.2	0.2	0.2
NEAAs	0.1	0.1	0.1	0.1	0.1
Taurine	0.1	0	0	0	0
EAAs	0	0.1	0.1	0.1	0.1
Vitamins	0	0.1	0.1	0.1	0.1
Hyaluronan	0.4	0.4	0.4	0.2	0.8
HSA	0.5	0.5	0.5	0	0
G-MM	0	0	0	0	0.5
Gentamicin	0.00375	0.00375	0.00375	0.00375	0.00375
MOPS	0	0	0	1	0

Appendix B

Ferrick, L., Lee, Y. S., & Gardner, D. K. Reducing time to pregnancy and facilitating the birth of healthy children through functional analysis of embryo physiology. *Biol Reprod*. 2019;6;1124-39.

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Reducing time to pregnancy and facilitating the birth of healthy children through functional analysis of embryo physiology†

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Abstract

An ever-increasing number of couples rely on assisted reproductive technologies (ART) in order to conceive a child. Although advances in embryo culture have led to increases in the success rates of clinical ART, it often takes more than one treatment cycle to conceive a child. Ensuring patients conceive as soon as possible with a healthy embryo is a priority for reproductive medicine. Currently, selection of embryos for transfer relies predominantly on the morphological assessment of the preimplantation embryo; however, morphology is not an absolute link to embryo physiology, nor the health of the resulting child. Non-invasive quantitation of individual embryo physiology, a key regulator of both embryo viability and health, could provide valuable information to assist in the selection of the most viable embryo for transfer, hence reducing the time to pregnancy. Further, according to the Barker Hypothesis, the environment to which a fetus is exposed to during gestation affects subsequent offspring health. If the environment of the preimplantation period is capable of affecting metabolism, which in turn will affect gene expression through the metaboloepigenetic link, then assessment of embryo metabolism should represent an indirect measure of future offspring health. Previously, the term viable embryo has been used in association with the potential of an embryo to establish a pregnancy. Here, we propose the term healthy embryo to reflect the capacity of that embryo to lead to a healthy child and adult.

Summary Sentence

Functional analysis of preimplantation embryo physiology is a valuable tool for assessment of embryo health.

Key words: assisted reproductive technology, biomarkers, epigenetics, fertility, metaboloepigenetics, metabolism, pregnancy.

Introduction

The role of clinical ART is to help infertile couples establish a pregnancy through the transfer of a healthy embryo with the subsequent delivery of a healthy child, who has the biological potential to live a long and normal life. To date, clinical ART has resulted in the birth of over eight million babies worldwide, with an ever-increasing number of couples requiring such methodologies to conceive. Since the birth of the first test tube baby, Louise Brown, 40 years ago [1], there have been numerous advances in technologies to alleviate human infertility, including intracytoplasmic sperm injection and preimplantation genetic testing (PGT) of the preimplantation embryo [2–4]. Prior to the late 1990s, it was not feasible to culture the human embryo to the blastocyst stage, and out of necessity the transfer of cleavage

stage embryos asynchronously to the uterus became the norm, consequently exposing them to an environment they would not otherwise see in vivo. Of note, asynchronous embryo transfer has been shown to compromise pregnancy and result in negative fetal outcomes in domestic and laboratory mammals [5, 6]. Subsequently, cleavage stage embryo transfers led to low implantation and pregnancy rates in clinical ART. In an attempt to improve transfer outcomes, it was typical to transfer several embryos to the uterus in a given in vitro fertilization (IVF) cycle [7, 8], leading to an increase in high-order multiple gestations carrying significant risk to both mother and babies [9]. With the development of blastocyst culture, implantation and pregnancy rates have significantly increased, thereby paving the way for the routine introduction of single embryo transfer [10].

The ability to culture human embryos throughout the preimplantation period to the blastocyst stage can be attributed largely to extensive studies on embryo metabolism and maternal reproductive tract physiology [11]. This approach ensured that minimal metabolic stress was placed on the embryo during culture [12]. The Barker Hypothesis, now referred to as Developmental Origins of Health and Disease (DOHaD), postulates that the environment in vivo within the uterus, which can be dramatically affected by nutritional state, programs the developing fetus [13]. Subsequent studies on embryos conceived through IVF have identified that changes to the environment, such as nutrient availability, are capable of influencing long-term health [14]. Indeed, suboptimal environments, such as atmospheric oxygen and simple culture media formulations, during the preimplantation period have been linked to reduced cleavage and blastocyst development rates, perturbed metabolism, altered gene expression, and genomic imprinting defects [15-18]. Given the reports that children conceived following cleavage stage embryo transfer are at increased risk for cardiovascular conditions [19-21], there is an evident need to not only optimize the in vitro environment, but to ensure the long-term health of the children conceived through IVF.

Although there is a trend for increasing blastocyst transfer and success rates for infertile couples worldwide, there remains a profound negative impact of infertility on the psychological wellbeing of infertile couples as manifested by 19–36% of patients discontinuing treatments, with a further 8–23% of couples divorcing [22–26]. Consequently, in addition to improvements in embryo culture systems, it is imperative that we are capable of identifying healthy embryos for transfer in order to reduce the time to pregnancy and support ongoing pregnancies. Traditional embryo selection methods focus on preimplantation embryo morphology as an assessment of embryo viability; however, it is not an absolute link to overall embryonic physiology, a key regulator of both embryo viability and health [27, 28]. This review therefore discusses non-invasive approaches of embryonic selection based on embryo physiology (outlined in Figure 1). Such methodologies have identified potential biomarkers of embryo health that could be used in conjunction with current methods to provide more robust algorithms to reduce the time to pregnancy, but more importantly could plausibly identify the healthiest embryos that possess the greatest probability of giving rise to healthy children.

Morphology and kinetics of development

For 40 years, morphology has been the key determinant in selecting which human embryo(s) to transfer. Key morphological features such as pronuclear appearance, blastomere symmetry and number, degree of fragmentation, and inner cell mass (ICM) and trophectoderm (TE) development have been analyzed and related to subsequent embryo viability leading to the development of numerous morphological grading systems [29, 30]. Until recently, grading the morphology of preimplantation embryos required embryos to be removed from the incubator, exposing embryos to variations in oxygen, temperature and pH, all of which are detrimental to development and subsequent viability [31-34]. Furthermore, such morphological grading only provides a "snap-shot" of preimplantation embryo development at a given time and consequently limits the frequency of observations, when in actuality embryonic development is capable of markedly changing within a matter of hours [35, 36].

The introduction of time-lapse incubation systems has enabled continuous non-invasive assessment of embryo development, including the timing of cleavage divisions and other visible developmental events, referred to as morphokinetics. Morphokinetic parameters, such as the timing of the first cleavage division, have been measured and linked to embryo viability post-transfer [37–40]. Of interest, observations through time-lapse have revealed novel morphological events, such as direct cleavage, involving the immediate cleavage of one blastomere into three daughter cells, and reverse cleavage, where either daughter cell fuses after cleavage division [41, 42], both associated with poor implantation potential, and hence are valuable parameters to consider during embryo selection. Further, morphokinetic data has facilitated the development of algorithms in an attempt to predict embryo development and implantation potential [43–46]. However, debate continues as to whether such algorithms are capable of predicting embryo viability [47, 48]. Recently, the creation of extensive digital libraries of embryo development, made possible through time-lapse analysis, has created the opportunity to employ Artificial Intelligence (AI) to predict embryo viability, by analyzing which parameters are associated with positive transfer outcomes [49]. Although time-lapse and AI technologies offer the ability to increase our knowledge of morphological events, and ultimately assist in improving embryo selection, thereby decreasing time to pregnancy, such analyses of images do not provide direct quantification of embryo physiology or genetic composition that are more closely associated with embryo health.

DNA analysis and genomic composition

PGT is widely utilized to identify aneuploid embryos and is also able to diagnose specific gene defects prior to embryo transfer and implantation. PGT is currently an invasive procedure requiring the removal of a few cells from the TE. However, in the future it may become possible to obtain an accurate analysis of the embryo's genomic composition and to diagnose specific gene defects through non-invasive analysis of embryo-derived DNA in the surrounding culture medium [50, 51]. In the context of this discussion, it is important to understand that embryos that are considered genetically "normal" are not guaranteed to be viable, and subsequently result in the development of a healthy fetus. Therefore, even for embryos that have undergone genetic screening, quantitative functional analysis of embryo physiology could provide valuable information regarding embryonic health, aiding the selection of healthy embryos and reducing time to pregnancy.

Secretome profiling (secretomics)

Protein profiling of human embryos, while not currently utilized clinically, does provide insight into how embryos utilize proteins to support preimplantation development. The human genome consists of

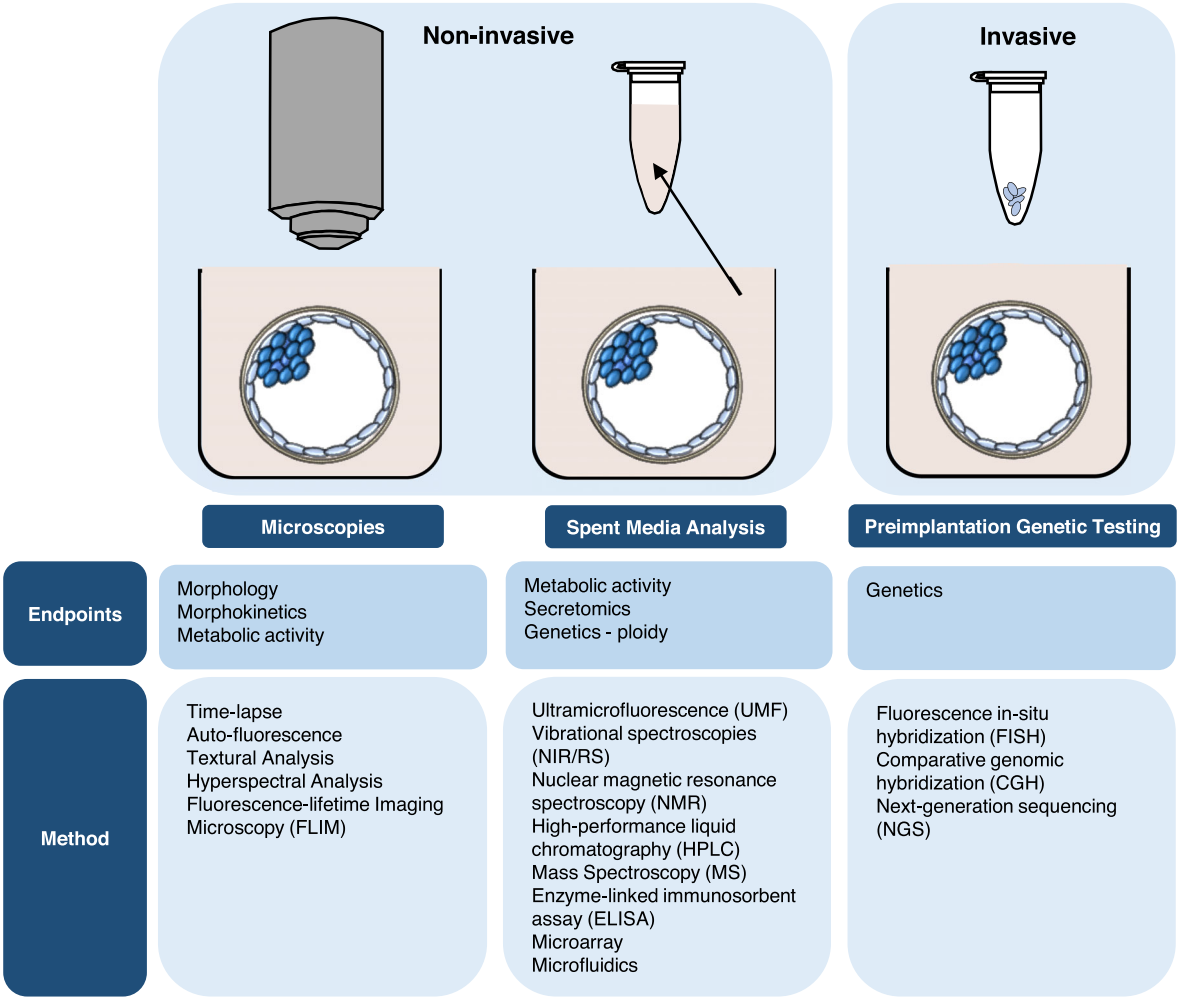

Figure 1. Summary of existing technologies utilized to analysis preimplantation embryo physiology.

25 000–30 000 genes, yet the total number of human proteins present within the human proteome is estimated to be between 500 000 and 1 500 000. As such, post-translational modifications can extend the chemical repertoire of each protein. Analysis of the human proteome provides a "snap-shot" of essential components driving cellular function and physiological state of embryos at any given time, such as metabolic enzymes, transporters, and signaling proteins in the cell cycle. Assessment of protein profiles throughout the preimplantation period would therefore demonstrate the proteomic requirements of the embryo to support viable development. Potential protein targets identified within preimplantation embryo spent culture media, i.e., the embryonic secretome, have subsequently been related to embryo viability (summarized in Table 1) and may be able to aid in the selection of healthy embryos by providing further insight into embryo physiology [52].

Of note, the conditions in which an embryo is conceived and developed can have a profound effect on its physiology, and subsequently the biomarker profile being assessed. For example, mouse preimplantation embryos cultured in the presence of either atmospheric (20%) or physiological (5%) oxygen concentrations exhibit significantly different proteome profiles [53]. Additionally, the secretome of embryos cultured in the presence of physiological oxygen concentrations closely resembled that of in vivo-derived embryos, while those cultured in atmospheric oxygen revealed marked differences in their protein secretion and was associated with poor developmental potential [54].

One of the first embryonic factors identified in the secretome was 2-acetyl-1-alkyl-sn-glycero-3-phosphocholine (PAF), which acts on preimplantation embryos in an autocrine loop and is involved in maternal immune function and platelet activation. PAF was identified within mouse preimplantation embryo spent culture media and in the presence of a PAF receptor antagonist (SRI 63-441) inhibitors, preimplantation embryo development was detrimentally affected, suggesting PAF is linked to embryo viability and could be a good biomarker for embryo selection [55, 56]. However, due to technical complexities associated with the quantification of PAF, it has never been validated as a biomarker of human embryonic health [57]. Leptin, a small pleotropic protein, is involved in embryo-endometrial epithelial interactions at the time of implantation. An increase in leptin within the secretome was found to be associated with improved development in human blastocysts [58]. Human leukocyte antigen G (HLA-G) is another targeted protein that may be indicative of viability. HLA-G was first reported to be expressed by preimplantation human embryos in 1996 by Jurisicova and colleagues, who demonstrated that embryos expressing higher levels of HLA-G exhibited higher cleavage rates [59]. HLA-G has a role in implantation [60]

Table 1. Secretomic analysis of human preimplantation embryos.

Study	Protein	No. of embryos	Analysis conditions	Day of analysis	Technology	Study findings
Gonzalez et al. 2000 [58]	Leptin	30	20% O_2, CCM Medium	Day 5–6	ELISA	Leptin levels were significantly associated with embryonic development.
Noci et al. 2005 [61]	sHLA-G	318	20% O_2, M3 Medium	Day 1–2, 2–3	ELISA	sHLA-G within embryonic secretome was significantly associated with pregnancy rates.
Katz-Jaffe et al. 2006 [54]	Ubiquitin	120	5% O_2, G1/G2 Media	Day 1–5 (24 h periods)	SEDLI-TOF-MS	Protein profiles of embryos differed with every 24 h of development. A 8.5 kDa protein (ubiquitin) was significantly increased in developing embryos versus embryos that arrest.
Dominguez et al. 2008 [68]	CXCL13, GM-CSF	35	20% O_2, CCM Medium	Day 3–6	Protein Microarray	CXCL13 and GM-CSF significantly decreased in media of blastocysts that successfully implanted compared to blastocysts with similar morphology that failed to implant.
Dominguez et al. 2010 [151]	IL-6	12 377	20% O_2, CCM Medium	Day 4–5	ELISA	IL-6, secreted by endometrial epithelial cells, significantly diminished within embryonic secretome of implanted blastocysts.

and since its discovery, soluble HLA-G (sHLA-G) has been investigated as a possible biomarker of embryo viability [61-64]. Results to date however have been conflicting [65, 66], and in the absence of sHLA-G embryos are still capable of developing into a viable pregnancy [67], placing its relevance as a biomarker into question.

For secretomic technologies to be utilized in a clinical setting, they need to be accurate, sensitive, quantitative and cost-effective. Enzyme-linked immunosorbent assay (ELISA) has been a common method to measure proteins within the secretome of preimplantation embryos, such as HLA-G, yet a lack of sensitivity in such technology may explain the inability to provide reproducible data and limits its application clinically [67]. Protein arrays have been utilized for secretomic analysis of human preimplantation embryos that successfully implanted and have revealed these embryos exhibit a decrease in chemokine (C-X-C motif) ligand 13 (CXCL13) and granulocyte-macrophage colony-stimulating factor (GM-CSF) compared to those embryos that failed to implant [68]. Protein microarrays are however limited by the high cost of the array and the number of targets that can be assessed at one time.

Advances in mass spectroscopy (MS) have led to the development of fast, cost-effective, high-throughput, and sensitive technologies capable of measuring single preimplantation embryo protein profiles within the secretome or spent culture media. These advances in MS technology include coupling to an ion source, such as electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI) and surface-enhanced laser desorption/ionization (SELDI), and time of flight (TOF) mass spectrometry that separates molecules according to mass to charge ratio (m/z). Human serum albumin (HSA) in culture media is bound with many factors from blood, which masks the presence of embryo-derived proteins in the medium. However, the use of recombinant HSA resolves this issue, and has been successfully used in combination with SELDI-TOF MS to analyze the secretome of both mouse and human embryos [54]. Analysis of the human blastocyst secretome has revealed differences in protein profiles associated with developmental potential, and furthermore blastocysts with similar morphological grades exhibited different protein profiles, indicating that cell function and embryo grade are not tightly linked [54]. Correlation of day 5 human secretome data with blastocyst development identified a 8.5 kDa protein (tentatively identified as Ubiquitin) that was up-regulated only in the secretome of developing blastocysts compared to degenerate embryos. Ubiquitin is known to have a role during implantation, and hence ubiquitin levels within the secretome may help identify embryos exhibiting normal physiology and therefore higher viability [69]. Of interest, degenerate embryos exhibited an up-regulation of a number of possible protein biomarkers involved in apoptotic and growth-inhibition pathways.

Secretomics and aneuploidy

Analysis of the embryonic secretome in relation to ploidy status has been undertaken, with euploid blastocysts exhibiting notably different secretome profiles to aneuploid blastocysts, while degenerating aneuploid blastocysts exhibited significantly different secretomes to hatching aneuploid blastocysts [70]. Lipocalin-1 was the first protein identified within the human embryo secretome associated with chromosomal aneuploidy [71]. Spent media from aneuploid embryos has significantly higher levels of lipocalin-1 compared to euploid embryos. Consequently, lipocalin-1 may be a non-invasive predictor of which embryos are at greatest risk for genetic abnormalities.

Secretomics and morphokinetics

A retrospective analysis of morphokinetic parameters, combined with the secretome, revealed the presence/absence of IL-6, and the duration of the second cell cycle (cc2) assists embryo selection. The presence of IL-6 and an interval of cc2 between 5 and 12 h were

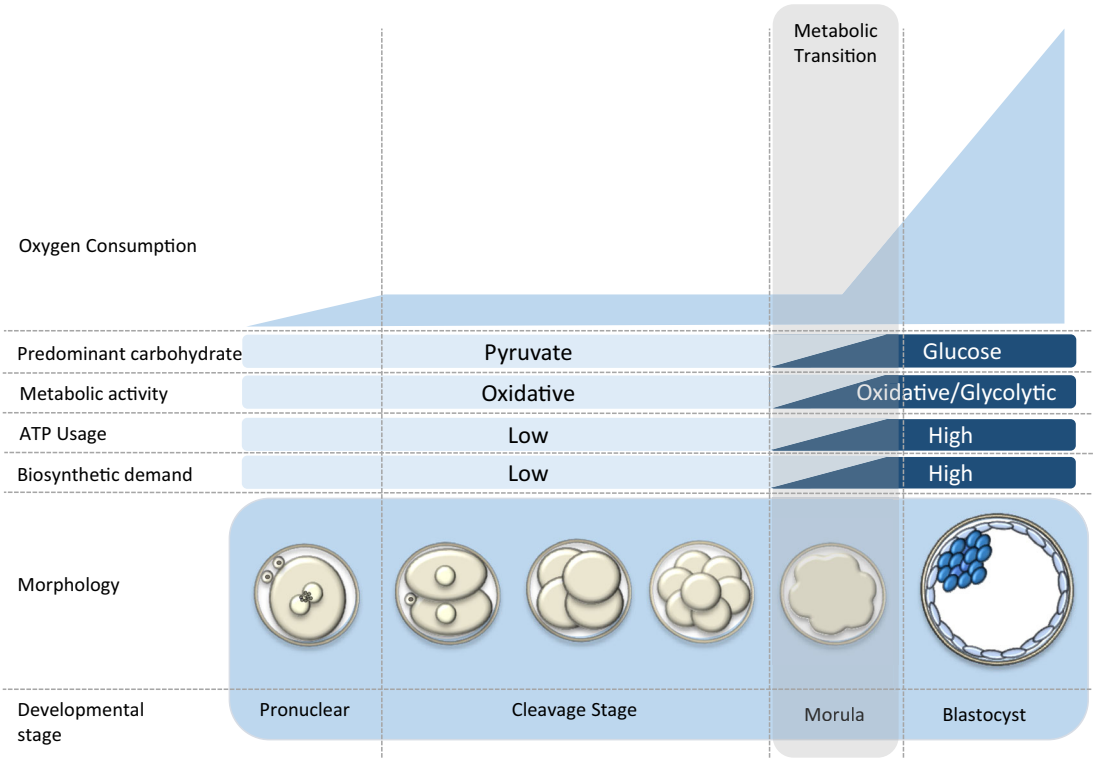

Figure 2. Metabolic changes during mammalian preimplantation development. Prior to compaction, the pronucleate oocyte and cleavage stage embryo consume pyruvate predominantly and through oxidative metabolism produce appropriate levels of ATP required to support cellular division. After compaction, the embryo exhibits exponential growth, requiring higher levels of ATP synthesis and biosynthetic precursors. This increase in activity is supported by an increase in the overall oxidative capacity of the embryo and also a transition to a highly glycolytic metabolism, whereby higher levels of glucose are consumed and converted to lactate in the presence of oxygen.

correlated with significantly higher implantation rates [72]. For example, 83% of embryos with the presence of IL-6 and 77% of embryos with cc2 between 5 and 12 h went on to implant, and when these markers were used in combination, 88.8% of embryos with the presence of IL-6 and cc2 between 5 and 12 h implanted. Although this study was based on a limited number of embryos, the data suggest that assessment of both embryonic secretome and embryo morphokinetic development provides a more accurate method of embryo selection than morphokinetics alone. Unfortunately, while MS technologies have been successful in assessing embryo physiology (Table 1) the significant cost and reproducibility of these technologies has limited their use in a clinical setting. Additionally, the space, training, and time required for such technologies have restricted clinical application. However, the development of new techniques, such as single-cell fiber optic-based nanosensors, may be able to provide a precise, real-time analysis of individual [73, 74] or a group of protein targets present within the embryonic secretome providing a multi-format approach capable of assessing embryonic physiology.

Metabolic activity

During the preimplantation period, the embryo undergoes marked changes in nutrient utilization. The metabolism of the pronucleate oocyte and cleavage stage embryo is characterized by low levels of pyruvate oxidation, whereas the blastocyst is characterized by high levels of glucose uptake and lactate production through aerobic glycolysis, although the blastocyst remains oxidative as reflected in its high oxygen consumption (summarized in Figure 2). A link between embryo metabolism and the ability to develop in vitro was established [75] and led a number of laboratories to investigate the concept of metabolic normality as a biomarker of embryo viability, summarized in Table 2, and conversely, identifying embryos that exhibit aberrant physiology.

As early as 1980, glucose metabolism was considered as a biomarker of embryo viability. Renard and colleagues demonstrated that high glucose uptake by day 10 cattle embryos was associated with higher pregnancy rates [76]. Subsequent optimization of ultramicrofluorescence (UMF), capable of accurately analyzing nano- and pico-liter volumes of spent culture media, enabled the rate of carbohydrate metabolism to be non-invasively measured throughout the entire preimplantation period [77]. It was subsequently determined that glucose consumption is higher in mouse blastocysts exhibiting greater developmental potential post-transfer [78].

Analysis of carbohydrate metabolism by human preimplantation embryos has confirmed that metabolic activity is associated with both embryo developmental potential in vitro and viability post-transfer [52, 79–81]. Human embryos that developed to the blastocyst stage exhibited significantly higher levels of pyruvate and glucose uptake than embryos that arrest during earlier stages of development [52]. Furthermore, glucose uptake by morphologically identical embryos from the same patient revealed metabolism to be independent of morphology yet is correlated to developmental potential. It was subsequently determined that glucose uptake by human embryos on both day 4 and 5 of embryo development was significantly higher

Table 2. Metabolites as biomarkers of preimplantation embryo viability.

Study	Species	Metabolite/s	No. of embryos	Analysis conditions	Day of analysis	Technology	Study findings
Renard et al. 1980 [76]	Cattle	Glucose	59	5% O_2, B2 medium	Day 10–11	Spectroscopies	Glucose uptake was significantly associated with blastocyst development and pregnancy rates.
Leese and Barton 1984 [77]	Mouse	Pyruvate, Glucose	9	20% O_2, Medium M2	Day 1–5	UMF	Pyruvate uptake was high during early development. A significant increase in glucose uptake was observed later in development.
Gardner and Leese 1986 [152]	Mouse	Pyruvate, Glucose	14	20% O_2, Medium M2	Day 1–5 (24 h periods)	UMF	Pyruvate uptake exceeded glucose uptake until 8-cell/morula, when a dramatic increase in glucose was observed.
Gardner and Leese 1987 [78]	Mouse	Glucose	50	20% O_2, Medium M2	Day 4	UMF	Glucose uptake was significantly lower in embryos that did not develop. Glucose uptake was also higher in females than males.
Hardy et al. 1989 [79]	Human	Pyruvate, Glucose	73	20% O_2, mT6 medium	Day 1–6 (24 h periods)	UMF	Pyruvate and glucose uptake were significantly associated with blastocyst development. Arrested embryos exhibited lower levels of pyruvate and glucose uptake.
Gott et al. 1990 [86]	Human	Pyruvate, Glucose, Lactate	40	20% O_2, mT6 medium	Day 2–6 (24 h periods)	UMF	Both pyruvate and glucose uptake were important for day 2–5 of development. Day 6 glucose became the predominant metabolite. Lower uptake of nutrients was associated with embryos arresting during development.
Conaghan et al. 1993 [80]	Human	Pyruvate	590	20% O_2, Earle's balanced salt solution (GIBCO)	Day 1–3 (24 h periods)	UMF	Lower mean value of pyruvate uptake was associated with implantation, yet overall no significant difference to values associated with embryos that failed to implant.
Turner et al. 1994 [153]	Human	Pyruvate	80	20% O_2, HTF medium	Day 1	UMF	An intermediate range of pyruvate uptake was associated with higher clinical pregnancy rates.
Lane and Gardner 1996 [87]	Mouse	Glycolytic rate (Glucose, Lactate)	279	20% O_2, mMTF medium	Day 4	UMF	Embryos exhibiting lower glycolytic rates (<88%) were associated with higher implantation rates. Selecting embryos on the basis of glycolytic rate resulted in 80% fetal development.
Gardner et al. 2001 [52]	Human	Pyruvate, Glucose	42	5% O_2, mG2 media	Day 4–6 (24 h periods)	UMF	Higher glucose and pyruvate uptake were significantly associated with blastocyst development. Glucose uptake was also higher in blastocysts with higher morphological grades.
Houghton et al. 2002 [104]	Human	Amino acids	42	20% O_2, mEBSS medium	Day 2–3, 3–4, 4–5	HPLC	Day 2–3 amino acid profile was significantly associated with blastocyst development and viability post-transfer. Amino acid turnover was significantly higher in embryos arrested prior to blastocyst stage.
Brison et al. 2004 [105]	Human	Amino acids	221	20% O_2, mEBSS medium	Day 1–2	HPLC	Amino acid turnover was significantly correlated to clinical pregnancy and live birth.
Picton et al. 2010 [84]	Human	Amino acids	188	20% O_2, mEBSS medium	Day 1–4 (24 h periods)	HPLC	Amino acid turnover was significantly different due to embryonic stage and ploidy status.
Gardner et al. 2011 [81]	Human	Glucose	50	5% O_2, mG2 media	Day 3–4, 4–5	UMF	Glucose uptake was significantly higher in embryos that developed into a viable pregnancy. Glucose uptake was 28% higher in females compared with males.

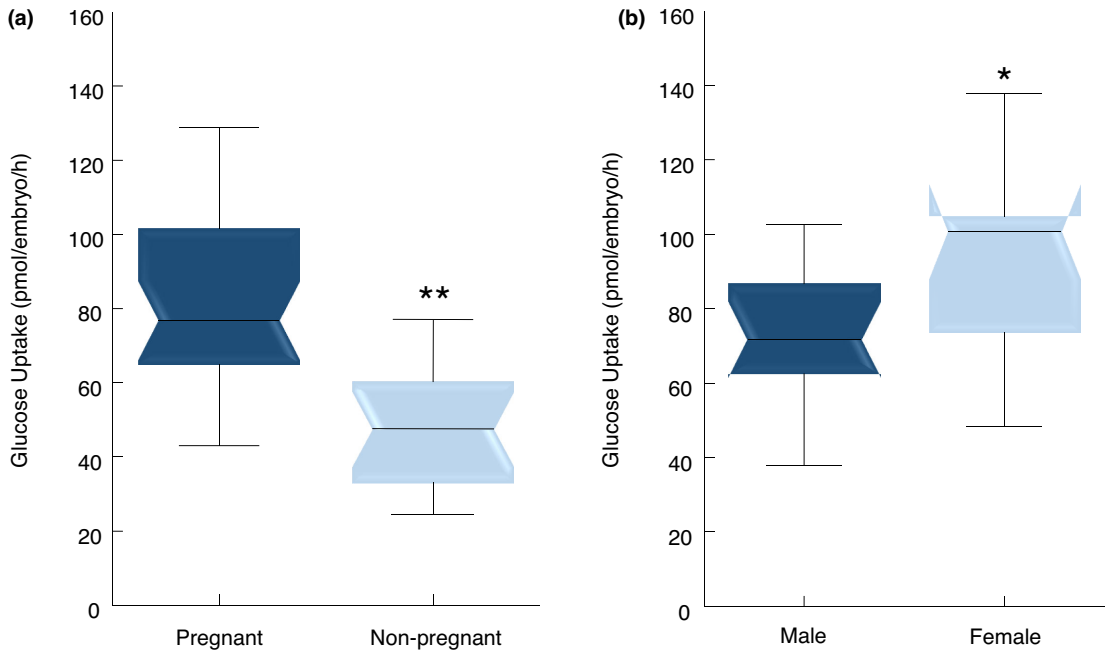

Figure 3. Glucose uptake by day 4 human embryos and the relationship to pregnancy and embryonic sex. (a) glucose uptake of embryos that result in pregnancy (positive fetal heart beat) is significantly different to non-pregnant (**$P < 0.01$). (b) Glucose uptake by male embryos on day 4 of development significantly differs to female embryos (*$P < 0.05$). Notches represent the confidence intervals of the medians, the depths of the boxes represent the interquartile ranges (50% of the data), and whiskers represent 5% and 95% quartiles. The lines across the boxes are the median glucose utilization. (Adapted from Gardner DK et al. *Hum Reprod* 2011; 26(8): 1981–1986.)

in those embryos that went on to establish a viable pregnancy [81] (Figure 3a).

In 1978, it was discovered that female embryos have higher levels of X-linked proteins, due to the fact that there is a window during the preimplantation period when both X-chromosomes are active [82, 83]. Given that glucose-6-phosphate dehydrogenase, a key enzyme in the metabolism of glucose, is X-linked, it is plausible that female embryos would consume more glucose than males at the same stage of development. Analysis of glucose utilization by mouse preimplantation embryos subsequently determined that glucose uptake was indeed higher in female embryos [83]. This intriguing observation has been repeated in the human, in which day 4 female embryos consumed 28% more glucose than males (Figure 3b) [81]. Gender differences have also been observed in amino acid metabolism of human cleavage stage embryos. Male embryos exhibit higher consumption rates of tryptophan and leucine than female embryos, which consume higher levels of asparagine [84]. As such these data indicate that just as in the adult, sex-related differences exist in the first few days of life. The significance of this warrants further investigation.

The significance of measuring glycolytic activity is that not only is the amount (rate) of metabolite consumption or production important but also the pathway (fate) that is active when utilizing that metabolite. Together, these observations gave rise to the "Rate and Fate" hypothesis [85]. For example, lactate production from glucose reflects the relative rate of glycolysis. A high glycolytic rate, calculated on the basis that 2 mol lactate is produced per mol of glucose consumed, exists when an embryo converts majority of glucose to lactate, irrespective of the amount of glucose consumed. A conversion of ~50% of glucose to lactate in the mouse blastocyst is associated with the development of viable embryo [86, 87]. Mouse embryos in culture exhibit a wide range of glycolytic rates. It was determined that those embryos in the lowest 15% of the glycolytic distribution (<88% conversion) exhibited higher implantation potential (80%) compared to embryos in the top 15% of glycolytic distribution (>160%; resulting in a mere 6% implantation rate) [87]. When embryos were randomly selected for transfer, implantation rates were just 20%, four-fold lower than when selecting embryos based on their glycolytic rate, indicating selection of embryos based on embryo physiology is more accurate than morphology alone. Importantly, blastocysts calculated to have a glycolytic rate greater than 100% indicate the use of endogenous substrates, such as glycogen. The premature utilization of glycogen could compromise implantation, during which the embryo is transiently exposed to an avascular and relatively anaerobic environment, which requires the use of endogenous glucose stores, such as glycogen, metabolized through glycolysis [88]. Should the implantation process be impaired through compromised metabolic function, there is a very real potential that even if a pregnancy ensues, placental function downstream could be compromised.

Metabolic activity as an epigenetic regulator (metaboloepigenetics)

A link between the levels of specific metabolic intermediates, a reflection of metabolic state and fate, and the epigenome has recently been established [89, 90], termed "metaboloepigentics." One metabolic co-factor nicotinamide adenine dinucleotide (NAD^+), consumed and produced during glycolysis, is linked to histone acetylation by regulating the activity of sirtuins, a class of histone deacetylases [91, 92], which hence regulates chromatin configuration. Therefore, the levels of NAD^+ can affect levels of gene expression (Figure 4) [93–97].

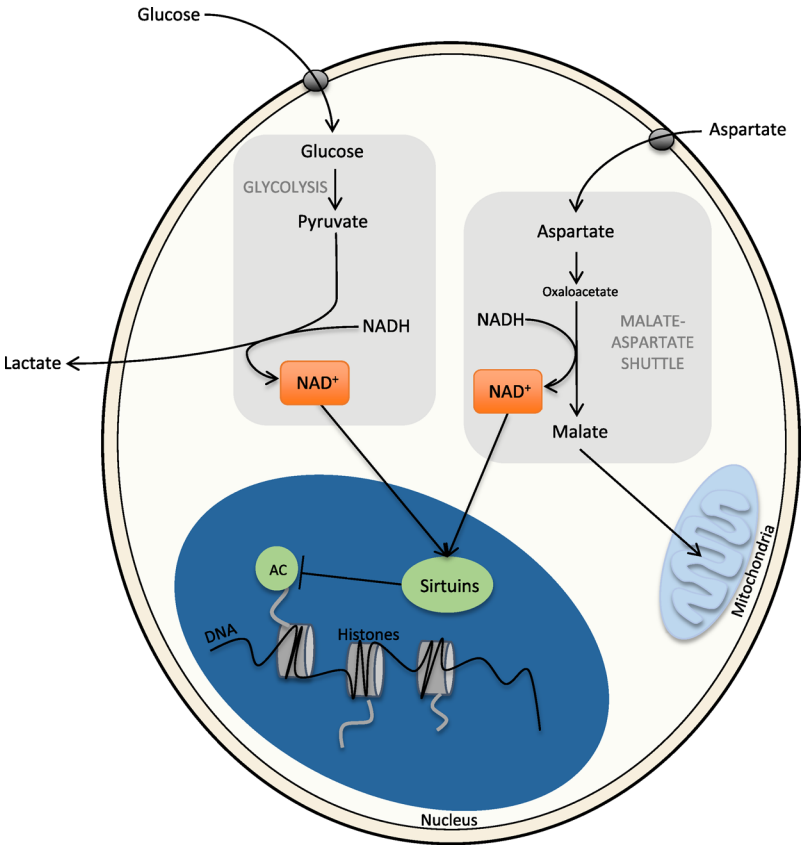

Figure 4. The metaboloepigenetic link between cytosolic NAD^+ and the acetylation state of cells within the blastocyst. Carbohydrates and amino acids can influence the cytosolic levels of NAD^+ through the relative activities of glycolysis and the malate-aspartate shuttle (MAS) respectively. Cytosolic NAD^+ is an activator of sirtuins (a family of deacetylase proteins), which when activated removes acetyl groups (AC) from the tail of histone proteins.

Lactate concentration has been shown to affect the redox potential of embryos through its effect on NAD^+ availability, and as such helps to explain the importance of the relative activity of glycolysis, and hence cytosolic NAD^+, in determining blastocyst health [90, 97, 98]. We have now established that varying the lactate levels in embryo culture medium not only affects metabolism directly, but can alter acetylation levels of the blastocyst (Whatley, Harvey, and Gardner, unpublished observations). Ongoing studies will help elucidate the roles of NAD^+ in genetic regulation and subsequent embryo health.

Amino acids are also key regulators of mammalian embryo development, metabolism, and viability [99, 100]. There is data to indicate that amino acid metabolism also impacts the epigenetic state of the cell [101, 102]. Aspartate is a key regulator of the malate-aspartate shuttle (MAS) during the early stages of the mouse preimplantation development, and subsequently regulates lactate utilization and cytosolic NAD^+ availability [103]. The metaboloepigenetic link between NAD^+ and the acetylation landscape of the embryonic genome suggest that amino acids may also influence the epigenetic state of the embryo and plausibly the health of the resulting child (Figure 4). Indeed, in the presence of a MAS inhibitor (amino-oxyacetate), which consequently reduced the levels of NAD^+, blastocyst development was significantly impaired culminating in impaired fetal growth [103]. It is therefore highly plausible that metabolomic profiling of preimplantation embryos will provide insight into epigenetic state, and how this impacts the long-term health of the resulting offspring.

Amino acid turnover of human embryos has been assessed (Table 2) [104] and found to significantly change not only between developmental stages, in particular cleavage stage compared to blastocyst stage, but also as a factor of embryo quality. Houghton and colleagues determined that metabolism of alanine, arginine, glutamine, methionine, and asparagine on day 2–3 of human embryo development was associated with blastocyst formation [104]. A follow up study, examining the relationship between pronucleate oocyte amino acid use and fate of post-transfer on day 2 [105], found that a lower amino acid turnover was associated with a higher implantation potential and subsequent clinical pregnancy rates and live births. However, such observations, recorded under atmospheric oxygen, need to be repeated under physiological oxygen concentrations. The addition of amino acids to mouse preimplantation embryo culture media improved aberrant embryonic glucose and lactate metabolism whereby the glycolytic rate of embryos cultured in the presence of amino acids closely resembled the metabolism of in vivo-derived embryos compared to control embryos [106]. These data infer that the addition of an activator or inhibitor of a specific pathway could act in a therapeutic manner to "correct" aberrant metabolic flux, although this approach should be combined with the epigenetic analysis of the embryo given the metaboloepigenetic link.

Metabolic activity and morphokinetics

To date, techniques for assessing embryo viability have been used in isolation. It is proposed that the more parameters measured

concomitantly, the greater the accuracy of the diagnosis. To this end, a comprehensive mouse study utilizing time-lapse microscopy and analysis of spent media samples was undertaken, which enabled the assessment of morphokinetics and both carbohydrate and amino acid metabolism within individual preimplantation embryos [107]. Embryos were grouped into "fast" or "slow" developing embryos, according to the time of the first cleavage division to the two-cell stage. "Fast" embryos exhibited a significant increase in TE and ICM cell numbers and higher levels of glucose uptake and lactate production. The relative proportion of glucose that was converted to lactate was lower in "fast" embryos, which is expressed as a lower overall glycolytic rate of 49.6%, compared to "slow" embryos exhibiting a mean glycolytic rate of 59.7%. Amino acid analysis revealed significant differences in profiles between both groups of preimplantation embryos, and a significantly lower overall amino acid turnover by "fast" embryos. The transfer of preimplantation embryos confirmed "fast" embryos are associated with greater developmental potential post-transfer. This study is consistent with previous studies demonstrating that a high glucose uptake, combined with a glycolytic rate of ~50% is associated with higher implantation potential in preimplantation embryos. Consequently, these data support the combined use of morphological, morphokinetic, and physiological parameters for the selection of human embryos in the IVF clinic, thereby providing a more comprehensive assessment of embryo viability and health.

Investigation, into the timing of blastocoel expansion (tEB-tSB), has recently been linked to euploidy whereby the euploidy rate was 47.7% in embryos completing blastocoel expansion within 13 h. In contrast, embryos whose blastocoel expansion took longer than 13 h exhibited a reduced euploidy rate of 31.5% [108]. Blastocoel expansion requires a considerable amount of energy, therefore it is plausible that morphokinetic analysis of blastocoel expansion (tEB-tSB) reflects the metabolic activity of the embryo. We have now confirmed that human embryos with the fastest time from cavitation to full blastocyst expansion do display significantly higher glucose uptakes (Ferrick, Lee, and Gardner, unpublished). It is evident from data in the field of "OMICS" (genomics, proteomics, and metabolomics), preimplantation embryos with identical morphology can have distinct physiologies [52, 76, 86, 87]. Directly measuring preimplantation embryo physiology in conjunction with morphology should provide a more extensive assessment of preimplantation embryo health, ultimately reducing the time to pregnancy, and aiding in the birth of healthy babies.

Metabolomic profiling

In a discussion paper on physiology, it would be remiss not to mention attempts to examine the embryonic metabolome. Unlike direct quantification of specific metabolic pathways, metabolomics is the systematic analysis of multiple metabolites present in spent culture media, which collectively represent the end product of cellular metabolic processes. Hence, rather than focusing on a specific metabolite, the metabolomic profile of preimplantation embryos provides a general measure of embryo physiology at any given stage during development. Several vibrational spectroscopies, such as Raman spectroscopy (RS), and near-infrared (NIR) spectroscopy, have been evaluated, as has nuclear magnetic resonance (NMR) (Table 3).

In the first study to report a correlation between the metabolome and embryo viability, Seli and colleagues assigned each embryo a viability index score on day 3 of development, which was then correlated with pregnancy outcome. Using both RS and NIR spectroscopy, selection algorithms were developed, based on the presence/absence of functional groups (as opposed to entire molecules), which were associated with subsequent implantation with the sensitivity of 86.0% and 75.0%, and specificity of 76.5% and 83.5%, respectively [109]. Such an algorithm was tested by Scott and colleagues who observed a correlation between embryos with a higher viability score and successful implantation outcomes of both cleavage and blastocyst stage embryos [110]. In a follow-up study using NIR spectroscopy, the examination of preimplantation embryo metabolic profiles on day 2/3 of development, viability score was correlated with live birth rate. Interestingly, the accuracy of NIR spectroscopy in predicting reproductive potential was 53.6%, which was higher than morphological selection (38.5%) based on blastomere number and percentage of fragmentation alone [111]. Follow-up studies confirmed metabolomic analysis of spent media from day 3–5 embryos was independent of morphology, and embryos with a high viability index score were associated with a higher pregnancy rate [112–115]. Importantly, these studies again highlight that morphology does not necessarily convey all of the biological information related to embryo physiology.

Randomized clinical trials (RCT) followed, however, these trials were not able to replicate the results observed in the proof-of-principle studies [114], and pregnancy results were not significantly different from embryos that were selected on a morphological basis. Both control and NIR arms, however, included only good quality embryos based on morphological assessment, and therefore, the study was not a true representation of the difference between morphologically scored embryos and NIR scored embryos and their transfer outcomes. Subsequently, the study was terminated by the data safety monitoring board (DSMB) on the grounds that a significant difference in implantation rates was not likely [114]. It was concluded that NIR metabolomic profiling was not capable of selecting embryos with increased implantation potential due to a lack in sensitivity in the technology. A further RCT was conducted, this time assigning a viability score to embryos transferred on day 3 of development [115]. Live birth rates were similar between the control and NIR group, despite demonstrating that 75.4% of embryos that were assigned top morphological grades did not have the highest viability score.

Reasons for the apparent failure of such studies, and hence obstacles for future clinical trials, include ensuring that technical errors are minimized. For example, the incubation volume an embryo is cultured in is crucial, and any deviations can have a large effect on the data. Hence, if an embryo is cultured in 20 μl of medium, changing the volume by just 1–2 μl significantly alters the relative availability of nutrients, translating to a 5–10% error in measurement. Metabolic trials, therefore, require high levels of accuracy and precision in all aspects, especially embryo culture, to ensure success. NIR spectroscopies instruments can also be variable in their performance, which in turn masks the low intensity of the signal generated from the embryo within the spent culture media [116]. In addition to instrument variability, threshold of analytical signal in the culture media, instrument signal to noise ratio, and variability between clinics could all have impacted on the development of a robust algorithm and subsequent failure of the application. The challenge remains ensuring that technologies are sensitive enough to measure the small changes in metabolite levels in embryos that consist of >100 cells, within small volumes of spent media in which the embryo has been cultured for a matter of hours.

Table 3. Metabolomic analysis of human preimplantation embryos.

Study	Study type	No. of embryos	Analysis conditions	Day of analysis	Technology	Study findings
Seli et al. 2007 [109]	Prospective study	69	20% O_2, G1/Quinn's Advantage Cleavage medium	Day 1–3	RS/NIR	RS and NIR predicted embryo viability with 86% and 75% sensitivity, and 76.5% and 83.5% specificity, respectively.
Scott et al. 2008 [110]	Prospective study	41	5% O_2 Quinn's Advantage Cleavage/BlastAssist medium	Day 1–3, 3–5	RS	Embryos that successfully implanted were associated with higher viability scores on both day 3 and day 5. Diagnostic accuracy of RS metabolomic profiling was 80.5%.
Seli et al. 2008 [154]	Retrospective study	34	20% O_2, G1 medium	Day 1–3	NMR	Glutamate concentrations were higher in spent media samples of embryos that resulted in pregnancy compared to those that failed to implant. NMR metabolomic profiling predicted embryo viability with 88.2% sensitivity, and 88.2% specificity.
Vergouw et al. 2008 [111]	Prospective study	333	20% O_2, HTF medium	Day 1–2/3	NIR	Viability scores were significantly different between positive and negative pregnancy outcomes. NIR metabolomic profiling assessed embryo viability with an accuracy of 53.6% compared to 38.5% when assessing morphology.
Seli et al. 2010 [112]	Retrospective study	485	5% O_2, HTF/Sage Cleavage medium	Day 1–2/3	NIR	NIR metabolic profiling distinguished between embryos of higher reproductive potential and those that failed to implant independent of morphology.
Alstrom et al. 2011 [113]	Retrospective study	137	5/6% O_2, CCM/Sage Blastocyst medium	Day 2–5, 4–5	NIR	Metabolic profiles of embryos were able to predict implantation potential of blastocyst and may not be restricted to a specific set of culture conditions.
Hardarson et al. 2012 [114]	Randomized clinical trial	327	5% O_2, Cook Cleavage/CCM medium	Day1–2, 2–5	NIR	No significant improvement to implantation rates compared with control group. Study was terminated by DSMB.
Vergouw et al. 2012 [115]	Randomized clinical trial	307	20% O_2, Sage Blastocyst medium	Day 1–3	NIR	No significant improvements in implantation rates when selecting embryos on the basis of their viability score. 75.4% of embryos with the best morphology did not have the greatest viability score.
Padakalakatti et al. 2012 [155]	Prospective study	127	20% O_2 ISM1 medium	Day 1–3	NMR	Embryos that successfully implanted had significantly lower pyruvate/alanine ratios than those embryos that failed to implant.
Rinaudo et al. 2012 [156]	Retrospective study	228	G1 medium	Day 1–3	NMR	Assessing five different culture conditions, NMR based profiling of spent culture media was not able to predict implantation for embryos transferred on day 3 of development.
Wallace et al. 2014 [157]	Prospective study	58	Quinn's Advantage Cleavage medium	Day 1–2	NMR	NMR profiling of spent media identified significant metabolite differences between samples from embryos that led to a positive pregnancy result versus negative results.
Kirkegaard et al. 2014 [158]	Prospective study	148	20% O_2, Sydney IVF Cleavage/Blastocyst medium	Day 1–3, 3–5	NMR	No correlation between NMR profiling of spent media and pregnancy outcome.

Metabolic activity analysis through fluorescent microscopies

Fluorescent microscopies have been used to investigate embryo physiology for several years. In particular, autofluorescence is a useful tool to investigate preimplantation embryo metabolism [117]. Nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) are key metabolic components that exhibit fluorescence. It is possible to measure the intensity of NADH and FAD fluorescence and subsequently measure the reduction-oxidative ratio of the preimplantation embryo [118, 119]. Of note, NADH is inversely related to NAD^+ availability. Hence, using fluorescent microscopy techniques, it may be possible to investigate the epigenetic status of the embryo due to the metaboloepigenetic link that exists between NAD^+ and histone acetylation.

Fluorescent lifetime imaging microscopy (FLIM) can measure free NADH, from which the ratio between bound and free NADH can

be calculated as a measure of glycolysis or oxidative phosphorylation [120]. The effectiveness of FLIM-based metabolic imaging to measure metabolic states has been established during the differentiation of various cancer cell types [121], and stem cells [122], and has now been used to analyze mitochondrial function in oocytes [123]. Metabolic imaging of gametes and embryos holds promise as it provides an objective, rapid, non-invasive, and quantitative measurement of the physiological state of their mitochondria [124]. While this approach is promising, a causal relationship between mitochondrial functions and embryo viability has yet to be established. Consequently, further studies are required to validate whether such an approach can be used as an indirect measure of intracellular metabolic function.

More recently, the application of hyperspectral autofluorescent analysis, which enables a broad range of autofluorescent signals to be investigated simultaneously, has provided insight into the heterogeneity of cellular metabolism of preimplantation embryos [125, 126]. Additionally, textural analysis of light and fluorescent microscopies may provide additional information on the viability of preimplantation embryos. These techniques have been successful in investigating cancer cells [127-129] and more recently have been used to examine bovine cumulus-oocyte complexes (COCs) and cleavage stage embryos, where textural differences in fluorescent staining could be detected between experimental groups [119]. Further work is now required to elucidate the true quantitative value of these microscopies in providing information on embryonic physiology and their association with subsequent embryo health. As exposure to light for extended periods has been shown to increase DNA damage, ROS production, and hence be detrimental to embryo and fetal development [130–132], it is evidently important to also determine the safety of light exposure, intensity, and wavelength on embryo and fetal development prior to clinical introduction.

Microfluidics (lab-on-a-chip) as a means of automating embryo analysis

The challenge in the translation of proteomics and metabolomics to a clinical setting lies in developing and validating a technology that is capable of reliably, quantitatively, and sensitively measuring preimplantation embryo physiology throughout the entire preimplantation embryo development period. Technologies such as microfluidic devices are capable of handling fluids in the submicroliter volumes, and through the use of appropriate on-chip valving, fluids can be moved and measured with a high degree of accuracy [133]. Microfluidics devices have successfully been developed and utilized in clinic settings to sort sperm according to morphology, motility, functionality, and DNA integrity, which have been summarized by Knowlton and colleagues [134]. Additionally, the ability to analyze nanoliter volumes of fluid makes microfluidic devices ideally suited for single embryo analysis, and analytical technologies are already available, which can be incorporated into chips [135]. Microdevices have been developed to analyze nucleic acids, proteins and peptides, amino acids and small metabolites [136, 137], and proof-of-principle studies for the assessment of a more reliable and accurate means of measuring the embryonic genome [138], secretome [139–145], and metabolome [133, 135] have been performed and are promising.

Fortunately, another advantage of microfluidic technology is the ability to provide a more automated system. Not only can multiple targets be measured simultaneously, but these can also be measured throughout the preimplantation period at predetermined times. Additionally, it should be possible for such devices to be integrated with time-lapse microscopy allowing for morphology, morphokinetics, and physiology of preimplantation embryos to be analyzed throughout culture. On-chip imaging can already produce images with resolutions below 1 μm [146]. Consequently, such measurements could then be used to generate algorithms, or for input into AI programs, which can assess multiple parameters relating to embryo viability and pregnancy outcomes. Hence, from one chip device it would be possible to obtain morphokinetic and cellular data (from image analysis) and physiological data (from media analysis) in real time, all the while having AI interpret every parameter to help identify and rank the healthiest embryos during their development, while conversely identifying those embryos with limited developmental potential (Figure 5).

Conclusion

Morphological and morphokinetic analyses have assisted in the exclusion of embryos exhibiting abnormal phenotypes, while PGT has been of value in identifying euploid embryos for transfer. However, functional analysis of embryo physiology would not only assist in reducing the time to pregnancy but should also facilitate the identification of the healthiest embryos, which in turn will provide the best transfer outcomes, i.e., the live birth of healthy child. With regards to the assessment of embryo physiology, research has and should continue to focus on the non-invasive analysis of spent culture media to quantitate the embryonic secretome and metabolome during the preimplantation period. Metabolic profiles of preimplantation embryos that are capable of developing in vitro and those blastocysts that go on to establish pregnancy after transfer exhibit marked differences from those embryos with limited developmental potential. Given the relationship between metabolic activity, such as cytosolic NAD^+ availability, and the regulation of the epigenome through metaboloepigenetics [89, 97, 147], analysis of metabolic function may not only relate to embryonic viability but also to the epigenetic state of the embryo during development, thereby conveying information regarding developmental programing and subsequently the health of resulting children. Importantly, however, the conditions in which embryos are maintained can also have a profound effect on cellular physiology, hence on the very biomarkers one maybe assessing. This is especially true for metabolism, where oxygen concentration, among other factors, can significantly affect the relative activity of metabolic pathways and the epigenome [34, 148–150].

To date, the technologies required for embryo analyses have been sophisticated and expensive. However, with the development of novel nanoscale detection systems, such as microfluidic devices, assessing metabolic biomarkers, such as glucose and lactate, could become available to a greater number of IVF laboratories, and will no longer be the domain of a handful of research facilities. Over the next few years, it should therefore become possible to not only quantitate the potential viability of an embryo in culture, but plausibly to assess the health potential of the child conceived through a combination of metabolomics, microscopies, and microfluidic technologies, thereby, reducing time to pregnancy and improving the health of the resulting children.

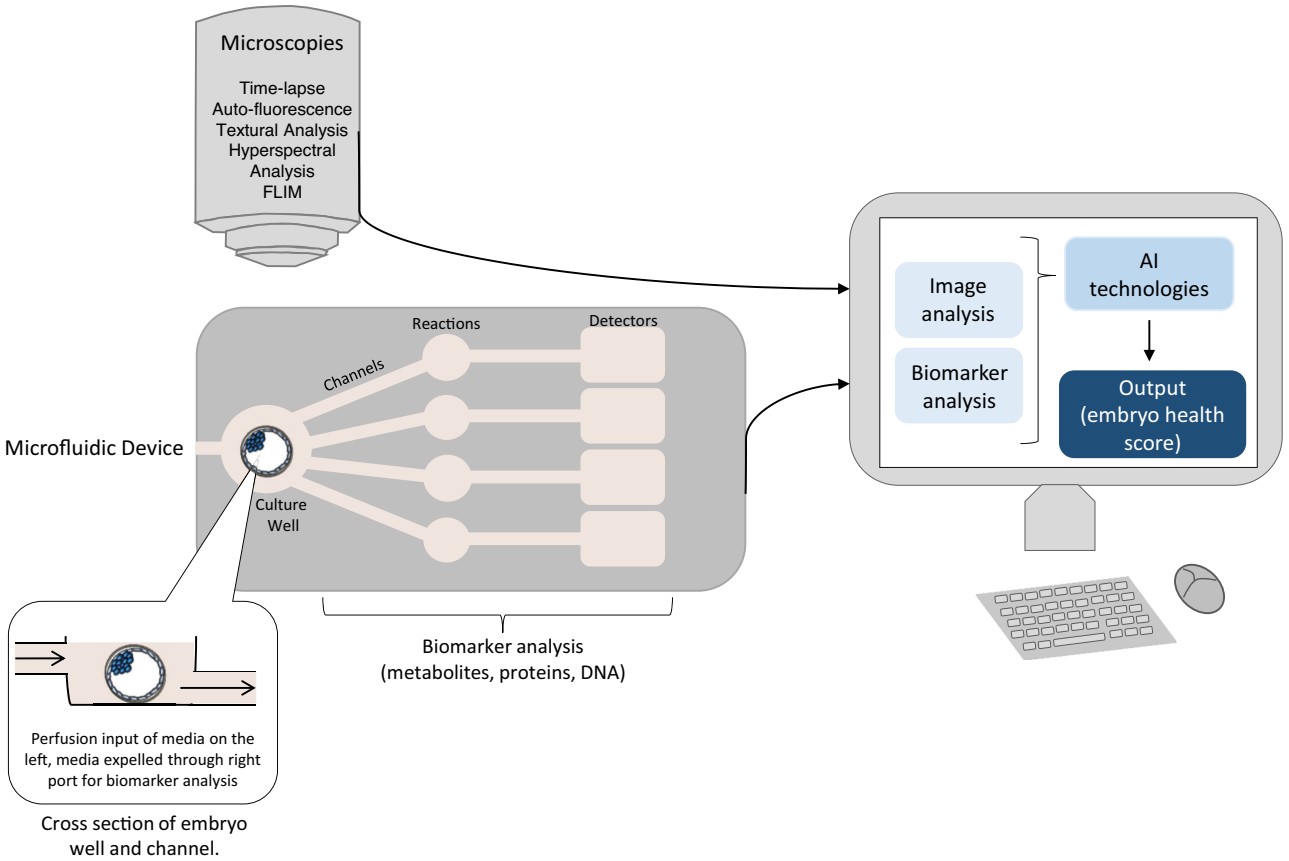

Figure 5. Integration of microscopies and microfluidics to quantitate embryo physiology and health in a clinical setting. Embryos are cultured in microfluidic devices where media flow is regulated to facilitate sampling. Concomitantly, microscopy technologies are acquired and together these data are sent to a central computer system where AI could be used to help create a health score for each individual embryo.

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References

1. Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet North Am Ed* 1978; **2**:366.
2. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet North Am Ed* 1992; **340**:17–18.
3. Munne S, Alikani M, Tomkin G, Grifo J, Cohen J. Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil Steril* 1995; **64**:382–391.
4. Verlinsky Y, Cieslak J, Ivakhnenko V, Lifchez A, Strom C, Kuliev A. Birth of healthy children after preimplantation diagnosis of common aneuploidies by polar body fluorescent in situ hybridization analysis. *Fertil Steril* 1996; **66**:126–129.
5. Barnes FL. The effects of the early uterine environment on the subsequent development of embryo and fetus. *Theriogenology* 2000; **53**:649–658.
6. Walker KJ, Green MP, Gardner DK. Spatial asynchronous transfer of cleavage-stage mouse embryos to the uterus compromises fetal development. *Mol Reprod Dev* 2015; **82**:80.
7. Lancaster P, Shafir E, Huang J. *Assisted Conception, Australia and New Zealand, 1992 and 1993*. Kensington: AIHW, National Perinatal Epidemiology & Statistics Unit; 1995; 1–71.
8. European IVF-Monitoring Programme. Assisted reproductive technology in Europe, 1997. Results generated from European registers by ESHRE. *Hum Reprod* 2001; **16**:384–391.
9. Adashi EY, Barri PN, Berkowitz R, Braude P, Bryan E, Carr J, Cohen J, Collins J, Devroey P, Frydman R, Gardner D, Germond M et al. Infertility therapy-associated multiple pregnancies (births): an ongoing epidemic. *Reprod Biomed Online* 2003; **7**:515–542.
10. Gardner DK, Lane M. Towards a single embryo transfer. *Reprod Biomed Online* 2003; **6**:470–481.
11. Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update* 1997; **3**:367–382.
12. Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 1998; **49**:83–102.
13. Barker DJ. The Wellcome Foundation Lecture, 1994. The fetal origins of adult disease. *Proc R Soc Lond B* 1995; **262**:37–43.
14. Feuer S, Rinaudo P. From embryos to adults: a DOHaD perspective on in vitro fertilization and other assisted reproductive technologies. *Healthcare* 2016; **4**:E51
15. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996; **65**:349–353.
16. Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. *Reprod Fertil Dev* 2005; **17**:361–370.
17. Giritharan G, Talbi S, Donjacour A, Di Sebastiano F, Dobson AT, Rinaudo PF. Effect of in vitro fertilization on gene expression and

development of mouse preimplantation embryos. *Reproduction* 2007; **134**:63–72.
18. Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2016; **22**:2–22.
19. Ceelen M, Van Weissenbruch MM, Prein J, Smit JJ, Vermeiden JP, Spreeuwenberg M, van Leeuwen FE, Delemarre-van De Waal HA. Growth during infancy and early childhood in relation to blood pressure and body fat measures at age 8–18 years of IVF children and spontaneously conceived controls born to subfertile parents. *Hum Reprod* 2009; **24**:2788–2795.
20. Scherrer U, Rimoldi SF, Rexhaj E, Stuber T, Duplain H, Garcin S, de Marchi SF, Nicod P, Germond M, Allemann Y, Sartori C. Systemic and pulmonary vascular dysfunction in children conceived by assisted reproductive technologies. *Circulation* 2012; **125**:1890–1896.
21. Von Arx R, Allemann Y, Sartori C, Rexhaj E, Cerny D, de Marchi SF, Soria R, Germond M, Scherrer U, Rimoldi SF. Right ventricular dysfunction in children and adolescents conceived by assisted reproductive technologies. *J Appl Physiol* 2015; **118**:1200–1206.
22. Olivius C, Friden B, Borg G, Bergh C. Why do couples discontinue in vitro fertilization treatment? A cohort study. *Fertil Steril* 2004; **81**:258–261.
23. Gameiro S, Boivin J, Peronace L, Verhaak CM. Why do patients discontinue fertility treatment? A systematic review of reasons and predictors of discontinuation in fertility treatment. *Hum Reprod Update* 2012; **18**:652–669.
24. Walschaerts M, Bujan L, Parinaud J, Mieusset R, Thonneau P. Treatment discontinuation in couples consulting for male infertility after failing to conceive. *Fertil Steril* 2013; **99**:1319–1323.
25. Lande Y, Seidman DS, Maman E, Baum M, Hourvitz A. Why do couples discontinue unlimited free IVF treatments? *Gynecol Endocrinol* 2015; **31**:233–236.
26. Van Dongen A, Huppelschoten AG, Kremer JA, Nelen WL, Verhaak CM. Psychosocial and demographic correlates of the discontinuation of in vitro fertilization. *Hum Fert* 2015; **18**:100–106.
27. Sakkas D, Gardner DK. Evaluation of embryo quality: Analysis of morphology and physiology. In Gardner DK, Weissman A, Howles CM, Shoham Z (eds.), *Textbook of Assisted Reproductive Techniques*. Boca Raton: CRC Press; 2018:225–242.
28. Gardner DK. Human embryo development and assessment of viability. In:Skinner, MK (ed.), *Encyclopedia of Reproduction*. New York: Elsevier; 2018:176–185.
29. Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring human embryo growth rates in in vitro fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *J Assist Reprod Genet* 1986; **3**:284–295.
30. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000; **73**:1155–1158.
31. Wang W-H, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling–rewarming observed using polarized light microscopy. *Hum Reprod* 2001; **16**:2374–2378.
32. Sun X-F, Zhang W-H, Chen X-J, Xiao G-H, Mai W-Y, Wang W-H. Spindle dynamics in living mouse oocytes during meiotic maturation, ageing, cooling and overheating: a study by polarized light microscopy. *Zygote* 2004; **12**:241–249.
33. Zander-Fox DL, Mitchell M, Thompson JG, Lane M. Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. *Reprod Biomed Online* 2010; **21**:219–229.
34. Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod* 2012; **87**:21–28.
35. Mio Y, Maeda K. Time-lapse cinematography of dynamic changes occurring during in vitro development of human embryos. *Am J Obstet Gynecol* 2008; **199**:660.e1–660.e5.
36. Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011; **26**:2658–2671.
37. Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, Steptoe PC, Webster JM. Factors influencing the success of in vitro fertilization for alleviating human infertility. *J Assist Reprod Genet* 1984; **1**:3–23.
38. Lundin K, Bergh C, Hardarson T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Hum Reprod* 2001; **16**:2652–2657.
39. Sakkas D, Percival G, D'Arcy Y, Sharif K, Afnan M. Assessment of early cleaving in vitro fertilized human embryos at the 2-cell stage before transfer improves embryo selection. *Fertil Steril* 2001; **76**:1150–1156.
40. Salumets A, Hydén-Granskog C, Mäkinen S, Suikkari AM, Tiitinen A, Tuuri T. Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Hum Reprod* 2003; **18**:821–825.
41. Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ, Bellver J, Meseguer M. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. *Fertil Steril* 2012; **98**:1458–1463.
42. Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. *Fertil Steril* 2014; **102**:1295–1300.e2.
43. Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. *Fertil Steril* 2014; **102**:1287–1294.e5.
44. Milewski R, Milewska AJ, Kuczyńska A, Stankiewicz B, Kuczyński W. Do morphokinetic data sets inform pregnancy potential? *J Assist Reprod Genet* 2016; **33**:357–365.
45. Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohi J, Meseguer M. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril* 2016; **105**:376–384.e9.
46. Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Hum Reprod* 2016; **31**:2231–2244.
47. Pribenszky C, Nilselid AM, Montag M. Response: time-lapse systems for ART. *Reprod Biomed Online* 2018; **36**:290–292.
48. Armstrong S, Bhide P, Jordan V, Pacey A, Farquhar C. Time-lapse systems for embryo incubation and assessment in assisted reproduction. *Cochrane Database Syst Rev* 2018; **5**:CD011320.
49. Tran A, Cooke S, Illingworth PJ, Gardner DK. Artificial intelligence as a novel approach for embryo selection. *Fertil Steril* 2018; **110**:E430.
50. Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, Mercader A, Meseguer M, Blesa D, Moreno I, Valbuena D, Rubio C et al. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod* 2018; **33**:745–756.
51. Xu J, Fang R, Chen L, Chen D, Xiao JP, Yang W, Wang H, Song X, Ma T, Bo S, Shi C, Ren J et al. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci USA* 2016; **113**:11907–11912.
52. Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril* 2001; **76**:1175–1180.
53. Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. A proteomic analysis of mammalian preimplantation embryonic development. *Reproduction* 2005; **130**:899–905.
54. Katz-Jaffe MG, Schoolcraft WB, Gardner DK. Analysis of protein expression (secretome) by human and mouse preimplantation embryos. *Fertil Steril* 2006; **86**:678–685.
55. Spinks N, O'Neill C. Antagonists of embryo-derived platelet-activating factor prevent implantation of mouse embryos. *Reproduction* 1988; **84**:89–98.

56. Ryan J, Spinks N, O'Neill C, Wales R. Implantation potential and fetal viability of mouse embryos cultured in media supplemented with platelet-activating factor. *Reproduction* 1990; **89**:309–315.
57. O'Neill C. The role of paf in embryo physiology. *Hum Reprod Update* 2005; **11**:215–228.
58. González RR, Caballero-Campo P, Jasper M, Mercader A, Devoto L, Pellicer A, Simon C. Leptin and leptin receptor are expressed in the human endometrium and endometrial leptin secretion is regulated by the human blastocyst. *J Clin Endocrinol Metab* 2000; **85**:4883–4888.
59. Jurisicova A, Casper RF, MacLusky NJ, Librach CL. Embryonic human leukocyte antigen-G expression: possible implications for human preimplantation development. *Fertil Steril* 1996; **65**:997–1002.
60. Roussev RG, Coulam CB. HLA-G and its role in implantation (review). *J Assist Reprod Genet* 2007; **24**:288–295.
61. Noci I, Fuzzi B, Rizzo R, Melchiorri L, Criscuoli L, Dabizzi S, Biagiotti R, Pellegrini S, Menicucci A, Baricordi O. Embryonic soluble HLA-G as a marker of developmental potential in embryos. *Hum Reprod* 2005; **20**:138–146.
62. Desai N, Filipovits J, Goldfarb J. Secretion of soluble HLA-G by day 3 human embryos associated with higher pregnancy and implantation rates: assay of culture media using a new ELISA kit. *Reprod Biomed Online* 2006; **13**:272–277.
63. Fisch JD, Keskintepe L, Ginsburg M, Adamowicz M, Sher G. Graduated Embryo Score and soluble human leukocyte antigen-G expression improve assisted reproductive technology outcomes and suggest a basis for elective single-embryo transfer. *Fertil Steril* 2007; **87**:757–763.
64. Rebmann V, Switala M, Eue I, Schwahn E, Merzenich M, Grosse-Wilde H. Rapid evaluation of soluble HLA-G levels in supernatants of in vitro fertilized embryos. *Hum Immunol* 2007; **68**:251–258.
65. Rebmann V, Switala M, Eue I, Grosse-Wilde H. Soluble HLA-G is an independent factor for the prediction of pregnancy outcome after ART: a German multi-centre study. *Hum Reprod* 2010; **25**:1691–1698.
66. Warner CM, Lampton PW, Newmark JA, Cohen J. Soluble human leukocyte antigen-G and pregnancy success. *Reprod Biomed Online* 2008; **17**:470–485.
67. Vercammen M, Verloes A, Haentjens P, Van de Velde H. Can soluble human leucocyte antigen-G predict successful pregnancy in assisted reproductive technology? *Curr Opin Obstet Gynecol* 2009; **21**:285–290.
68. Domínguez F, Gadea B, Esteban FJ, Horcajadas JA, Pellicer A, Simón C. Comparative protein-profile analysis of implanted versus non-implanted human blastocysts. *Hum Reprod* 2008; **23**:1993–2000.
69. Wang HM, Zhang X, Qian D, Lin HY, Li QL, Liu DL, Liu GY, Yu XD, Zhu C. Effect of ubiquitin-proteasome pathway on mouse blastocyst implantation and expression of matrix metalloproteinases-2 and-9. *Biol Reprod* 2004; **70**:481–487.
70. Katz-Jaffe M, Stevens J, Kearns W, Gardner D, Schoolcraft W. Relationship between embryonic secretome and chromosomal abnormalities in human IVF. *Fertil Steril* 2006; **86**:S57.
71. McReynolds S, Vanderlinden L, Stevens J, Hansen K, Schoolcraft WB, Katz-Jaffe MG. Lipocalin-1: a potential marker for noninvasive aneuploidy screening. *Fertil Steril* 2011; **95**:2631–2633.
72. Dominguez F, Meseguer M, Aparicio-Ruiz B, Piqueras P, Quiñonero A, Simón C. New strategy for diagnosing embryo implantation potential by combining proteomics and time-lapse technologies. *Fertil Steril* 2015; **104**:908–914.
73. Liu G, Zhang K, Ma K, Care A, Hutchinson MR, Goldys EM. Graphene quantum dot based "switch-on" nanosensors for intracellular cytokine monitoring. *Nanoscale* 2017; **9**:4934–4943.
74. Liu G, Zhang K, Nadort A, Hutchinson MR, Goldys EM. Sensitive cytokine assay based on optical fiber allowing localized and spatially resolved detection of Interleukin-6. *ACS Sens* 2017; **2**:218–226.
75. Bowman P, McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. *J Embryol Exp Morphol* 1970; **24**:203–207.
76. Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. *Reproduction* 1980; **58**:161–164.
77. Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *Reproduction* 1984; **72**:9–13.
78. Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *J Exp Zool* 1987; **242**:103–105.
79. Hardy K, Hooper MA, Handyside AH, Rutherford AJ, Winston RM, Leese HJ. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum Reprod* 1989; **4**:188–191.
80. Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. *J Assist Reprod Genet* 1993; **10**:21–30.
81. Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod* 2011; **26**:1981–1986.
82. Epstein CJ, Smith S, Travis B, Tucker G. Both X chromosomes function before visible X-chromosome inactivation in female mouse embryos. *Nature* 1978; **274**:500–503.
83. Gardner DK, Larman MG, Thouas GA. Sex-related physiology of the preimplantation embryo. *Mol Hum Reprod* 2010; **16**:539–547.
84. Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, Leese HJ, Harris SE. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Mol Hum Reprod* 2010; **16**:557–569.
85. Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril* 2013; **99**:1062–1072.
86. Gott AL, Hardy K, Winston RM, Leese HJ. Non-invasive measurement of pyruvate and glucose uptake and lactate production by sigle human preimplantation embryos. *Hum Reprod* 1990; **5**:104–108.
87. Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod* 1996; **11**:1975–1978.
88. Rogers P, Murphy C, Gannon B. Changes in the spatial organization of the uterine vasculature during implantation in the rat. *Reproduction* 1982; **65**:211–214.
89. Donohoe DR, Bultman SJ. Metaboloepigenetics: interrelationships between energy metabolism and epigenetic control of gene expression. *J Cell Physiol* 2012; **227**:3169–3177.
90. Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev* 2015; **27**:638–654.
91. Imai S, Guarente L. NAD+ and sirtuins in aging and disease. *Trends Cell Biol* 2014; **24**:464–471.
92. Ryall JG, Dell'Orso S, Derfoul A, Juan A, Zare H, Feng X, Clermont D, Koulnis M, Gutierrez-Cruz G, Fulco M. The NAD+-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. *Cell Stem Cell* 2015; **16**:171–183.
93. Houtkooper RH, Cantó C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 2010; **31**:194–223.
94. Latham T, Mackay L, Sproul D, Karim M, Culley J, Harrison DJ, Hayward L, Langridge-Smith P, Gilbert N, Ramsahoye BH. Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. *Nucleic Acids Res* 2012; **40**:4794–4803.
95. Gardner DK. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? *Bioessays* 2015; **37**:364–371.
96. Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab* 2015; **21**:392–402.
97. Harvey AJ, Rathjen J, Gardner DK. Metaboloepigenetic regulation of pluripotent stem cells. *Stem Cells Int* 2016; **2016**:1–15.
98. Gardner DK, Pool TB, Lane M. Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability. *Semin Reprod Med* 2000; **18**:205–218.
99. Gardner DK. Dissection of culture media for embryos: the most important and less important components and characteristics. *Reprod Fertil Dev* 2008; **20**:9–18.

100. Gardner DK, Kelley RL. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis* 2017; **8**:418–435.
101. Washington JM, Rathjen J, Felquer F, Lonic A, Bettess MD, Hamra N, Semendric L, Tan BS, Lake JA, Keough RA, Morris MB, Rathjen PD. L-Proline induces differentiation of ES cells: a novel role for an amino acid in the regulation of pluripotent cells in culture. *Am J Physiol-Cell Physiol* 2010; **298**:C982–C992.
102. Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, Zhang J, Onder T, Unternaehrer JJ, Zhu H, Asara JM, Daley GQ et al. Influence of threonine metabolism on S-adenosylmethionine and histone methylation. *Science* 2013; **339**:222–226.
103. Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem* 2005; **280**:18361–18367.
104. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 2002; **17**:999–1005.
105. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 2004; **19**:2319–2324.
106. Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998; **13**:991–997.
107. Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod* 2015; **30**:543–552.
108. Desai N, Goldberg JM, Austin C, Falcone T. Are cleavage anomalies, multinucleation, or specific cell cycle kinetics observed with time-lapse imaging predictive of embryo developmental capacity or ploidy? *Fertil Steril* 2018; **109**:665–674.
109. Seli E, Sakkas D, Scott R, Kwok SC, Rosendahl SM, Burns DH. Noninvasive metabolomic profiling of embryo culture media using Raman and near-infrared spectroscopy correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil Steril* 2007; **88**:1350–1357.
110. Scott R, Seli E, Miller K, Sakkas D, Scott K, Burns DH. Noninvasive metabolomic profiling of human embryo culture media using Raman spectroscopy predicts embryonic reproductive potential: a prospective blinded pilot study. *Fertil Steril* 2008; **90**:77–83.
111. Vergouw CG, Botros LL, Roos P, Lens JW, Schats R, Hompes PG, Burns DH, Lambalk CB. Metabolomic profiling by near-infrared spectroscopy as a tool to assess embryo viability: a novel, non-invasive method for embryo selection. *Hum Reprod* 2008; **23**:1499–1504.
112. Seli E, Vergouw CG, Morita H, Botros L, Roos P, Lambalk CB, Yamashita N, Kato O, Sakkas D. Noninvasive metabolomic profiling as an adjunct to morphology for noninvasive embryo assessment in women undergoing single embryo transfer. *Fertil Steril* 2010; **94**:535–542.
113. Ahlstrom A, Wikland M, Rogberg L, Barnett JS, Tucker M, Hardarson T. Cross-validation and predictive value of near-infrared spectroscopy algorithms for day-5 blastocyst transfer. *Reprod Biomed Online* 2011; **22**:477–484.
114. Hardarson T, Ahlstrom A, Rogberg L, Botros L, Hillensjo T, Westlander G, Sakkas D, Wikland M. Non-invasive metabolomic profiling of Day 2 and 5 embryo culture medium: a prospective randomized trial. *Hum Reprod* 2012; **27**:89–96.
115. Vergouw CG, Kieslinger DC, Kostelijk EH, Botros LL, Schats R, Hompes PG, Sakkas D, Lambalk CB. Day 3 embryo selection by metabolomic profiling of culture medium with near-infrared spectroscopy as an adjunct to morphology: a randomized controlled trial. *Hum Reprod* 2012; **27**:2304–2311.
116. Uyar A, Seli E. Metabolomic assessment of embryo viability. *Semin Reprod Med* 2014; **32**:141–152
117. Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod* 2000; **62**:16–22.
118. Dumollard R, Hammar K, Porterfield M, Smith PJ, Cibert C, Rouvière C, Sardet C. Mitochondrial respiration and Ca2+ waves are linked during fertilization and meiosis completion. *Development* 2003; **130**:683–692.
119. Sutton-McDowall ML, Purdey M, Brown HM, Abell AD, Mottershead DG, Cetica PD, Dalvit GC, Goldys EM, Gilchrist RB, Gardner DK. Redox and anti-oxidant state within cattle oocytes following in vitro maturation with bone morphogenetic protein 15 and follicle stimulating hormone. *Mol Reprod Dev* 2015; **82**:281–294.
120. Cinco R, Digman MA, Gratton E, Luderer U. Spatial characterization of bioenergetics and metabolism of primordial to preovulatory follicles in whole ex vivo murine ovary. *Biol Reprod* 2016; **95**:129–129.
121. Walsh AJ, Cook RS, Manning HC, Hicks DJ, Lafontant A, Arteaga CL, Skala MC. Optical metabolic imaging identifies glycolytic levels, subtypes, and early-treatment response in breast cancer. *Cancer Res* 2013; **73**:6164–6174.
122. Quinn KP, Sridharan GV, Hayden RS, Kaplan DL, Lee K, Georgakoudi I. Quantitative metabolic imaging using endogenous fluorescence to detect stem cell differentiation. *Sci Rep* 2013; **3**:3432.
123. Sanchez T, Wang T, Pedro MV, Zhang M, Esencan E, Sakkas D, Needleman D, Seli E. Metabolic imaging with the use of fluorescence lifetime imaging microscopy (FLIM) accurately detects mitochondrial dysfunction in mouse oocytes. *Fertil Steril* 2018; **110**:1387–1397
124. Sanchez T, Seidler EA, Gardner DK, Needleman D, Sakkas D. Will noninvasive methods surpass invasive for assessing gametes and embryos? *Fertil Steril* 2017; **108**:730–737.
125. Gosnell ME, Anwer AG, Mahbub SB, Perinchery SM, Inglis DW, Adhikary PP, Jazayeri JA, Cahill MA, Saad S, Pollock CA. Quantitative non-invasive cell characterisation and discrimination based on multispectral autofluorescence features. *Sci Rep* 2016; **6**:23453.
126. Sutton-McDowall ML, Gosnell M, Anwer AG, White M, Purdey M, Abell AD, Goldys EM, Thompson JG. Hyperspectral microscopy can detect metabolic heterogeneity within bovine post-compaction embryos incubated under two oxygen concentrations (7% versus 20%). *Hum Reprod* 2017; **32**:2016–2025.
127. Murata S, Herman P, Lakowicz JR. Texture analysis of fluorescence lifetime images of AT-and GC-rich regions in nuclei. *J Histochem Cytochem* 2001; **49**:1443–1451.
128. Castellano G, Bonilha L, Li L, Cendes F. Texture analysis of medical images. *Clin Radiol* 2004; **59**:1061–1069.
129. Alvarenga AV, Pereira WC, Infantosi AFC, Azevedo CM. Complexity curve and grey level co-occurrence matrix in the texture evaluation of breast tumor on ultrasound images. *Med Phys* 2007; **34**:379–387.
130. Fischer B, Schumacher A, Hegele-Hartung C, Beier HM. Potential risk of light and room temperature exposure to preimplantation embryos. *Fertil Steril* 1988; **50**:938–944.
131. Oh SJ, Gong SP, Lee ST, Lee EJ, Lim JM. Light intensity and wavelength during embryo manipulation are important factors for maintaining viability of preimplantation embryos in vitro. *Fertil Steril* 2007; **88**:1150–1157.
132. Takenaka M, Horiuchi T, Yanagimachi R. Effects of light on development of mammalian zygotes. *Proc Natl Acad Sci USA* 2007; **104**:14289–14293.
133. Urbanski JP, Johnson MT, Craig DD, Potter DL, Gardner DK, Thorsen T. Noninvasive metabolic profiling using microfluidics for analysis of single preimplantation embryos. *Anal Chem* 2008; **80**:6500–6507.
134. Knowlton SM, Sadasivam M, Tasoglu S. Microfluidics for sperm research. *Trends Biotechnol* 2015; **33**:221–229.
135. Heo YS, Cabrera LM, Bormann CL, Smith GD, Takayama S. Real time culture and analysis of embryo metabolism using a microfluidic device with deformation based actuation. *Lab Chip* 2012; **12**:2240–2246.
136. West J, Becker M, Tombrink S, Manz A. Micro total analysis systems: latest achievements. *Anal Chem* 2008; **80**:4403–4419.
137. Liu P, Mathies RA. Integrated microfluidic systems for high-performance genetic analysis. *Trends Biotechnol* 2009; **27**:572–581.

138. Njoroge SK, Chen H-W, Witek MA, Soper SA. Integrated microfluidic systems for DNA analysis. *Top Curr Chem* 2011; **304**:203–260.
139. Chen S, Sung W, Lee G, Lin Z, Chen P, Liao P. A disposable poly(methylmethacrylate)-based microfluidic module for protein identification by nanoelectrospray ionization-tandem mass spectrometry. *Electrophoresis* 2001; **22**:3972–3977.
140. Gao J, Xu J, Locascio LE, Lee CS. Integrated microfluidic system enabling protein digestion, peptide separation, and protein identification. *Anal Chem* 2001; **73**:2648–2655.
141. Xue Q, Wainright A, Gangakhedkar S, Gibbons I. Multiplexed enzyme assays in capillary electrophoretic single-use microfluidic devices. *Electrophoresis* 2001; **22**:4000–4007.
142. Wheeler AR, Moon H, Kim C-JC, Loo JA, Garrell RL. Electrowetting-based microfluidics for analysis of peptides and proteins by matrix-assisted laser desorption/ionization mass spectrometry. *Anal Chem* 2004; **76**:4833–4838.
143. Herrmann M, Veres T, Tabrizian M. Enzymatically-generated fluorescent detection in micro-channels with internal magnetic mixing for the development of parallel microfluidic ELISA. *Lab Chip* 2006; **6**:555–560.
144. Young JB, Li L. An impulse-driven liquid-droplet deposition interface for combining LC with MALDI MS and MS/MS. *J Am Soc Mass Spectrom* 2006; **17**:325–334.
145. Hoegger D, Morier P, Vollet C, Heini D, Reymond F, Rossier JS. Disposable microfluidic ELISA for the rapid determination of folic acid content in food products. *Anal Bioanal Chem* 2006; **387**:267–275.
146. Cui X, Lee LM, Heng X, Zhong W, Sternberg PW, Psaltis D, Yang C. Lensless high-resolution on-chip optofluidic microscopes for *Caenorhabditis elegans* and cell imaging. *Proc Natl Acad Sci USA* 2008; **105**:10670–10675.
147. Cantó C, Menzies KJ, Auwerx J. NAD+ metabolism and the control of energy homeostasis: a balancing act between mitochondria and the nucleus. *Cell Metab* 2015; **22**:31–53.
148. Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev* 2005; **17**:371–378.
149. Chason RJ, Csokmay J, Segars JH, DeCherney AH, Armant DR. Environmental and epigenetic effects upon preimplantation embryo metabolism and development. *Trends Endocrinol Metab* 2011; **22**:412–420.
150. Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod* 2013; **89**:75.
151. Domínguez F, Gadea B, Mercader A, Esteban FJ, Pellicer A, Simón C. Embryologic outcome and secretome profile of implanted blastocysts obtained after coculture in human endometrial epithelial cells versus the sequential system. *Fertil Steril* 2010; **93**:774–782. e771.
152. Gardner DK, Leese HJ. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. *Hum Reprod* 1986; **1**:25–27.
153. Turner K, Martin K, Woodward B, Lenton E, Leese HJ. Fertilization and early embryology: Comparison of pyruvate uptake by embryos derived from conception and non-conception natural cycles. *Hum Reprod* 1994; **9**:2362–2366.
154. Seli E, Botros L, Sakkas D, Burns DH. Noninvasive metabolomic profiling of embryo culture media using proton nuclear magnetic resonance correlates with reproductive potential of embryos in women undergoing in vitro fertilization. *Fertil Steril* 2008; **90**:2183–2189.
155. Pudakalakatti SM, Uppangala S, D'Souza F, Kalthur G, Kumar P, Adiga SK, Atreya HS. NMR studies of preimplantation embryo metabolism in human assisted reproductive techniques: a new biomarker for assessment of embryo implantation potential. *NMR Biomed* 2012; **26**:20–27.
156. Rinaudo P, Shen S, Hua J, Qian S, Prabhu U, Garcia E, Cedars M, Sukumaran D, Szyperski T, Andrews C. 1 H NMR based profiling of spent culture media cannot predict success of implantation for day 3 human embryos. *J Assist Reprod Genet* 2012; **29**:1435–1442.
157. Wallace M, Cottell E, Cullinane J, McAuliffe FM, Wingfield M, Brennan L. 1H NMR based metabolic profiling of day 2 spent embryo media correlates with implantation potential. *Syst Biol Reprod Med* 2014; **60**:58–63.
158. Kirkegaard K, Svane A, Nielsen J, Hindkjaer J, Nielsen NC, Ingerslev HJ. Nuclear magnetic resonance metabolomic profiling of Day 3 and 5 embryo culture medium does not predict pregnancy outcome in good prognosis patients: a prospective cohort study on single transferred embryos. *Hum Reprod* 2014; **29**:2413–2420.

Appendix C

Ferrick L, Lee YSL, Gardner DK. Metabolic activity of human blastocysts correlates with their morphokinetics, morphological grade, KIDScore and artificial intelligence ranking. *Hum Reprod* 2020; In press.

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ORIGINAL ARTICLE *Embryology*

Metabolic activity of human blastocysts correlates with their morphokinetics, morphological grade, KIDScore and artificial intelligence ranking

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STUDY QUESTION: Is there a relationship between blastocyst metabolism and biomarkers of embryo viability?

SUMMARY ANSWER: Blastocysts with higher developmental potential and a higher probability of resulting in a viable pregnancy consume higher levels of glucose and exhibit distinct amino acid profiles.

WHAT IS KNOWN ALREADY: Morphological and morphokinetic analyses utilized in embryo selection provide insight into developmental potential, but alone are unable to provide a direct measure of embryo physiology and inherent health. Glucose uptake is a physiological biomarker of viability and amino acid utilization is different between embryos of varying qualities.

STUDY DESIGN, SIZE, DURATION: Two hundred and nine human preimplantation embryos from 50 patients were cultured in a time-lapse incubator system in both freeze all and fresh transfer cycles. A retrospective analysis of morphokinetics, morphology (Gardner grade), KIDScore, artificial intelligence grade (EmbryoScore), glucose and amino acid metabolism, and clinical pregnancies was conducted.

PARTICIPANTS/MATERIALS, SETTING, METHODS: ICSI was conducted in all patients, who were aged ≤37 years and previously had no more than two IVF cycles. Embryos were individually cultured in a time-lapse incubator system, and those reaching the blastocyst stage had their morphokinetics annotated and were each assigned a Gardner grade, KIDScore and EmbryoScore. Glucose and amino acid metabolism were measured. Clinical pregnancies were confirmed by the presence of a fetal heartbeat at 6 weeks of gestation.

MAIN RESULTS AND THE ROLE OF CHANCE: Glucose consumption was at least 40% higher in blastocysts deemed of high developmental potential using either the Gardner grade ($P < 0.01$, n = 209), KIDScore ($P < 0.05$, n = 207) or EmbryoScore ($P < 0.05$, n = 184), compared to less viable blastocysts and in blastocysts that resulted in a clinical pregnancy compared to those that failed to implant ($P < 0.05$, n = 37). Additionally, duration of cavitation was inversely related to glucose consumption ($P < 0.05$, n = 200). Total amino acid consumption was significantly higher in blastocysts with an EmbryoScore higher than the cohort median score ($P < 0.01$, n = 185). Furthermore, the production of amino acids was significantly lower in blastocysts with a high Gardner grade ($P < 0.05$, n = 209), KIDScore ($P < 0.05$, n = 207) and EmbryoScore ($P < 0.01$, n = 184).

LIMITATIONS, REASONS FOR CAUTION: Samples were collected from patients who had ICSI treatment and from only one clinic.

WIDER IMPLICATIONS OF THE FINDINGS: These results confirm that metabolites, such as glucose and amino acids, are valid biomarkers of embryo viability and could therefore be used in conjunction with other systems to aid in the selection of a healthy embryo.

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Key words: embryo / glucose / amino acids / non-invasive / selection / viability

Introduction

Historically, transferring multiple embryos in an IVF cycle was performed to attain acceptable success rates. However, transferring two or more embryos creates the possibility of a multiple gestation, which carries significantly greater medical risks to both mother and child (Albrecht and Tomich, 1996; Luke *et al.*, 1996; Adashi *et al.*, 2003; Ombelet *et al.*, 2005). Improvements in embryo culture systems facilitated single embryo transfers, reducing the incidence of multiple gestations (Gerris *et al.*, 1999) and in the last 20 years, there has been a shift to single blastocyst transfers (Gardner *et al.*, 1998; 2000; Harbottle *et al.*, 2015; Takeshima *et al.*, 2016; Penzias *et al.*, 2017). While pregnancy rates worldwide have increased largely due to improvements in culture conditions, our ability to decrease time to pregnancy is dependent upon our capabilities to select the most viable blastocyst for transfer.

Traditionally, embryos are graded on morphological features leading to the creation of scoring systems for each successive stage of development. Parameters assessed include blastomere number, symmetry and degree of fragmentation during the cleavage stages (Cummins *et al.*, 1986; Gerris *et al.*, 1999; Van Royen *et al.*, 1999), as well as size of blastocoel, and inner cell mass (ICM) and trophectoderm (TE) differentiation in the case of the blastocyst (Gardner *et al.*, 2000). Traditional morphological grading requires removal of the embryo culture dish from the incubator for assessment under the microscope, thereby exposing embryos to fluctuations in the environment such as light, temperature and pH, which can adversely impact embryo physiology and developmental potential (Lane and Gardner, 2005; Hansen, 2007; Zander-Fox *et al.*, 2010; Wale and Gardner, 2016; Gardner and Kelley, 2017). Additionally, such an approach to grading is, by necessity, restricted to only a handful of discrete time points during development, thereby failing to capture the true dynamic nature of preimplantation embryo development (Montag *et al.*, 2011).

With the advent of time-lapse systems, preimplantation embryo development can be captured continuously, whilst also providing an uninterrupted culture environment. Time-lapse has subsequently facilitated identification of novel morphological events, such as reverse and direct cleavage, both of which have been linked to decreased embryo viability (Rubio *et al.*, 2012; Liu *et al.*, 2014). Annotating the timing of key morphological events and the rate of development (i.e. the time between two morphological events) has in turn led to the development of algorithms for embryo selection incorporating both morphological and morphokinetic parameters (Rubio *et al.*, 2014; Basile *et al.*, 2015; Milewski *et al.*, 2015; Liu *et al.*, 2016; Petersen *et al.*, 2016; Reignier *et al.*, 2019). Annotating morphokinetic parameters for each individual embryo is, however, time-consuming and a level of inter-observer and intra-observer subjectivity exists impacting overall grading success (Storr *et al.*, 2017).

Artificial intelligence (AI) is relatively new to the field of assisted reproductive technologies and represents a potential means of standardizing and automating embryo selection. AI can be trained to assess embryo viability without preset assumptions, thereby removing the subjectivity previously associated with embryo grading (Curchoe and Bormann, 2019). Such deep-learning has been applied to a single static image during the preimplantation period (Chen *et al.*, 2009; Miyagi *et al.*, 2019; Thirumalaraju *et al.*, 2019), while other groups have utilized images from across the entire preimplantation period facilitated by time-lapse microscopy, thereby significantly increasing the amount of data used to identify embryos with the highest viability (Tran *et al.*, 2019). Such technologies hold great promise and the clinical introduction of AI awaits validation in prospective randomized controlled trials.

While assessment of morphology and morphokinetics is of value in deselection and selection of embryos for transfer, they are unable to provide a direct measure of embryo physiology (Paternot *et al.*, 2011; Santos Filho *et al.*, 2012; VerMilyea *et al.*, 2014). Metabolic regulation is essential for embryo development, and aberrations in preimplantation embryo metabolism are an indicator of lower postimplantation viability (Gardner, 1998; Gardner and Wale, 2013). Importantly, embryos that have been assigned the same morphological grade do not necessarily exhibit the same metabolic profile (Gardner *et al.*, 2001). Analysis of embryo metabolism can therefore provide additional information regarding embryo viability subsequently aiding embryo selection. To date, studies on human embryos have focused on the utilization of carbohydrates or amino acids (Gott *et al.*, 1990; Gardner *et al.*, 2001; Houghton *et al.*, 2002; Gardner *et al.*, 2011). However, other metabolites including lactate, lipids and oxygen (the consumption of which can provide an indirect measure of oxidative metabolism) which have been analyzed in animal models, may also be of value in establishing the metabolic state and health of the human embryo (Lane and Gardner, 1996; Thompson *et al.*, 1996; Sturmey *et al.*, 2009).

The metabolic profile of an embryo can be determined through the non-invasive analysis of changes in metabolite concentrations within spent culture medium (Leese and Barton, 1984; Houghton *et al.*, 2002). It has been demonstrated that high levels of glucose consumption are associated with higher development and implantation potential in human, mouse and cow blastocysts (Renard *et al.*, 1980; Gardner and Leese, 1987; Lane and Gardner, 1996; Gardner *et al.*, 2001; 2011; Lee *et al.*, 2015; Kelley and Gardner, 2019). Additionally, amino acid profiles are distinctly different between embryos of different developmental stages and quality (Houghton *et al.*, 2002; Brison *et al.*, 2004; Picton *et al.*, 2010; Wale and Gardner, 2012; Lee *et al.*, 2015; Kelley and Gardner, 2019). These data therefore support the possible use of both glucose and amino acids as biomarkers to evaluate embryo physiology and developmental potential prior to transfer.

Furthermore, metabolism is directly linked to gene expression and developmental programming through the metaboloepigenetic axis, in which metabolites and cofactors regulate the activity of epigenetic modifiers (Donohoe and Bultman, 2012; Gardner and Harvey, 2015; Harvey *et al.*, 2016). As distinct metabolic profiles of an embryo have been shown to manifest as changes in development post-transfer (Lane and Gardner, 1996; 1998; Mitchell *et al.*, 2009), the utilization and analysis of metabolic biomarkers would aid in the selection of embryos with normal preimplantation physiology and subsequently increase the likelihood of healthy offspring (Barker and Osmond, 1986; Barker *et al.*, 1989; Gardner and Kelley, 2017; Ferrick *et al.*, 2019). It is currently unknown if metabolism is linked to existing embryo viability markers. Consequently, the aim of this study was to determine how blastocyst glucose and amino acid metabolism, as a measure of embryonic viability and health, relates to embryonic developmental parameters and transfer outcomes.

Materials and methods

Patient selection and ovarian stimulation

Fifty patients aged ≤37 years, with no more than two previous IVF cycles, undergoing an ICSI cycle between January 2018 and July 2019 were included in this study. Women underwent a gonadotropin-releasing hormone (GnRH) antagonist stimulation protocol. Ovarian stimulation began on the second day of menstrual cycle with recombinant FSH injections (Gonal F, Merck Australia). A GnRH antagonist (Cetrotide, Merck Australia) was administered on Days 4–5 of stimulation to prevent premature ovulation. Transvaginal ultrasound and serum estradiol levels were used to monitor cycles. Upon leading follicles reaching ~17 mm in size, a trigger hCG injection (Ovidrel, Merck Australia) was administered and oocyte collection performed 36 h post-trigger.

Embryo culture

Two hours following oocyte retrieval oocytes were denuded of surrounding cumulus cells using a hyaluronidase solution (SynVitro Hyadase, CooperSurgical, CT, USA) and inseminated via ICSI 2 h later. Injected oocytes were immediately transferred to individual culture wells containing 25 µl G-TL medium overlaid with 1.4 ml Ovoil (Vitrolife AB, Sweden) in a pre-equilibrated EmbryoSlide® culture dish (Vitrolife AB, Sweden). Embryos were cultured in an EmbryoScope® time-lapse incubator (Vitrolife AB, Sweden) at 5% O_2, 6% CO_2, 89% N_2 at 37°C and time of ICSI was set as insemination time. At 90 and 114 h postinsemination, embryos were washed in culture medium and transferred to a new pre-equilibrated EmbryoSlide® culture dish containing 15 µl G-TL medium in each well overlaid with 1.4 ml Ovoil. On Day 5 of development, embryos were assessed for transfer or vitrification, or cultured until Day 6 where they were either vitrified or discarded accordingly. The 90–114 h culture dishes containing spent media were stored at −20°C until collection for metabolic analysis. Two hundred and nine embryos that reached the blastocyst stage during culture (i.e. by Day 6) were retrospectively analyzed for morphological grade, morphokinetics, metabolism and transfer success.

Analysis of morphology

Morphological assessment was conducted at 115 h postinsemination. Blastocyst expansion and ICM and TE quality were graded according to the Gardner grading system (Gardner et al., 2000). Embryos graded higher than BB were considered to be of higher viability than embryos graded BB or less (Gardner et al., 2004). Blastocyst diameter was measured (115 h postinsemination) using the EmbryoViewer® software diameter tool (version 7.5.201.20844, Vitrolife AB, Sweden) and calculated as the distance between the outer borders of the TE (Almagor et al., 2016).

Analysis of morphokinetics

Morphokinetic analysis was achieved using EmbryoViewer® software to assess images captured by the EmbryoScope® time-lapse incubator every 10 min in seven focal planes. The specific timings of two-cell cleavage (t2), three-cell cleavage (t3), four-cell cleavage (t4), five-cell cleavage (t5), six-cell cleavage (t6), seven-cell cleavage (t7), eight-cell cleavage (t8), morulae (tM), start of blastulation (tSB), blastocyst (tB), expanded blastocyst (tEB) and hatching blastocyst (tH) were recorded as hours postinsemination (Desai et al., 2014). Annotations were recorded for embryos which could be accurately assessed. KIDScore values (0–9.9) predict the probability that an embryo will successfully implant (Reignier et al., 2019) and are calculated utilizing the D5 KIDScore algorithm (version 2), which is integrated into the EmbryoViewer® software and available for use clinically. Embryos assigned a KIDScore greater than the median cohort score (6.9) were considered to be of higher viability than embryos assigned a lower KIDScore. Of the 209 embryos cultured, two embryos where annotations could not be accurately assessed were not included in analysis. Additionally, rate of expansion (tEB-tSB) was calculated and embryos were grouped into fast (≤13 h) or slow (>13 h) expansion rates as previously described (Desai et al., 2018). To minimize inter-user variability, only one scientist conducted the annotation assessments.

AI selection

Videos of preimplantation development were exported from EmbryoViewer® software (0–115 h postinsemination) and uploaded into IVY (version 1.0.0, Harrison AI, Sydney, AUS) an AI program, which assesses preimplantation embryo development across a single focal plane and calculates an EmbryoScore (%) which represents the probability that an embryo will develop a fetal heartbeat and can subsequently assist in ranking embryos for transfer (Tran et al., 2019). Embryos assigned an EmbryoScore greater than the median cohort score (29%) were considered to be of higher viability than embryos assigned a lower EmbryoScore. Of the 209 embryos cultured, 25 embryos could not be included in analysis as a result of an interruption in the time-lapse video, such as the embryo went out of focus or oil bubbles in the culture well. Such factors can impact the overall EmbryoScore.

Metabolic analysis

Day 5 (90–114 h) spent media samples were collected and analyzed for both glucose and amino acid metabolism to provide a detailed metabolic profile for each individual embryo that reached the blastocyst stage. Spent media samples (test) and no-embryo (control) media were collected for each patient and metabolite consumption/production was calculated by subtracting test samples from controls.

Glucose uptake

Ultramicrofluoresence (UMF), a miniaturization of conventional enzymatic analysis, was utilized to quantitate glucose metabolism (Leese and Barton, 1984; Gardner et al., 2001).

Amino acid utilization

Liquid chromatography-mass spectrometry (LC-MS) analysis using an Agilent 6490 Triple Quadrupole LC-MS with iFunnel Technology coupled with an Agilent 1290 liquid chromatography system (Agilent, Santa Clara, CA, USA) was utilized to measure amino acid concentrations when compared against a standard calibrated curve (Wale and Gardner, 2012). Pooled quality controls and pooled batch quality controls were conducted to ensure uniformity within and between batches and Agilent MassHunter Quantitative Analysis software (B.08.0, Agilent, CA, USA) was

used to calculate relative abundances (integrated area) of amine-containing metabolites.

Pregnancy assessment

Of the 209 embryos cultured, 37 single blastocysts were transferred in a fresh cycle and the remaining blastocysts were vitrified as part of a freeze-all cycle. The embryo with the best morphology, followed by the highest KIDScore amongst a cohort was selected for transfer. Only embryos transferred in a fresh cycle were included in pregnancy rate analysis. Biochemical pregnancy was recorded after an hCG blood test (positive pregnancy was an hCG level >5 IU/L), and clinical pregnancies were later confirmed at a 6 week ultrasound and observation of a fetal heartbeat.

Statistical analysis

For morphokinetic analysis blastocysts were grouped according to the median glucose and amino acid utilization. To analyse blastocyst metabolism in comparison to KIDScore and EmbryoScore, embryos were grouped based on the median scores (6.9 and 29%, respectively). Embryos were grouped as per Gardner grade (higher than BB or BB and less) for analysis of blastocyst metabolism and morphological grade. Data are expressed as mean ± SEM and tested for normality using the Shapiro–Wilk test. The difference between two groups was determined using parametric Student's *t*-test or nonparametric Mann–Whitney *U* test and the *P*-value threshold was set to 0.05. All analyses were conducted using Prism 8 (version 8.3.0, GraphPad, LLC).

Ethical approval

Ethical approval was obtained from Melbourne IVF Human Research Ethics Committee (53/117-MIVF), patients were recruited during clinical consultation.

Results

Blastocyst metabolism is positively related to embryo viability scores

Three different grading systems were used to retrospectively assess embryo viability prior to transfer: (i) morphological grading using the Gardner grading system, (ii) D5 KIDScore algorithm and (iii) EmbryoScores through an AI system (IVY). Regardless of which method embryo viability was measured, blastocysts with the highest viability consumed at least 40% more glucose than lower-quality blastocysts (Fig. 1). Blastocysts with a Gardner grade greater than a BB grade (n = 86) consumed significantly more glucose on Day 5 of development than less viable blastocysts which scored a BB grade or less (n = 123) (91.7 ± 8.5 versus 61.4 ± 5.9 pmol/embryo/h, $P < 0.01$) (Fig. 1A). Similarly, blastocysts that were assigned a KIDScore greater than the median cohort score (n = 100) consumed more glucose on Day 5 than lower scoring blastocysts (n = 107) (88.1 ± 7.8 versus 60.9 ± 6.3 pmol/embryo/h, $P < 0.05$) (Fig. 1B) and blastocysts with an EmbryoScore greater than the median cohort score (n = 91) (i.e. those with a higher probability of developing a fetal heartbeat) consumed more glucose on Day 5 of development compared to less viable blastocysts (n = 93) (87.9 ± 8.1 versus 61.7 ± 7.4 pmol/embryo/h, $P < 0.05$) (Fig. 1C). Of those embryos with a high KIDScore and an EmbryoScore (n = 83), 77% had a high EmbryoScore (n = 64). Of these embryos with both a high KIDScore and EmbryoScore, 64% consumed more glucose than the median cohort uptake (n = 41).

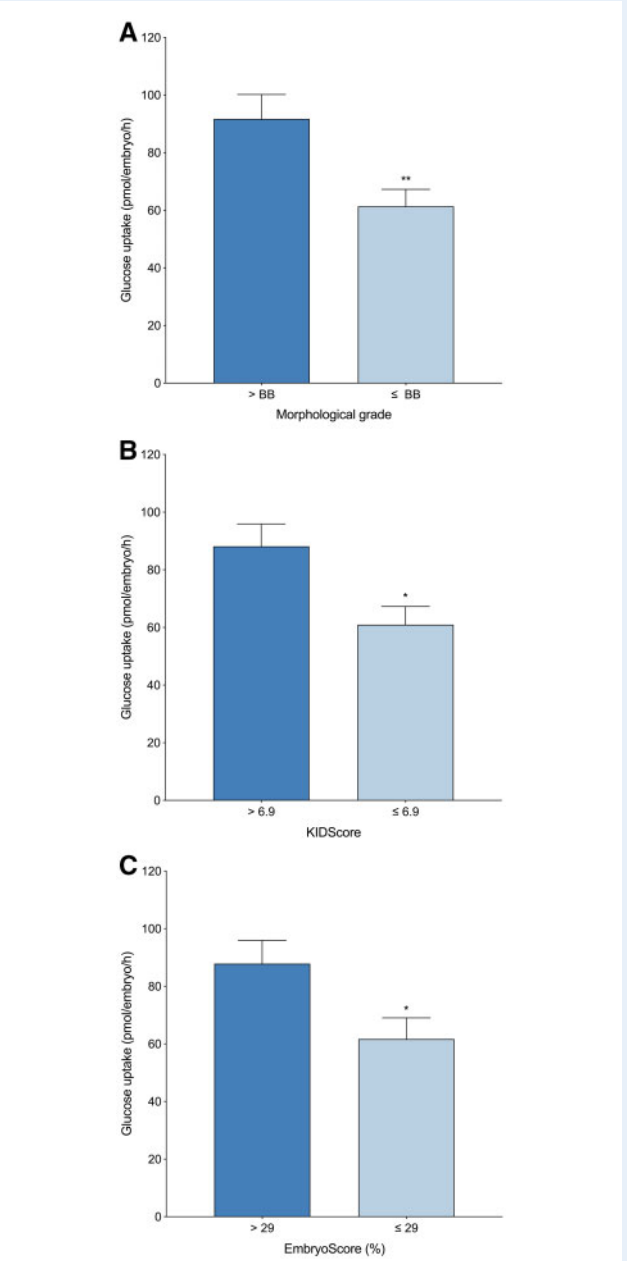

Figure 1. Blastocyst glucose uptake compared to embryo viability. (**A**) Gardner grade (>BB, n = 86; ≤BB, n = 123), (**B**) KIDScore (>6.9, n = 100; ≤6.9, n = 107) and (**C**) EmbryoScore (>29%, n = 91; ≤29%, n = 93). Each data set (A–C) is expressed as mean ± SEM (pmol/embryo/h). Significance is measured utilizing Mann–Whitney U test and denoted by asterisks, *$P < 0.05$, **$P < 0.01$.

Blastocyst glucose metabolism is related to clinical pregnancy rates

Blastocysts that resulted in a clinical pregnancy demonstrated a 2-fold increase in glucose consumption on Day 5 of the preimplantation period than blastocysts that failed to implant or maintain a successful pregnancy (77.4 ± 11.7 versus 38.4 ± 11.2 pmol/embryo/h, $P < 0.05$) (Fig. 2). Of note, blastocysts assigned an AA morphological grade which established a clinical pregnancy consumed the highest level of glucose (84.4 ± 17.3 pmol/embryo/h, n = 11). Additionally, while results were not statistically significant, blastocysts assigned an AA morphological grade which failed to establish a pregnancy (n = 7) consumed lower glucose than blastocysts assigned a B grade for ICM and/or TE (AB, BA or BB) that successfully implanted (n = 6) (22.2 ± 25.3 pmol/embryo/h versus 64.5 ± 9.1 pmol/embryo/h) ($P = 0.12$). No significant difference was observed in amino acid utilization profiles between embryos that established a clinical pregnancy and embryos that failed to implant (data not shown).

Amino acid profiles of blastocysts are distinctly different according to embryo viability scores

Blastocysts with high viability according to their Gardner grade consumed significantly higher levels of arginine ($P < 0.05$), aspartate ($P < 0.05$), glutamate ($P < 0.05$), isoleucine ($P < 0.05$), leucine ($P < 0.05$), lysine ($P < 0.05$) and threonine ($P < 0.05$) and produced significantly lower levels of alanine ($P < 0.01$) and glutamine ($P < 0.0001$), compared to blastocysts assigned a lower Gardner grade (Fig. 3A). Similarly, these same amino acids as well as asparagine, phenylalanine, taurine and tryptophan were utilized differently by blastocysts that had a high implantation potential, as assessed by D5 KIDScore algorithm, compared to less viable blastocysts (alanine, $P < 0.01$; arginine, $P < 0.05$; asparagine, $P < 0.05$; aspartate, $P < 0.05$; glutamine, $P < 0.01$; glutamate, $P < 0.05$; isoleucine, $P < 0.05$; leucine, $P < 0.05$; lysine, $P < 0.05$; phenylalanine, $P < 0.05$; taurine, $P < 0.05$; threonine, $P < 0.05$; tryptophan, $P < 0.05$) (Fig. 3B). Furthermore, each amino acid with the exception of alanyl-glutamine, glycine and valine were either consumed at higher levels or produced at lower levels by blastocysts with a higher EmbryoScore than less viable blastocysts (alanine, $P < 0.001$; arginine, $P < 0.01$; asparagine, $P < 0.01$; aspartate, $P < 0.01$; glutamine, $P < 0.001$; glutamate, $P < 0.001$; histidine, $P < 0.05$; isoleucine, $P < 0.05$; leucine, $P < 0.01$; lysine, $P < 0.01$; methionine, $P < 0.05$; phenylalanine, $P < 0.05$; proline, $P < 0.05$; serine, $P < 0.01$; taurine, $P < 0.01$; threonine, $P < 0.01$; tryptophan, $P < 0.01$; tyrosine, $P < 0.05$) (Fig. 3C).

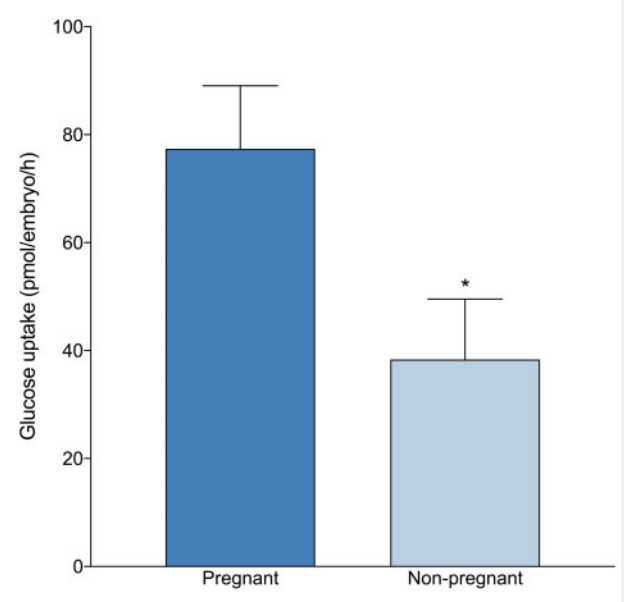

Figure 2. Glucose uptake of blastocysts that were transferred in a fresh single blastocyst transfer cycle and ongoing pregnancy rates. All data are expressed as mean ± SEM (pmol/embryo/h) for pregnant (n = 17) and non-pregnant (n = 20). Significance is measured utilizing Student's *t*-test and asterisks denote significance, *$P < 0.05$.

Blastocyst total amino acid consumption and production are related to embryo viability scores

Overall amino acid turnover (sum of consumption and production) was analyzed as a further measure of blastocyst metabolic activity. There was no significant difference in amino acid turnover between blastocysts of high and low viability when assessed using Gardner grade, KIDScore or EmbryoScore (Fig. 4). However, the total production of amino acids on Day 5 of preimplantation development was significantly lower in blastocysts with high viability, as determined by Gardner grade (171.6 ± 30.5 versus 210.2 ± 24.1 pmol/embryo/h, $P < 0.05$), D5 KIDScore algorithm (166.1 ± 27.0 versus 221.1 ± 26.6 pmol/embryo/h, $P < 0.05$) and EmbryoScore (160.5 ± 26.4 versus 235.9 ± 30.4 pmol/embryo/h, $P < 0.01$) (Fig. 4). Additionally, blastocysts with high EmbryoScores also consume significantly more amino acids overall compared to less viable blastocysts (245.2 ± 28.6 versus 134.7 ± 20.6 pmol/embryo/h, $P < 0.01$) (Fig. 4C).

Fast developing embryos have different Day 5 metabolic profiles compared to slow developing embryos

Embryos that consumed more than the median amount of glucose (61 pmol/embryo/h) on Day 5 of development developed faster across all morphokinetic parameters (with the exception of t3, t5, t8 and tH) compared to embryos that consumed lower levels of glucose on Day 5 of development (Fig. 5A). A delay of ~1 h during the early cleavage stages was observed in embryos that consumed lower levels of glucose on Day 5, and this delay was extended to 2–4 h by the blastocyst stage (Fig. 5A). Furthermore, embryos with an expansion rate (tEB-tSB) of 13 h or less consumed significantly higher levels of glucose on Day 5 of development compared to embryos that took longer than 13 h to fully expand (85.0 ± 6.9 versus 61.6 ± 9.2 pmol/embryo/h, $P < 0.05$) (Fig. 5B). Blastocysts with a diameter greater than the mean of 151 µm (n = 96) consumed significantly more glucose than smaller blastocysts (n = 104) (94.7 ± 8.1 versus 56.2 ± 6.6 pmol/embryo/h, $P < 0.001$), with a significant linear relationship between blastocyst size and glucose uptake ($P < 0.001$).

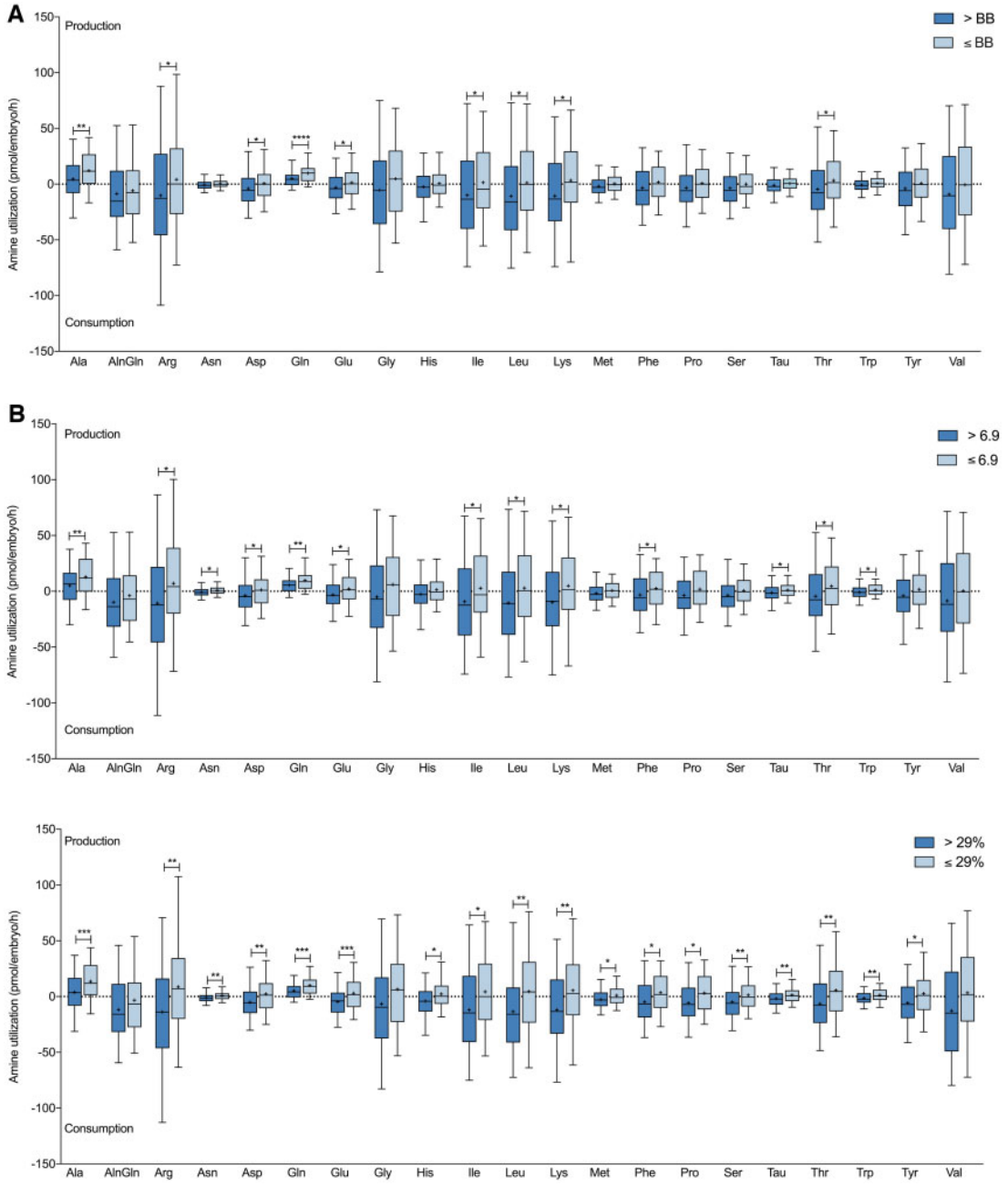

Figure 3. Blastocyst amino acid utilization profiles compared to embryo viability. (**A**) Gardner grade (>BB, n = 86; ≤BB, n = 123), (**B**) KIDScore (>6.9, n = 100; ≤6.9, n = 107) and (**C**) EmbryoScore (>29%, n = 91; ≤29%, n = 93). Negative values represent amino acid consumption and positive values represent amino acid production. Alanine (Ala), alanyl-glutamine (AlnGln), arginine (Arg), asparagine (Asn), aspartate (Asp), glutamine (Gln), glutamate (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), taurine (Tau), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val). Boxes represent 25th to 75th percentile, whiskers represent 5th to 95th percentile, '+' represents mean, and median represented by horizontal line. Significance is measured utilizing Student's *t*-test and denoted by asterisks, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

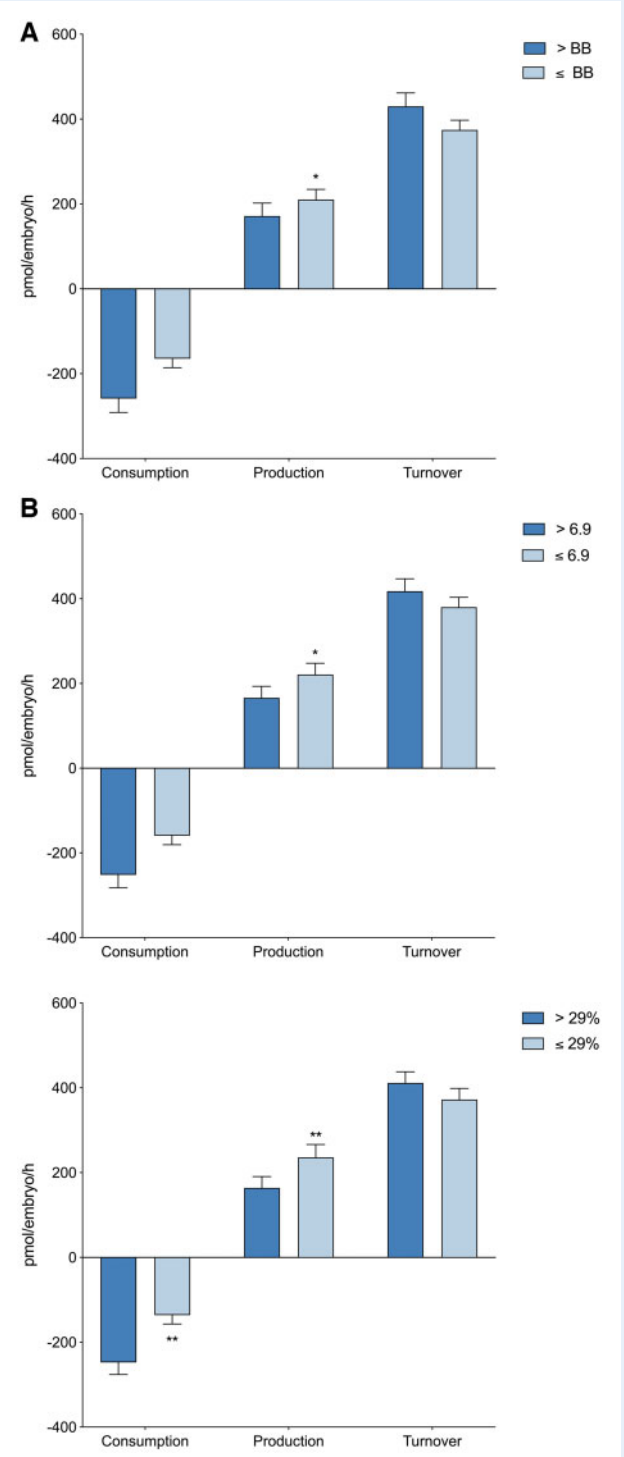

Figure 4. Total amino acid consumption, production and amino acid turnover in relation to embryo viability. (**A**) Gardner grade (>BB, n = 86; ≤BB, n = 123), (**B**) KIDScore (>6.9, n = 100; ≤6.9, n = 107) and (**C**) EmbryoScore (>29%, n = 91; ≤29%, n = 93). Each data set (A–C) is expressed as mean ± SEM (pmol/embryo/h). Significance is measured utilizing Mann–Whitney U test and denoted by asterisk, *$P < 0.05$, **$P < 0.01$.

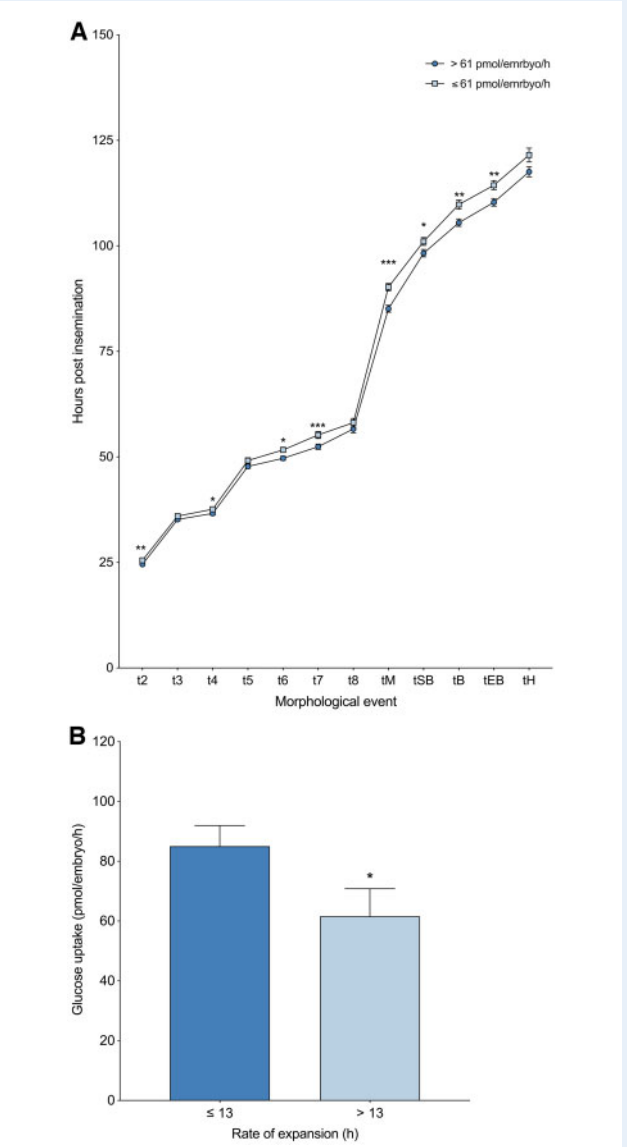

Figure 5. Blastocyst glucose uptake and the relationship to morphokinetic development of preimplantation embryos. (**A**) Morphokinetic development of embryos that consumed high or low levels of glucose. Dark, circular data points represent the timing of each key morphokinetic event of embryos that consumed greater than the median amount of glucose (61 pmol/embryo/h). Light, square data points represent the timing of each key morphokinetic event of embryos that consumed less than the median amount of glucose. Morphokinetic parameters calculated as hours post-insemination: two-cell cleavage, t2 (n = 209); three-cell cleavage, t3 (n = 209); four-cell cleavage, t4 (n = 209); five-cell cleavage t5 (n = 209); six-cell cleavage, t6 (n = 205); seven-cell cleavage, t7 (n = 200); eight-cell cleavage, t8 (n = 200); morulae, tM (n = 204); start of blastulation, tSB (n = 209); blastocyst, tB (n = 209); expanded blastocyst, tEB (n = 201); and hatching blastocyst, tH (n = 137). (**B**) Glucose uptake of embryos that fully expanded (tEB-tSB) in ≤13 h (n = 116) compared to embryos that fully expanded in >13 h (n = 84). Data are expressed as mean ± SEM, significance is measured utilizing Mann–Whitney U test and asterisks denote significance, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Consistent with glucose consumption, the amino acid profiles of faster developing embryos were significantly different. Specifically, embryos that consumed higher levels of arginine, asparagine, aspartate, isoleucine, lysine, taurine and/or valine developed faster to tM, tSB, tB and/or tEB ($P < 0.05$) (Table I). Additionally, alanine and glutamate were produced at lower levels by embryos that developed faster to tM, tSB, tB and/or tEB ($P < 0.05$) (Table II). No significant difference was observed between Day 5 amino acid profiles and the time between tSB and tEB (i.e. rate of expansion).

Discussion

In this study, blastocyst metabolism was shown to positively correlate with existing methods of embryo selection, Gardner grade, D5 KIDScore algorithm as well as the AI EmbryoScore. Of paramount significance, glucose consumption was found to be over 40% higher in blastocysts with a higher developmental and implantation potential. Indeed, when related to clinical pregnancy, glucose consumption was doubled in blastocysts that led to a successful pregnancy. Furthermore, blastocysts with a higher developmental potential and probability to succeed post-transfer also exhibited distinct amino acid profiles than blastocysts of lower viability, irrespective of which grading system was used.

Glucose is the predominant energy source utilized during blastocyst formation, supporting formation of the blastocoel, development of the first epithelium, and exponential cellular growth; processes which are energetically demanding (Biggers *et al.*, 1988; Watson *et al.*, 1990, 1992). High glucose consumption ensures carbon flux through the pentose phosphate pathway required to produce biosynthetic

Table I Morphokinetic development of embryos and their correlation to blastocyst consumption of individual amino acids.

Consumption of amino acids (pmol/embryo/h)		Timing of specific morphokinetic events (h)			
		tM	tSB	tB	tEB
Arginine	> Median	86.1 ± 1.1	98.3 ± 0.7*	106.4 ± 0.9	111.1 ± 0.9
	≤ Median	88.3 ± 1.1	101.0 ± 1.1	108.9 ± 1.1	113.7 ± 1.1
Asparagine	> Median	86.6 ± 0.7	98.5 ± 0.7	106.3 ± 0.9*	111.0 ± 0.8*
	≤ Median	88.7 ± 1.0	100.9 ± 1.0	109.0 ± 1.1	113.9 ± 1.1
Aspartate	> Median	86.4 ± 0.7*	98.3 ± 0.7*	106.4 ± 0.8	111.0 ± 0.8
	≤ Median	89.0 ± 1.0	101.1 ± 1.1	108.9 ± 1.1	113.8 ± 1.1
Isoleucine	> Median	85.8 ± 1.1	98.5 ± 0.7	106.2 ± 0.8*	110.9 ± 0.8*
	≤ Median	88.6 ± 1.0	100.9 ± 1.0	109.1 ± 1.1	114.0 ± 1.1
Lysine	> Median	86.0 ± 1.1	98.5 ± 0.7	106.3 ± 0.8*	111.0 ± 0.8*
	≤ Median	88.5 ± 1.0	100.8 ± 1.1	109.9 ± 1.2	113.9 ± 1.2
Taurine	> Median	86.9 ± 0.7	98.5 ± 0.8	106.0 ± 0.8*	111.2 ± 0.9
	≤ Median	88.4 ± 1.0	100.9 ± 1.0	109.3 ± 1.1	113.7 ± 1.1
Valine	> Median	87.0 ± 0.8	98.0 ± 0.7	106.1 ± 0.8*	110.8 ± 0.9
	≤ Median	88.3 ± 1.0	101.4 ± 1.0	109.2 ± 1.1	114.0 ± 1.1

Median consumption (pmol/embryo/h): arginine 5.10; asparagine 0.37; aspartate 1.40; isoleucine 6.10; lysine 0.15; taurine 0.20; valine 5.40. The timing of specific morphological events (time to morulae (tM), n = 204; start of blastulation (tSB), n = 209; blastocyst (tB), n = 209; and expanded blastocyst (tEB), n =201; expressed as mean ± SEM (hours postinsemination)). Significance is measured utilizing Student's *t*-test and asterisks denote level of significance.
*$P < 0.05$.

Table II Morphokinetic development of embryos and their correlation to blastocyst production of individual amino acids.

Production of amino acids (pmol/embryo/h)		Timing of specific morphokinetic events (h)			
		tM	tSB	tB	tEB
Alanine	> Median	88.3 ± 1.0	101.4 ± 1.0	109.2 ± 1.1	114.0 ± 1.1
	≤ Median	87.0 ± 0.8	98.0 ± 0.7**	106.1 ± 0.8*	110.8 ± 0.9*
Glutamate	> Median	89.1 ± 1.0	101.5 ± 1.1	109.8 ± 1.1	114.2 ± 1.1
	≤ Median	86.2 ± 0.7*	97.9 ± 0.7***	105.5 ± 0.8**	110.7 ± 0.8*

Median production (pmol/embryo/h): alanine 8.95; glutamate 6.70. The timing of specific morphological events (time to morulae (tM), n = 204; start of blastulation (tSB), n = 209; blastocyst (tB), n = 209; and expanded blastocyst (tEB), n = 201; expressed as mean ± SEM (hours postinsemination)). Significance is measured utilizing Student's *t*-test and asterisks denote level of significance.
*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

precursors for DNA, RNA and lipid synthesis (Reitzer et al., 1980; Morgan and Faik, 1981; Gardner, 1998). Furthermore, the blastocyst has the capacity to convert glucose to lactate, even in the presence of sufficient oxygen, through aerobic glycolysis (Gardner, 1998). Lactate, long perceived as a by-product or regulator of metabolism (Lane and Gardner, 1996, 2000), has more recently been implicated as a key signaling molecule and immunomodulator, thereby regulating initial events of implantation (Gardner, 2015). Additionally, lactate may directly affect embryonic epigenetic state and gene expression through lactylation of histone proteins (Zhang et al., 2019), an example of a metaboloepigenetic interaction, further highlighting the significance of blastocyst glucose metabolism in assessing embryonic physiology and health. Consistent with previous reports that embryos consuming higher levels of glucose are associated with a higher developmental potential (Renard et al., 1980; Gardner and Leese, 1987; Gardner et al., 2001; 2011; Lee et al., 2015), we show that blastocysts with higher viability consume more glucose during Day 5 of development than less viable blastocysts. Furthermore, embryos that experienced a delay of 2–4 h in development to the blastocyst stage consumed lower levels of glucose on Day 5 of the preimplantation period. This is consistent with data on the mouse embryo which determined that slow cleaving embryos give rise to blastocysts with reduced glucose consumption (Lee et al., 2015). Together, these results support the hypothesis that slower developing embryos, or embryos of lower viability, have a diminished ability to maintain the energy and biosynthetic requirements to support viable blastocyst formation, and subsequently viability post-transfer (Edwards et al., 1984; Wong et al., 2010; Meseguer et al., 2011; Lee et al., 2015).

Blastocyst size has been linked to metabolic activity and viability due to the high energy demands of blastocyst expansion (Gardner and Balaban, 2016; Niederberger et al., 2019). Blastocyst diameter, as an accurate measure of blastocyst size, is positively correlated to clinical pregnancy rates where larger embryos are more likely to establish a clinical pregnancy (Shapiro et al., 2008). Similarly, this study found a positive relationship between blastocyst diameter and glucose consumption. Consistently, blastocysts with a faster expansion rate (tEB-tSB) consumed more glucose than slower expanding blastocysts, plausibly due to these embryos having a higher capacity to maintain energy requirements, subsequently supporting high developmental potential. However, it has been established that even when blastocysts do have the same diameter (Lane and Gardner, 1996; Gardner et al., 2001), there is still a large metabolic variation between embryos, inferring that although the size of a blastocyst is related to metabolism, it is not the sole factor dictating the overall metabolic activity of the embryo.

Of further interest, Desai et al. (2018) found embryos that have a faster expansion rate were associated with a lower rate of aneuploidy. Changes to karyotypes associated with aneuploidy leads to alterations in gene expression, the proteome and metabolism a phenomenon termed aneuploid-stress (Zhu et al., 2018), suggesting embryonic metabolism may be directly related to the genetic composition of embryos in addition to embryo viability and health. Ongoing investigations are currently underway to determine whether metabolic biomarkers can provide a non-invasive measure of embryonic genetic composition.

Retrospective analysis of clinical pregnancy rates confirms that glucose consumption is significantly higher in blastocysts that successfully implant and result in a clinical pregnancy compared to blastocysts that fail to development post-transfer. These data are consistent with Gardner et al. (2011) who determined that glucose consumption of human blastocysts cultured at 5% oxygen was higher in blastocysts with positive transfer outcomes. It was further noted that while data were not significantly different, plausibly due to low sample numbers, blastocysts with a top Gardner grade (AA) that failed to lead to a successful pregnancy had a lower glucose uptake than lower graded blastocysts which were successful post-transfer. This supports the findings of Gardner et al. (2011) who concluded glucose metabolism was a stronger predictor of pregnancy success than morphological grade.

Higher developmental potential and probability of forming a viable pregnancy was also related to amino acid profiles, which may provide an additional means of measuring embryo physiology and health. Specifically, alanine, arginine, aspartate, isoleucine, leucine, lysine, glutamate, glutamine and threonine were utilized differently by blastocysts with high viability, regardless of the embryo selection method used. Of significance, arginine and leucine are both implicated in regulating the mammalian target of rapamycin (mTOR) pathway (González et al., 2012). mTOR is responsible for maintaining cellular homeostasis by responding to nutrient, energy and/or oxygen availability and regulates translation machinery and metabolic processes accordingly. A significant increase in arginine and leucine consumption by blastocysts with higher viability suggests less viable blastocysts are unable to maintain cellular homeostasis as effectively thereby impacting embryo developmental potential. Additionally, arginine and leucine, together and individually, also promote TE motility, which assists TE invasion and is a requirement for penetration of the endometrium during implantation (Martin et al., 2003; Gangloff et al., 2004; Guertin et al., 2006; González et al., 2012). Of further significance, blastocysts that consumed high levels of glucose also consumed significantly more aspartate than less viable embryos. Aspartate is the rate-limiting factor in the malate-aspartate shuttle that facilitates the regeneration of cytosolic nicotinamide adenine dinucleotide (NAD^+) required for glucose metabolism (Gardner, 1998; Lane and Gardner, 2005; Mitchell et al., 2009). More recently NAD^+ has been recognized as a metaboloepigenetic signaling molecule via activation of sirtuins, a class of NAD^+-dependent histone deacetylases (Haigis and Sinclair, 2010; Tatone et al., 2018). Hence, the relative availability of aspartate and subsequently NAD^+ may also influence embryonic gene regulation and subsequent health (Houtkooper et al., 2010; Donohoe and Bultman, 2012; Gardner and Harvey, 2015; Harvey et al., 2016; Ferrick et al., 2019; Lees et al., 2020). Given the wide array of metabolic and regulatory pathways amino acids are involved in, it is evident that between blastocysts of high and low viability, individual amino acid profiles represent distinct physiologies and provide a means of assessing important regulators of cellular homeostasis, cell-signaling, implantation and embryonic health. Of note, the amino acid analysis performed in this study assessed amino acid profiles over a 24 h period during blastocyst formation and is therefore limited in its ability to identify differences in flux and interactions between amino acid pathways. Targeted amino acid assays, designed to quantitate the utilization of an individual amino acid with high accuracy will plausibly provide further insight into amino utilization, and further studies are required for the development and validation of such assays.

The results of this study demonstrate that a blastocyst with a high probability of developing into viable and healthy pregnancy is one that consumes high levels of glucose and specific amino acids. Furthermore, this study shows no significant difference in total amino acid turnover,

despite the distinct patterns of amino acid consumption and production between blastocysts with different levels of viability. Consequently, these data do not align with the Quiet Hypothesis, which states that the most viable preimplantation embryo has the lowest overall metabolism and the lowest amino acid turnover (Leese, 2002). Of note, studies that lay the foundation for this hypothesis utilized 20% oxygen for culture or metabolic analysis (Bowman and McLaren, 1970; Leese, 1991; Turner *et al.*, 1994; Houghton *et al.*, 2002). Given that 20% oxygen directly perturbs both carbohydrate and amino acid metabolism leading to an altered physiology of the preimplantation embryo, it is plausible that the differences observed in this study and those which formed the basis of the Quiet Hypothesis are due to the concentration of oxygen utilized (Umaoka *et al.*, 1992; Guérin *et al.*, 2001; Wale and Gardner, 2012; Gardner, 2016; Kelley and Gardner, 2016; Gardner and Kelley, 2017; Kelley and Gardner, 2017, 2019). It was proposed by Gardner and Wale (2013) that viable embryos metabolize an optimal range of metabolites, i.e. one that is not suppressed nor unduly elevated (Gardner and Wale, 2013). This concept has gained traction and has been coined the 'Goldilocks Principle' (Leese *et al.*, 2016). Our data have shown that within this optimal range the most viable blastocyst is a blastocyst that is characterized by an 'active' metabolism and utilizes metabolites in a distinct manner. This metabolic profile represents the embryos ability to maintain sufficient energy to support both cellular homeostasis and biosynthesis required to support embryo development, viability and health of the resulting offspring.

Due to the retrospective nature of this study and low numbers of transferred embryos it was not feasible to establish the cumulative predictive power of metabolism combined with either morphological grades, KIDScore or EmbryoScore. However, as it was shown by Gardner *et al.* (2011) that metabolism is a stronger predictor of transfer success than morphological grades, it is tempting to speculate that the assessment of blastocyst metabolism (and indirectly the metaboloepigenome) will be complimentary to any system used to assess developmental potential based on morphological parameters such as the KIDScore and EmbryoScore. For example, of the 64 blastocysts with both a high KIDScore and EmbryoScore, inferring high viability, over a third had reduced glucose uptake indicating an impaired physiology which may therefore prevent such top-scoring embryos from resulting in a successful pregnancy. We therefore propose that a combination of metabolic activity together with morphological analysis will culminate in a single algorithm that provides greater power in predicting success post-transfer as well as subsequent fetal health.

This study is the first of its kind to incorporate analysis of both glucose and amino acid metabolism of individual preimplantation embryos under physiological (5%) oxygen concentrations. Of significance, while UMF is a highly sensitive technology and LC-MS is a great discovery tool as it allows analysis of all amino acids in a single sample, neither are translatable to a clinical setting. Bench top assay technologies, however, have previously been used to measure mouse embryo carbohydrate metabolism (Guerif *et al.*, 2013; Kelley and Gardner, 2019) and amino acids (Lee and Gardner, unpublished) and hence provide a means of assessing metabolic biomarkers clinically within an hour, making assessment of metabolic activity prior to a fresh transfer feasible. Consequently, we are now evaluating the predictive power of metabolic biomarkers to create an 'embryo health' selection algorithm incorporating morphology, morphokinetics (optimized through AI) and metabolism in order to reduce the time to pregnancy and facilitate the birth of healthy children.

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Authors' roles

D.K.G prepared the study design and contributed to the manuscript. Y.S.L.L assisted in the study design, coordination of sample collections and the preparation of the manuscript. L.F assisted in the study design, performed all viability assessments, carbohydrate analyses and statistical analysis and contributed to the manuscript.

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Conflict of interest

Virtus Health and Harrison AI have patented the AI technology used. D.K.G is contracted with Virtus Health. The other authors have no conflict of interest.

References

Adashi EY, Barri PN, Berkowitz R, Braude P, Bryan E, Carr J, Cohen J, Collins J, Devroey P, Frydman R *et al.* Infertility therapy-associated multiple pregnancies (births): an ongoing epidemic. *Reprod Biomed Online* 2003;**7**:515–542.

Albrecht JL, Tomich PG. The maternal and neonatal outcome of triplet gestations. *Am J Obstet Gynecol* 1996;**174**:1551–1556.

Almagor M, Harir Y, Fieldust S, Or Y, Shoham Z. Ratio between inner cell mass diameter and blastocyst diameter is correlated with successful pregnancy outcomes of single blastocyst transfers. *Fertil Steril* 2016;**106**:1386–1391.

Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1986;**1**:1077–1081.

Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 1989;**298**:564–567.

Basile N, Vime P, Florensa M, Aparicio Ruiz B, Garcia Velasco JA, Remohi J, Meseguer M. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod* 2015;**30**:276–283.

Biggers JD, Bell JE, Benos DJ. Mammalian blastocyst: transport functions in a developing epithelium. *Am J Physiol* 1988;**255**: C419–C432.

Bowman P, McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. *J Embryol Exp Morphol* 1970;**24**:203–207.

Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 2004;**19**:2319–2324.

Chen X-K, Wen SW, Bottomley J, Smith GN, Leader A, Walker MC. In vitro fertilization is associated with an increased risk for preeclampsia. *Hypertens Pregnancy* 2009;**28**:1–12.

Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring human embryo growth rates in in vitro fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *J In Vitro Fert Embryo Transf* 1986;**3**:284–295.

Curchoe CL, Bormann CL. Artificial intelligence and machine learning for human reproduction and embryology presented at ASRM and ESHRE 2018. *J Assist Reprod Genet* 2019;**36**:591–600.

Desai N, Goldberg JM, Austin C, Falcone T. Are cleavage anomalies, multinucleation, or specific cell cycle kinetics observed with time-lapse imaging predictive of embryo developmental capacity or ploidy? *Fertil Steril* 2018;**109**:665–674.

Desai N, Ploskonka S, Goodman LR, Austin C, Goldberg J, Falcone T. Analysis of embryo morphokinetics, multinucleation and cleavage anomalies using continuous time-lapse monitoring in blastocyst transfer cycles. *Reprod Biol Endocrinol* 2014;**12**:54.

Donohoe DR, Bultman SJ. Metaboloepigenetics: interrelationships between energy metabolism and epigenetic control of gene expression. *J Cell Physiol* 2012;**227**:3169–3177.

Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, Steptoe PC, Webster JM. Factors influencing the success of in vitro fertilization for alleviating human infertility. *J In Vitro Fert Embryo Transf* 1984;**1**:3–23.

Ferrick L, Lee YSL, Gardner DK. Reducing time to pregnancy and facilitating the birth of healthy children through functional analysis of embryo physiology. *Biol Reprod* 2019;**101**:1124–1139.

Gangloff Y-G, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz J-F, Um SH, Brown EJ, Cereghini S, Thomas G *et al*. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. *Mol Cell Biol* 2004;**24**: 9508–9516.

Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 1998;**49**:83–102.

Gardner DK. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? *Bioessays* 2015;**37**:364–371.

Gardner DK. The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human IVF. *Reprod Biomed Online* 2016;**32**:137–141.

Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important? *Mol Hum Reprod* 2016; **22**:704–718.

Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev* 2015;**27**:638–654.

Gardner DK, Kelley RL. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis* 2017;**8**:418–435.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;**73**:1155–1158.

Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril* 2001;**76**:1175–1180.

Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *J Exp Zool* 1987;**242**:103–105.

Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J. A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum Reprod* 1998;**13**:3434–3440.

Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: a prospective randomized trial. *Fertil Steril* 2004;**81**:551–555.

Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril* 2013;**99**:1062–1072.

Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod* 2011;**26**:1981–1986.

Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerssche M, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. *Hum Reprod* 1999;**14**:2581–2587.

González IM, Martin PM, Burdsal C, Sloan JL, Mager S, Harris T, Sutherland AE. Leucine and arginine regulate trophoblast motility through mTOR-dependent and independent pathways in the preimplantation mouse embryo. *Dev Biol* 2012;**361**:286–300.

Gott A, Hardy K, Winston R, Leese H. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod* 1990;**5**:104–108.

Guerif F, McKeegan P, Leese HJ, Sturmey RG. A simple approach for COnsumption and RElease (CORE) analysis of metabolic activity in single mammalian embryos. *PLoS One* 2013;**8**:e67834.

Guérin P, El Mouatassim S, Ménézo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001;**7**:175–189.

Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCα, but not S6K1. *Dev Cell* 2006;**11**:859–871.

Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* 2010;**5**:253–295.

Hansen PJ. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. *Theriogenology* 2007; **68**:S242–S249.

Harbottle S, Hughes C, Cutting R, Roberts S, Brison D, Association Of Clinical Embryologists & The (ACE) British Fertility Society

(BFS). Elective single embryo transfer: an update to UK best practice guidelines. *Hum Fertil* 2015;**18**:165–183.

Harvey AJ, Rathjen J, Gardner DK. Metaboloepigenetic regulation of pluripotent stem cells. *Stem Cells Int* 2016;**2016**:1816525.

Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 2002; **17**:999–1005.

Houtkooper RH, Cantó C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 2010;**31**:194–223.

Kelley RL, Gardner DK. Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. *Reprod Biomed Online* 2016;**33**:537–549.

Kelley RL, Gardner DK. In vitro culture of individual mouse preimplantation embryos: the role of embryo density, microwells, oxygen, timing and conditioned media. *Reprod Biomed Online* 2017;**34**: 441–454.

Kelley RL, Gardner DK. Individual culture and atmospheric oxygen during culture affect mouse preimplantation embryo metabolism and post-implantation development. *Reprod Biomed Online* 2019; **39**:3–18.

Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod* 1996;**11**: 1975–1978.

Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998;**13**:991–997.

Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod* 2000;**62**: 16–22.

Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev* 2005;**17**:371–378.

Lee YSL, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod* 2015;**30**:543–552.

Lees JG, Gardner DK, Harvey AJ. Nicotinamide adenine dinucleotide induces a bivalent metabolism and maintains pluripotency in human embryonic stem cells. *Stem Cells* 2020;**38**:624–638.

Leese HJ. Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol* 1991;**13**:35–72.

Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* 2002;**24**:845–849.

Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil* 1984;**72**:9–13.

Leese HJ, Guerif F, Allgar V, Brison DR, Lundin K, Sturmey RG. Biological optimization, the Goldilocks principle, and how much is lagom in the preimplantation embryo. *Mol Reprod Dev* 2016;**83**: 748–754.

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. *Fertil Steril* 2016;**105**:656–662.e1.

Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. *Fertil Steril* 2014;**102**:1295–1300.e2.

Luke B, Bigger HR, Leurgans S, Sietsema D. The cost of prematurity: a case-control study of twins vs singletons. *Am J Public Health* 1996;**86**:809–814.

Martin PM, Sutherland AE, Van Winkle LJ. Amino acid transport regulates blastocyst implantation. *Biol Reprod* 2003;**69**:1101–1108.

Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011;**26**:2658–2671.

Milewski R, Kuć P, Kuczyńska A, Stankiewicz B, Lukaszuk K, Kuczyński W *et al*. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. *J Assist Reprod Genet* 2015;**32**:571–579.

Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod* 2009;**80**:295–301.

Miyagi Y, Habara T, Hirata R, Hayashi N. Feasibility of predicting live birth by combining conventional embryo evaluation with artificial intelligence applied to a blastocyst image in patients classified by age. *Reprod Med Biol* 2019;**18**:344–356.

Montag M, Liebenthron J, Köster M. Which morphological scoring system is relevant in human embryo development? *Placenta* 2011; **32**:S252–S256.

Morgan MJ, Faik P. Carbohydrate metabolism in cultured animal cells. *Biosci Rep* 1981;**1**:669–686.

Niederberger C, Pellicer A, Simon C, Kathrins M, Goldstein M, Sigman M, Schlegel PN, Munné S, Gardner DK, Cobo A. 25 historic papers: an ASRM 75th birthday gift from fertility and sterility. *Fertil Steril* 2019;**112**:e2–e27.

Ombelet W, De Sutter P, Van der Elst J, Martens G. Multiple gestation and infertility treatment: registration, reflection and reaction-the Belgian project. *Hum Reprod Update* 2005;**11**:3–14.

Paternot G, Debrock S, D'Hooghe T, Spiessens C. Computer-assisted embryo selection: a benefit in the evaluation of embryo quality? *Reprod Biomed Online* 2011;**23**:347–354.

Penzias A, Bendikson K, Butts S, Coutifaris C, Fossum G, Falcone T, Gitlin S, Gracia C, Hansen K, La Barbera A. Guidance on the limits to the number of embryos to transfer: a committee opinion. *Fertil Steril* 2017;**107**:901–903.

Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Hum Reprod* 2016;**31**:2231–2244.

Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, Leese HJ, Harris SE. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Mol Hum Reprod* 2010;**16**:557–569.

Reignier A, Girard J-M, Lammers J, Chtourou S, Lefebvre T, Barriere P, Freour T. Performance of Day 5 KIDScore™ morphokinetic prediction models of implantation and live birth after single blastocyst transfer. *J Assist Reprod Genet* 2019;**36**:2279–2285.

Reitzer LJ, Wice B, Kennell D. The pentose cycle. Control and essential function in HeLa cell nucleic acid synthesis. *J Biol Chem* 1980;**255**:5616–5626.

Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. *J Reprod Fertil* 1980;**58**:161–164.

Rubio I, Galán A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. *Fertil Steril* 2014;**102**:1287–1294.e5.

Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escribá MJ, Bellver J, Meseguer M. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. *Fertil Steril* 2012;**98**:1458–1463.

Santos Filho E, Noble JA, Poli M, Griffiths T, Emerson G, Wells D. A method for semi-automatic grading of human blastocyst microscope images. *Hum Reprod* 2012;**27**:2641–2648.

Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Thomas S. Large blastocyst diameter, early blastulation, and low preovulatory serum progesterone are dominant predictors of clinical pregnancy in fresh autologous cycles. *Fertil Steril* 2008;**90**:302–309.

Storr A, Venetis CA, Cooke S, Kilani S, Ledger W. Inter-observer and intra-observer agreement between embryologists during selection of a single Day 5 embryo for transfer: a multicenter study. *Hum Reprod* 2017;**32**:307–314.

Sturmey RG, Reis A, Leese HJ, McEvoy TG. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod Domest Anim* 2009;**44**:50–58.

Takeshima K, Jwa SC, Saito H, Nakaza A, Kuwahara A, Ishihara O, Irahara M, Hirahara F, Yoshimura Y, Sakumoto T. Impact of single embryo transfer policy on perinatal outcomes in fresh and frozen cycles—analysis of the Japanese Assisted Reproduction Technology registry between 2007 and 2012. *Fertil Steril* 2016;**105**:337–346.e3.

Tatone C, Di Emidio G, Barbonetti A, Carta G, Luciano AM, Falone S, Amicarelli F. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. *Hum Reprod Update* 2018;**24**:267–289.

Thirumalaraju P, Hsu J, Bormann C, Kanakasabapathy M, Souter I, Dimitriadis I, Dickinson K, Pooniwala R, Gupta R, Yogesh V. Deep learning-enabled blastocyst prediction system for cleavage stage embryo selection. *Fertil Steril* 2019;**111**:e29.

Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. *J Reprod Fertil* 1996;**106**:299–306.

Tran D, Cooke S, Illingworth P, Gardner DK. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. *Hum Reprod* 2019;**34**:1011–1018.

Turner K, Martin KL, Woodward BJ, Lenton EA, Leese HJ. Comparison of pyruvate uptake by embryos derived from conception and non-conception natural cycles. *Hum Reprod* 1994;**9**:2362–2366.

Umaoka Y, Noda Y, Narimoto K, Mori T. Effects of oxygen toxicity on early development of mouse embryos. *Mol Reprod Dev* 1992;**31**:28–33.

Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W, Gerris J. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod* 1999;**14**:2345–2349.

VerMilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M, Boostanfar R, Baker VL, Suraj V, Chen AA *et al*. Computer-automated time-lapse analysis results correlate with embryo implantation and clinical pregnancy: a blinded, multi-centre study. *Reprod Biomed Online* 2014;**29**:729–736.

Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod* 2012;**87**:21–28.

Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2016;**22**:2–22.

Watson AJ, Kidder GM, Schultz GA. How to make a blastocyst. *Biochem Cell Biol* 1992;**70**:849–855.

Watson AJ, Pape C, Emanuel JR, Levenson R, Kidder GM. Expression of NA, K-ATpase α and β subunit genes during preimplantation development of the mouse. *Dev Genet* 1990;**11**:41–48.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Pera RAR. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010;**28**:1115.

Zander-Fox DL, Mitchell M, Thompson JG, Lane M. Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. *Reprod Biomed Online* 2010;**21**:219–229.

Zhang D, Tang Z, Huang H, Zhou G, Cui C, Weng Y, Liu W, Kim S, Lee S, Perez-Neut M *et al*. Metabolic regulation of gene expression by histone lactylation. *Nature* 2019;**574**:575–580.

Zhu J, Tsai H-J, Gordon MR, Li R. Cellular stress associated with aneuploidy. *Dev Cell* 2018;**44**:420–431.

References

Abdelrazik H, Sharma R, Mahfouz R, Agarwal A. L-carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. *Fertil Steril* 2009;91: 589-596.

Adashi EY, Barri PN, Berkowitz R, Braude P, Bryan E, Carr J, Cohen J, Collins J, Devroey P, Frydman R *et al.* Infertility therapy-associated multiple pregnancies (births): an ongoing epidemic. *Reprod Biomed Online* 2003;7: 515-542.

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. *Reprod Biol Endocrinol* 2012;10: 49.

Agarwal A, Durairajanayagam D, Du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. *Reprod Biol Endocrinol* 2014;12: 112.

Akarsu S, Gode F, Isik AZ, Dikmen ZG, Tekindal MA. The association between coenzyme Q10 concentrations in follicular fluid with embryo morphokinetics and pregnancy rate in assisted reproductive techniques. *J Assist Reprod Genet* 2017;34: 599-605.

Albrecht JL, Tomich PG. The maternal and neonatal outcome of triplet gestations. *Am J Obstet Gynecol* 1996;174: 1551-1556.

Alfarawati S, Fragouli E, Colls P, Stevens J, Gutiérrez-Mateo C, Schoolcraft WB, Katz-Jaffe MG, Wells D. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. *Fertil Steril* 2011;95: 520-524.

Ali A, Bilodeau J, Sirard M. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology* 2003;59: 939-949.

Almagor M, Harir Y, Fieldust S, Or Y, Shoham Z. Ratio between inner cell mass diameter and blastocyst diameter is correlated with successful pregnancy outcomes of single blastocyst transfers. *Fertil Steril* 2016;106: 1386-1391.

Anderson ME, Meister A. Transport and direct utilization of gamma-glutamylcyst (e) ine for glutathione synthesis. *Proc Natl Acad Sci U S A* 1983;80: 707-711.

Andriani GA, Almeida VP, Faggioli F, Mauro M, Tsai WL, Santambrogio L, Maslov A, Gadina M, Campisi J, Vijg J. Whole Chromosome Instability induces senescence and promotes SASP. *Sci Rep* 2016;6: 35218.

Armstrong S, Vail A, Mastenbroek S, Jordan V, Farquhar C. Time-lapse in the IVF-lab: how should we assess potential benefit? *Hum Reprod* 2015: deu250.

Baart EB, Martini E, van den Berg I, Macklon NS, Galjaard RJ, Fauser BC, Van Opstal D. Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. *Hum Reprod* 2006;21: 223-233.

Baird DT, Collins J, Egozcue J, Evers LH, Gianaroli L, Leridon H, Sunde A, Templeton A, Van Steirteghem A, Cohen J *et al.* Fertility and ageing. *Hum Reprod Update* 2005;11: 261-276.

Balaban B, Urman B, Ata B, Isiklar A, Larman MG, Hamilton R, Gardner DK. A randomized controlled study of human Day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. *Hum Reprod* 2008;23: 1976-1982.

Balakier H, Sojecki A, Motamedi G, Librach C. Impact of multinucleated blastomeres on embryo developmental competence, morphokinetics, and aneuploidy. *Fertil Steril* 2016;106: 608-614. e602.

Barbehenn EK, Wales RG, Lowry OH. The explanation for the blockade of glycolysis in early mouse embryos. *Proc Natl Acad Sci U S A* 1974;71: 1056-1060.

Barcroft LC, Offenberg H, Thomsen P, Watson AJ. Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation. *Dev Biol* 2003;256: 342-354.

Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1986;1: 1077-1081.

Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 1989;298: 564-567.

Barker DJP. The Welcome Foundation Lecture, 1994. The fetal origins of adult disease. *Proc Biol Sci* 1995;262: 37-43.

Barnes FL. The effects of the early uterine environment on the subsequent development of embryo and fetus. *Theriogenology* 2000;53: 649-658.

Barron DJ, Valdimarsson G, Paul DL, Kidder GM. Connexin32, a gap junction protein, is a persistent oogenetic product through preimplantation development of the mouse. *Genesis* 1989;10: 318-323.

Bartmann AK, Romão GS, Ramos Eda S, Ferriani RA. Why do older women have poor implantation rates? A possible role of the mitochondria. *J Assist Reprod Genet* 2004;21: 79-83.

Basile N, del Carmen Nogales M, Bronet F, Florensa M, Riqueiros M, Rodrigo L, García-Velasco J, Meseguer M. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. *Fertil Steril* 2014;101: 699-704. e691.

Basile N, Vime P, Florensa M, Aparicio Ruiz B, Garcia Velasco JA, Remohi J, Meseguer M. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod* 2015;30: 276-283.

Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. *Reprod Fertil Dev* 1991;3: 601-607.

Bavister BD, Arlotto T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. *Mol Reprod Dev* 1990;25: 45-51.

Becker M, Becker A, Miyara F, Han Z, Kihara M, Brown DT, Hager GL, Latham K, Adashi EY, Misteli T. Differential in vivo binding dynamics of somatic and oocyte-specific linker histones in oocytes and during ES cell nuclear transfer. *Mol Biol Cell* 2005;16: 3887-3895.

Bentov Y, Casper RF. The aging oocyte--can mitochondrial function be improved? *Fertil Steril* 2013;99: 18-22.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc Natl Acad Sci U S A* 2010;107: 3394-3399.

Berthelot F, Terqui M. Effects of oxygen, CO2/pH and medium on the in vitro development of individually cultured porcine one- and two-cell embryos. *Reprod Nutr Dev* 1996;36: 241-251.

Bhusane K, Bhutada S, Chaudhari U, Savardekar L, Katkam R, Sachdeva G. Secrets of Endometrial Receptivity: Some Are Hidden in Uterine Secretome. *Am J Reprod Immunol* 2016;75: 226-236.

Biggers J, Bell JE, Benos DJ. Mammalian blastocyst: transport functions in a developing epithelium. *Am J Physiol* 1988;255: C419-C432.

Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in KSOM(AA) medium: is a two-step protocol necessary? *Reprod Biomed Online* 2002;5: 133-140.

Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Natl Acad Sci U S A* 1967;58: 560-567.

Bilska A, Wlodek L. Lipoic acid-the drug of the future. *Pharmacol Rep* 2005;57: 570-577.

Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Database Syst Rev* 2012: CD008950.

Boomsma CM, Kavelaars A, Eijkemans MJ, Amarouchi K, Teklenburg G, Gutknecht D, Fauser BJ, Heijnen CJ, Macklon NS. Cytokine profiling in endometrial secretions: a non-invasive window on endometrial receptivity. *Reprod Biomed Online* 2009;18: 85-94.

Bowman P, McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. *J Embryol Exp Morphol* 1970;24: 203-207.

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four-and eight-cell stages of preimplantation development. *Nature* 1988;332: 459-461.

Brinster RL. Studies on the development of mouse embryo in vitro. II. The effect of energy source. *J Exp Zool* 1965;158: 59-68.

Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 2004;19: 2319-2324.

Bunton C. Oxidation of α-Diketones andα-Keto-Acids by Hydrogen Peroxide. *Nature* 1949;163: 444-444.

Burton GJ, Hempstock J, Jauniaux E. Oxygen, early embryonic metabolism and free radical-mediated embryopathies. *Reprod Biomed Online* 2003;6: 84-96.

Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, Motrenko T, Scaravelli G, Wyns C, Goossens V. Assisted reproductive technology in Europe, 2013: results generated from European registers by ESHRE. *Hum Reprod* 2017;32: 1957-1973.

Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Hickman CFL. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. *Reprod Biomed Online* 2013;26: 477-485.

Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Thornton S. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. *Reprod Biomed Online* 2013;27: 140-146.

Campbell A, Fishel S, Laegdsmand M. Aneuploidy is a key causal factor of delays in blastulation: author response to 'A cautionary note against aneuploidy risk assessment using time-lapse imaging'. *Reprod Biomed Online* 2014;28: 279-283.

Canto´ C, Menzies KJ, Auwerx J. NAD+ metabolism and the control of energy homeostasis: a balancing act between mitochondria and the nucleus. *Cell Metab* 2015;22: 31-53.

Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, Nagy ZP, Ubaldi FM. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. *Hum Reprod* 2014;29: 1173-1181.

Casado-Vela J, Rodriguez-Suarez E, Iloro I, Ametzazurra A, Alkorta N, García-Velasco JA, Matorras R, Prieto B, González S, Nagore D *et al.* Comprehensive proteomic analysis of human endometrial fluid aspirate. *J Proteome Res* 2009;8: 4622-4632.

Casslén BG. Free amino acids in human uterine fluid. Possible role of high taurine concentration. *J Reprod Med* 1987;32: 181-184.

Castillo-Martín M, Bonet S, Morató R, Yeste M. Supplementing culture and vitrification-warming media with l-ascorbic acid enhances survival rates and redox status of IVP porcine blastocysts via induction of GPX1 and SOD1 expression. *Cryobiology* 2014;68: 451-458.

Chason RJ, Csokmay J, Segars JH, DeCherney AH, Armant DR. Environmental and epigenetic effects upon preimplantation embryo metabolism and development. *Trends Endocrinol Metab* 2011;22: 412-420.

Chavez SL, Loewke KE, Han J, Moussavi F, Colls P, Munne S, Behr B, Pera RAR. Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. *Nat Commun* 2012;3: 1-12.

Chawla M, Fakih M, Shunnar A, Bayram A, Hellani A, Perumal V, Divakaran J, Budak E. Morphokinetic analysis of cleavage stage embryos and its relationship to aneuploidy in a retrospective time-lapse imaging study. *J Assist Reprod Genet* 2015;32: 69-75.

Choe C, Shin Y-W, Kim E-J, Cho S-R, Kim H-J, Choi S-H, Han M-H, Han J, Son D-S, Kang D. Synergistic effects of glutathione and β-mercaptoethanol treatment during in vitro maturation of porcine oocytes on early embryonic development in a culture system supplemented with L-cysteine. *J Reprod Dev* 2010;56: 575-582

Christianson MS, Zhao Y, Shoham G, Granot I, Safran A, Khafagy A, Leong M, Shoham Z. Embryo catheter loading and embryo culture techniques: results of a worldwide Web-based survey. *J Assist Reprod Genet* 2014;31: 1029-1036.

Ciemerych MA, Sicinski P. Cell cycle in mouse development. *Oncogene* 2005;24: 2877-2898.

Clegg KB, Pikó L. Poly (A) length, cytoplasmic adenylation and synthesis of poly (A)+ RNA in early mouse embryos. *Dev Biol* 1983;95: 331-341.

Coello A, Meseguer M, Galán A, Alegre L, Remohí J, Cobo A. Analysis of the morphological dynamics of blastocysts after vitrification/warming: defining new predictive variables of implantation. *Fertil Steril* 2017;108: 659-666.e654.

Cole R. Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst. *J Embryol Exp Morphol* 1967;17: 481-490.

Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. *J Assist Reprod Genet* 1993;10: 21-30.

Croxatto HB, Díaz S, Fuentealba B, Croxatto HD, Carrillo D, Fabres C. Studies on the duration of egg transport in the human oviduct. I. The time interval between ovulation and egg recovery from the uterus in normal women. *Fertil Steril* 1972;23: 447-458.

Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring human embryo growth rates in in vitro fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *J In Vitro Fert Embryo Transf* 1986;3: 284-295.

Curchoe CL, Bormann CL. Artificial intelligence and machine learning for human reproduction and embryology presented at ASRM and ESHRE 2018. *J Assist Reprod Genet* 2019;36: 591-600.

D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 2007;8: 813-824.

De Matos D, Furnus C. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: effect of β-mercaptoethanol, cysteine and cystine. *Theriogenology* 2000;53: 761-771.

De Matos DG, Furnus CC, Moses DF, Baldassarre H. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in vitro. *Mol Reprod Dev* 1995;42: 432-436.

De Sutter P, Gerris J, Dhont M. A health-economic decision-analytic model comparing double with single embryo transfer in IVF/ICSI. *Hum Reprod* 2002;17: 2891-2896.

Debrock S, Peeraer K, Fernandez Gallardo E, De Neubourg D, Spiessens C, D'Hooghe TM. Vitrification of cleavage stage day 3 embryos results in higher live birth rates than conventional slow freezing: a RCT. *Hum Reprod* 2015;30: 1820-1830.

Desai N, Goldberg JM, Austin C, Falcone T. Are cleavage anomalies, multinucleation, or specific cell cycle kinetics observed with time-lapse imaging predictive of embryo developmental capacity or ploidy? *Fertil Steril* 2018;109: 665-674.

Desai N, Ploskonka S, Goodman LR, Austin C, Goldberg J, Falcone T. Analysis of embryo morphokinetics, multinucleation and cleavage anomalies using continuous time-lapse monitoring in blastocyst transfer cycles. *Reprod Biol Endocrinol* 2014;12: 54.

Desai NN, Goldberg JM, Austin C, Falcone T. The new Rapid-i carrier is an effective system for human embryo vitrification at both the blastocyst and cleavage stage. *Reprod Biol Endocrinol* 2013;11: 41.

Desler C, Munch-Petersen B, Stevnsner T, Matsui S, Kulawiec M, Singh KK, Rasmussen LJ. Mitochondria as determinant of nucleotide pools and chromosomal stability. *Mutat Res* 2007;625: 112-124.

Diebold L, Chandel NS. Mitochondrial ROS regulation of proliferating cells. *Free Radic Biol Med* 2016;100: 86-93.

Donohoe DR, Bultman SJ. Metaboloepigenetics: interrelationships between energy metabolism and epigenetic control of gene expression. *J Cell Physiol* 2012;227: 3169-3177.

Dos Santos E, Varchetta R, de Lima C, Ispada J, Martinho H, Fontes P, Nogueira M, Gasparrini B, Milazzotto M. The effects of crocetin supplementation on the blastocyst outcome, transcriptomic and metabolic profile of in vitro produced bovine embryos. *Theriogenology* 2019;123: 30-36.

Dosouto C, Haahr T, Humaidan P. Gonadotropin-releasing hormone agonist (GnRHa) trigger - State of the art. *Reprod Biol* 2017;17: 1-8.

Du QY, Wang EY, Huang Y, Guo XY, Xiong YJ, Yu YP, Yao GD, Shi SL, Sun YP. Blastocoele expansion degree predicts live birth after single blastocyst transfer for fresh and vitrified/warmed single blastocyst transfer cycles. *Fertil Steril* 2016;105: 910-919.e911.

Ducibella T, Anderson E. Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Dev Biol* 1975;47: 45-58.

Dumoulin JC, Meijers CJ, Bras M, Coonen E, Geraedts JP, Evers JL. Effect of oxygen concentration on human in-vitro fertilization and embryo culture. *Hum Reprod* 1999;14: 465-469.

Duncan FE, Hornick JE, Lampson MA, Schultz RM, Shea LD, Woodruff TK. Chromosome cohesion decreases in human eggs with advanced maternal age. *Aging Cell* 2012;11: 1121-1124.

Eagle H. Amino acid metabolism in mammalian cell cultures. *Science* 1959;130: 432-437.

Ebner T, Vanderzwalmen P, Shebl O, Urdl W, Moser M, Zech NH, Tews G. Morphology of vitrified/warmed day-5 embryos predicts rates of implantation, pregnancy and live birth. *Reprod Biomed Online* 2009;19: 72-78.

Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13: 3441-3448.

Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature* 1969;221: 632-635.

Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, Steptoe PC, Webster JM. Factors influencing the success of in vitro fertilization for alleviating human infertility. *J In Vitro Fert Embryo Transf* 1984;1: 3-23.

Edwards RG, Steptoe PC, Purdy JM. Fertilization and cleavage in vitro of preovulator human oocytes. *Nature* 1970;227: 1307-1309.

Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. Spindles, mitochondria and redox potential in ageing oocytes. *Reprod Biomed Online* 2004;8: 45-58.

El Mouatassim S, Guérin P, Ménézo Y. Mammalian oviduct and protection against free oxygen radicals: expression of genes encoding antioxidant enzymes in human and mouse. *Eur J Obstet Gynecol Reprod Biol* 2000;89: 1-6.

Enderasn A, Schlafke S. The fine structure of the blastocyst: some comparative studies. *Preimplantation Stages of Pregnancy*. 1965: 29-59.

Epstein CJ, Smith SA. Amino acid uptake and protein synthesis in preimplantation mouse embryos. *Dev Biol* 1973;33: 171-184.

Ferreux L, Bourdon M, Sallem A, Santulli P, Barraud-Lange V, Le Foll N, Maignien C, Chapron C, de Ziegler D, Wolf JP *et al.* Live birth rate following frozen-thawed blastocyst transfer is higher with blastocysts expanded on Day 5 than on Day 6. *Hum Reprod* 2018;33: 390-398.

Ferrick L, Lee YSL, Gardner DK. Reducing time to pregnancy and facilitating the birth of healthy children through functional analysis of embryo physiology. *Biol Reprod* 2019;101: 1124-1139.

Ferrick L, Lee YSL, Gardner DK. Metabolic activity of human preimplantation blastocysts correlates to their morphokinetics, morphological grade, KIDScore and artificial intelligence ranking. *Hum Reprod* 2020;In press.

Feuer S, Rinaudo P. Preimplantation stress and development. *Birth Defects Res C Embryo Today* 2012;96: 299-314.

Feuer S, Rinaudo P. From Embryos to Adults: A DOHaD Perspective on In Vitro Fertilization and Other Assisted Reproductive Technologies. *Healthcare (Basel)* 2016;4.

Feugang JM, de Roover R, Moens A, Léonard S, Dessy F, Donnay I. Addition of beta-mercaptoethanol or Trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. *Theriogenology* 2004;61: 71-90.

Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil* 1993;99: 673-679.

Fissore RA, Kurokawa M, Knott J, Zhang M, Smyth J. Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction* 2002;124: 745-754.

Fitzgerald O, Harris K, Paul RC, Chambers GM. Assisted reproductive technology in Australia and New Zealand 2015. *National Perinatal Epidemiology and Statistics Unit, the University of New South Wales Sydney* 2017.

Fleming T, Velazquez M, Eckert J. Embryos, DOHaD and david barker. *J Dev Orig Health Dis* 2015;6: 377-383.

Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, Treff NR, Scott Jr RT. In vitro fertilization with single euploid blastocyst transfer: a randomized controlled trial. *Fertil Steril* 2013;100: 100-107. e101.

Franasiak JM, Forman EJ, Hong KH, Werner MD, Upham KM, Treff NR, Scott Jr RT. The nature of aneuploidy with increasing age of the female partner: a review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil Steril* 2014;101: 656-663. e651.

Gangloff Y-G, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz J-F, Um SH, Brown EJ, Cereghini S, Thomas G. Disruption of the mouse mTOR gene leads to early

postimplantation lethality and prohibits embryonic stem cell development. *Mol Cell Biol* 2004;24: 9508-9516.

Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int* 1994;18: 1163-1179.

Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 1998;49: 83-102.

Gardner DK. Noninvasive metabolic assessment of single cells. *Methods Mol Med* 2007;132: 1-9.

Gardner DK. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? *Bioessays* 2015;37: 364-371.

Gardner DK. The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human IVF. *Reprod Biomed Online* 2016;32: 137-141.

Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important? *Mol Hum Reprod* 2016;22: 704-718.

Gardner DK, Clarke RN, Lechene CP, Biggers JD. Development of a noninvasive ultramicrofluorometric method for measuring net uptake of glutamine by single preimplantation mouse embryos. *Gamete Res* 1989;24: 427-438.

Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev* 2015;27: 638-654.

Gardner DK, Kelley RL. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis* 2017;8: 418-435.

Gardner DK, Kuramoto T, Tanaka M, Mitzumoto S, Montag M, Yoshida A. Prospective randomized multicentre comparison on sibling oocytes comparing G-Series media system with antioxidants versus standard G-Series media system. *Reprod Biomed Online* 2020;40: 637-644.

Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 1993;48: 377-385.

Gardner DK, Lane M. Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. *Hum Reprod* 1996;11: 2703-2712.

Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update* 1997;3: 367-382.

Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. *Reprod Fertil Dev* 2005;17: 361-370.

Gardner DK, Lane M. Mammalian preimplantation embryo culture. *Methods Mol Med* 2014: 167-182.

Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996;65: 349-353.

Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996;65: 349-353.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;73: 1155-1158.

Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril* 2001;76: 1175-1180.

Gardner DK, Larman MG, Thouas GA. Sex-related physiology of the preimplantation embryo. *Mol Hum Reprod* 2010;16: 539-547.

Gardner DK, Leese HJ. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. *Hum Reprod* 1986;1: 25-27.

Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *J Exp Zool* 1987;242: 103-105.

Gardner DK, Leese HJ. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. *Development* 1988;104: 423-429.

Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil* 1990;88: 361-368.

Gardner DK, Pawelczynski M, Trounson AO. Nutrient uptake and utilization can be used to select viable day 7 bovine blastocysts after cryopreservation. *Mol Reprod Dev* 1996;44: 472-475.

Gardner DK, Pool TB, Lane M. Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability. *Semin Reprod Med* 2000;18: 205-218.

Gardner DK, Reed L, Linck D, Sheehan C, Lane M. Quality control in human in vitro fertilization. *Semin Reprod Med* 2005;23: 319-324.

Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: role of medium composition. *Hum Reprod* 1993;8: 288-295.

Gardner DK, Sakkas D. Assessment of embryo viability: the ability to select a single embryo for transfer--a review. *Placenta* 2003;24: S5-12.

Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J. A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum Reprod* 1998;13: 3434-3440.

Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: a prospective randomized trial. *Fertil Steril* 2004;81: 551-555.

Gardner DK, Truong TT. Culture of the Mouse Preimplantation Embryo. *Methods Mol Biol* 2019;2006: 13-32.

Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB. Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertil Steril* 1998;69: 84-88.

Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril* 2013;99: 1062-1072.

Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod* 2011;26: 1981-1986.

Gazzo E, Peña F, Valdéz F, Chung A, Bonomini C, Ascenzo M, Velit M, Escudero E. The KidscoreTM D5 algorithm as an additional tool to morphological assessment and PGT-A in embryo selection: a time-lapse study. *JBRA Assist Reprod* 2020;24: 55-60.

Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerssche M, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. *Hum Reprod* 1999;14: 2581-2587.

Ghosh J, Coutifaris C, Sapienza C, Mainigi M. Global DNA methylation levels are altered by modifiable clinical manipulations in assisted reproductive technologies. *Clin Epigenetics* 2017;9: 14.

Golbus MS, Calarco PG, Epstein CJ. The effects of inhibitors of RNA synthesis (α-amanitin and actinomycin D) on preimplantation mouse embryogenesis. *J Exp Zool* 1973;186: 207-216.

González IM, Martin PM, Burdsal C, Sloan JL, Mager S, Harris T, Sutherland AE. Leucine and arginine regulate trophoblast motility through mTOR-dependent and independent pathways in the preimplantation mouse embryo. *Dev Biol* 2012;361: 286-300.

González R, Ferrín G, Hidalgo AB, Ranchal I, López-Cillero P, Santos-Gónzalez M, López-Lluch G, Briceño J, Gómez MA, Poyato A *et al.* N-acetylcysteine, coenzyme Q10 and superoxide dismutase mimetic prevent mitochondrial cell dysfunction and cell death induced by d-galactosamine in primary culture of human hepatocytes. *Chem Biol Interact* 2009;181: 95-106.

Gook DA, Choo B, Bourne H, Lewis K, Edgar DH. Closed vitrification of human oocytes and blastocysts: outcomes from a series of clinical cases. *J Assist Reprod Genet* 2016;33: 1247-1252.

Gott A, Hardy K, Winston R, Leese H. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod* 1990;5: 104-108.

Grifo JA, Hodes-Wertz B, Lee H-L, Amperloquio E, Clarke-Williams M, Adler A. Single thawed euploid embryo transfer improves IVF pregnancy, miscarriage, and multiple gestation outcomes and has similar implantation rates as egg donation. *J Assist Reprod Genet* 2013;30: 259-264.

Guerif F, McKeegan P, Leese HJ, Sturmey RG. A simple approach for COnsumption and RElease (CORE) analysis of metabolic activity in single mammalian embryos. *PLoS One* 2013;8: e67834.

Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001;7: 175-189.

Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCα, but not S6K1. *Dev Cell* 2006;11: 859-871.

Gülcin İ. Antioxidant and antiradical activities of L-carnitine. *Life Sci* 2006;78: 803-811.

Gupta S, Malhotra N, Sharma D, Chandra A, Ashok A. Oxidative stress and its role in female infertility and assisted reproduction: clinical implications. *Intl J Fertil Steril* 2009;2: 147-167.

Gutiérrez-Mateo C, Colls P, Sánchez-García J, Escudero T, Prates R, Ketterson K, Wells D, Munné S. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. *Fertil Steril* 2011;95: 953-958.

Guzman L, Nuñez D, López R, Inoue N, Portella J, Vizcarra F, Noriega-Portella L, Noriega-Hoces L, Munné S. The number of biopsied trophectoderm cells may affect pregnancy outcomes. *J Assist Reprod Genet* 2019;36: 145-151.

Haas J, Meriano J, Laskin C, Bentov Y, Barzilay E, Casper RF, Cadesky K. Clinical pregnancy rate following frozen embryo transfer is higher with blastocysts vitrified on day 5 than on day 6. *J Assist Reprod Genet* 2016;33: 1553-1557.

Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* 2010;5: 253-295.

Hamanaka RB, Chandel NS. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem Sci* 2010;35: 505-513.

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, Ko MS. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum Mol Genet* 2004;13: 2263-2278.

Hannan N, Paiva P, Meehan K, Rombauts L, Gardner D, Salamonsen L. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology* 2011;152: 4948-4956.

Hansen P. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. *Theriogenology* 2007;68: S242-S249.

Harbottle S, Hughes C, Cutting R, Roberts S, Brison D, Embryologists AOC, Society TBF. Elective single embryo transfer: an update to UK best practice guidelines. *Hum Fertil* 2015;18: 165-183.

Hardy K, Hooper M, Handyside A, Rutherford A, Winston R, Leese H. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum Reprod* 1989;4: 188-191.

Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology* 2005;64: 992-1006.

Harvey AJ. The role of oxygen in ruminant preimplantation embryo development and metabolism. *Anim Reprod Sci* 2007;98: 113-128.

Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG. Oxygen-regulated gene expression in bovine blastocysts. *Biol Reprod* 2004;71: 1108-1119.

Harvey AJ, Kind KL, Thompson JG. REDOX regulation of early embryo development. *Reproduction* 2002;123: 479-486.

Harvey AJ, Rathjen J, Gardner DK. Metaboloepigenetic Regulation of Pluripotent Stem Cells. *Stem Cells Int* 2016;2016: 1816525.

Hashimoto S, Morimoto N, Yamanaka M, Matsumoto H, Yamochi T, Goto H, Inoue M, Nakaoka Y, Shibahara H, Morimoto Y. Quantitative and qualitative changes of mitochondria in human preimplantation embryos. *J Assist Reprod Genet* 2017;34: 573-580.

Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* 2001;2: 280-291.

Hérubel F, El Mouatassim S, Guérin P, Frydman R, Ménézo Y. Genetic expression of monocarboxylate transporters during human and murine oocyte maturation and early embryonic development. *Zygote* 2002;10: 175-181.

Hewitson LC, Leese HJ. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. *J Exp Zool* 1993;267: 337-343.

Heydarnejad A, Ostadhosseini S, Varnosfaderani SR, Jafarpour F, Moghimi A, Nasr-Esfahani MH. Supplementation of maturation medium with CoQ10 enhances developmental competence of ovine oocytes through improvement of mitochondrial function. *Mol Reprod Dev* 2019;86: 812-824.

Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, Bendikson KA, Paulson RJ, McGinnis LK. Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos. *Fertil Steril* 2018;110: 467-475. e462.

Ho Y, Wigglesworth K, Eppig JJ, Schultz RM. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 1995;41: 232-238.

Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 2002;17: 999-1005.

Houghton FD, Thompson JG, Kennedy CJ, Leese HJ. Oxygen consumption and energy metabolism of the early mouse embryo. *Mol Reprod Dev* 1996;44: 476-485.

Houtkooper RH, Cantó C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 2010;31: 194-223.

Huang L, Bogale B, Tang Y, Lu S, Xie XS, Racowsky C. Noninvasive preimplantation genetic testing for aneuploidy in spent medium may be more reliable than trophectoderm biopsy. *Proc Natl Acad Sci U S A* 2019;116: 14105-14112.

Huang TT, Huang DH, Ahn HJ, Arnett C, Huang CT. Early blastocyst expansion in euploid and aneuploid human embryos: evidence for a non-invasive and quantitative marker for embryo selection. *Reprod Biomed Online* 2019;39: 27-39.

Igarashi H, Takahashi T, Nagase S. Oocyte aging underlies female reproductive aging: biological mechanisms and therapeutic strategies. *Reprod Med Biol* 2015;14: 159-169.

Imai S-i, Guarente L. NAD+ and sirtuins in aging and disease. *Trends in cell biology* 2014;24: 464-471.

Janny L, Menezo YJ. Maternal age effect on early human embryonic development and blastocyst formation. *Mol Reprod Dev* 1996;45: 31-37.

Jiang W, Li Y, Zhao Y, Gao Q, Jin Q, Yan C, Xu Y. l-carnitine supplementation during in vitro culture regulates oxidative stress in embryos from bovine aged oocytes. *Theriogenology* 2020;143: 64-73.

Kaing A, Kroener LL, Tassin R, Li M, Liu L, Buyalos R, Hubert G, Shamonki M. Earlier day of blastocyst development is predictive of embryonic euploidy across all ages: essential data

for physician decision-making and counseling patients. *J Assist Reprod Genet* 2018;35: 119-125.

Kang H-J, Melnick AP, Stewart JD, Xu K, Rosenwaks Z. Preimplantation genetic screening: who benefits? *Fertil Steril* 2016;106: 597-602.

Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. A proteomic analysis of mammalian preimplantation embryonic development. *Reproduction* 2005;130: 899-905.

Kelley RL, Gardner DK. Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. *Reprod Biomed Online* 2016;33: 537-549.

Kelley RL, Gardner DK. In vitro culture of individual mouse preimplantation embryos: the role of embryo density, microwells, oxygen, timing and conditioned media. *Reprod Biomed Online* 2017;34: 441-454.

Kelley RL, Gardner DK. Individual culture and atmospheric oxygen during culture affect mouse preimplantation embryo metabolism and post-implantation development. *Reprod Biomed Online* 2019;39: 3-18.

Khurana NK, Wales RG. Effects of oxygen concentration on the metabolism of [U-14C]glucose by mouse morulae and early blastocysts in vitro. *Reprod Fertil Dev* 1989;1: 99-106.

Kim MK, Park EA, Kim HJ, Choi WY, Cho JH, Lee WS, Cha KY, Kim YS, Lee DR, Yoon TK. Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS? *Reprod Biomed Online* 2013;26: 22-29.

Kim MK, Park JK, Paek SK, Kim JW, Kwak IP, Lee HJ, Lyu SW, Lee WS. Effects and pregnancy outcomes of L-carnitine supplementation in culture media for human embryo development from in vitro fertilization. *J Obstet Gynaecol Res* 2018;44: 2059-2066.

Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril* 2013;99: 738-744 e734.

Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. *Theriogenology* 2004;62: 1186-1197.

Kramer YG, Kofinas JD, Melzer K, Noyes N, McCaffrey C, Buldo-Licciardi J, McCulloh DH, Grifo JA. Assessing morphokinetic parameters via time lapse microscopy (TLM) to predict euploidy: are aneuploidy risk classification models universal? *J Assist Reprod Genet* 2014;31: 1231-1242.

Krisher RL, Prather RS. A role for the Warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. *Mol Reprod Dev* 2012;79: 311-320.

Kupka M, D'Hooghe T, Ferraretti A, de Mouzon J, Erb K, Castilla J, Calhaz-Jorge C, De Geyter C. Assisted reproductive technology in Europe, 2011: results generated from European registers by ESHRE. *Hum Reprod* 2016;31: 233-248.

Lamb V, Leese H. Uptake of a mixture of amino acids by mouse blastocysts. *J Reprod Fertil* 1994;102: 169-175.

Lane M, Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil* 1994;102: 305-312.

Lane M, Gardner DK. Removal of embryo-toxic ammonium from the culture medium by in situ enzymatic conversion to glutamate. *J Exp Zool* 1995;271: 356-363.

Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod* 1996;11: 1975-1978.

Lane M, Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. *J Reprod Fertil* 1997;109: 153-164.

Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998;13: 991-997.

Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod* 2000;62: 16-22.

Lane M, Gardner DK. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biol Reprod* 2003;69: 1109-1117.

Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem* 2005;280: 18361-18367.

Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev* 2005;17: 371-378.

Lane M, Maybach JM, Gardner DK. Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Hum Reprod* 2002;17: 2686-2693.

Lane M, Schoolcraft WB, Gardner DK, Phil D. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 1999;72: 1073-1078.

Larman MG, Katz-Jaffe MG, McCallie B, Filipovits JA, Gardner DK. Analysis of global gene expression following mouse blastocyst cryopreservation. *Hum Reprod* 2011;26: 2672-2680.

Latham KE. Mechanisms and control of embryonic genome activation in mammalian embryos. *Int Rev Cytol* 1999;193: 71-124.

Latham T, Mackay L, Sproul D, Karim M, Culley J, Harrison DJ, Hayward L, Langridge-Smith P, Gilbert N, Ramsahoye BH. Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. *Nucleic Acids Res* 2012;40: 4794-4803.

Lawitts JA, Biggers JD. Culture of preimplantation embryos. *Methods Enzymol* 1993;225: 153-164.

Leary C, Sturmey R. Metabolic profile of in-vitro derived human embryos is not affected by the mode of fertilization. *Mol Hum Reprod* 2020.

Leaver M, Wells D. Non-invasive preimplantation genetic testing (niPGT): the next revolution in reproductive genetics? *Hum Reprod Update* 2020;26: 16-42.

Lee MT, Bonneau AR, Giraldez AJ. Zygotic genome activation during the maternal-to-zygotic transition. *Annu Rev Cell Dev Biol* 2014;30: 581-613.

Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod* 2015;30: 543-552.

Lees JG, Gardner DK, Harvey AJ. Pluripotent stem cell metabolism and mitochondria: beyond ATP. *Stem Cells Int* 2017;2017:2874283.

Lees JG, Gardner DK, Harvey AJ. Nicotinamide adenine dinucleotide induces a bivalent metabolism and maintains pluripotency in human embryonic stem cells. *Stem Cells* 2020;38: 624-638.

Leese H. The formation and function of oviduct fluid. *J Reprod Fertil* 1988;82: 843-856.

Leese H. Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol* 1991;13: 35.

Leese H, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *Reproduction* 1984;72: 9-13.

Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* 2002;24: 845-849.

Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil* 1984;72: 9-13.

Leese HJ, Guerif F, Allgar V, Brison DR, Lundin K, Sturmey RG. Biological optimization, the Goldilocks principle, and how much is lagom in the preimplantation embryo. *Mol Reprod Dev* 2016;83: 748-754.

Leese HJ, Sturmey RG, Baumann CG, McEvoy TG. Embryo viability and metabolism: obeying the quiet rules. *Hum Reprod* 2007;22: 3047-3050.

Leonavicius K, Royer C, Preece C, Davies B, Biggins JS, Srinivas S. Mechanics of mouse blastocyst hatching revealed by a hydrogel-based microdeformation assay. *Proc Natl Acad Sci U S A* 2018;115: 10375-10380.

Lessey BA. Endometrial receptivity and the window of implantation. *Baillieres Best Pract Res Clin Obstet Gynaecol* 2000;14: 775-788.

Liang B, Gao Y, Xu J, Song Y, Xuan L, Shi T, Wang N, Hou Z, Zhao Y-L, Huang WE. Raman profiling of embryo culture medium to identify aneuploid and euploid embryos. *Fertil Steril* 2019;111: 753-762. e751.

Liang S, Niu YJ, Shin KT, Cui XS. Protective Effects of Coenzyme Q10 on Developmental Competence of Porcine Early Embryos. *Microsc Microanal* 2017;23: 849-858.

Limoli CL, Giedzinski E, Morgan WF, Swarts SG, Jones GD, Hyun W. Persistent oxidative stress in chromosomally unstable cells. *Cancer Res* 2003;63: 3107-3111.

Linck D, Larman M, Gardner D. α-Lipoic acid: an antioxidant that improves embryo development and protects against oxidative stress. *Fertil Steril* 2007;88: S36-S37.

Liu W, Liu J, Du H, Ling J, Sun X, Chen D. Non-invasive pre-implantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. *Ann Med* 2017;49: 319-328.

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. *Fertil Steril* 2016;105: 656-662 e651.

Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. *Fertil Steril* 2014;102: 1295-1300 e1292.

Liu Z, Foote RH. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O2. *Biol Reprod* 1995;53: 786-790.

Lord T, Aitken RJ. Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction* 2013;146: R217-227.

Luke B, Bigger HR, Leurgans S, Sietsema D. The cost of prematurity: a case-control study of twins vs singletons. *Am J Public Health* 1996;86: 809-814.

Lundin K, Bergh C, Hardarson T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Hum Reprod* 2001;16: 2652-2657.

Magnusson C, Hillensjö T, Hamberger L, Nilsson L. Oxygen consumption by human oocytes and blastocysts grown in vitro. *Hum Reprod* 1986;1: 183-184.

Manjunatha B, Devaraj M, Gupta P, Ravindra J, Nandi S. Effect of taurine and melatonin in the culture medium on buffalo in vitro embryo development. *Reprod Domest Anim* 2009;44: 12-16.

Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol* 2001;240: 182-193.

Martin PM, Sutherland AE, Van Winkle LJ. Amino Acid Transport Regulates Blastocyst Implantation. *Biol Reprod* 2003;69: 1101-1108.

Martino NA, Dell'aquila ME, Cardone RA, Somoskoi B, Lacalandra GM, Cseh S. Vitrification preserves chromatin integrity, bioenergy potential and oxidative parameters in mouse embryos. *Reprod Biol Endocrinol* 2013;11: 27.

Mastroianna L, Jones R. Oxygen tension within the rabbit fallopian tube. *J Reprod Fertil* 1965;9: 99-102.

Maxwell SM, Grifo JA. Should every embryo undergo preimplantation genetic testing for aneuploidy? A review of the modern approach to in vitro fertilization. *Best Pract Res Clin Obstet Gynaecol* 2018;53: 38-47.

McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RP. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertil Steril* 2005;84: 1628-1636.

Mehta TS, Kiessling AA. Development potential of mouse embryos conceived in vitro and cultured in ethylenediaminetetraacetic acid with or without amino acids or serum. *Biol Reprod* 1990;43: 600-606.

Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, Barnett BD, Madden JD. A controlled randomized trial evaluating the effect of lowered incubator

oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod* 2009;24: 300-307.

Menke TM, McLaren A. Mouse blastocysts grown in vivo and in vitro: carbon dioxide production and trophoblast outgrowth. *J Reprod Dev* 1970;23: 117-127.

Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011;26: 2658-2671.

Milewski R, Kuc P, Kuczynska A, Stankiewicz B, Lukaszuk K, Kuczynski W. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. *J Assist Reprod Genet* 2015;32: 571-579.

Milewski R, Milewska AJ, Kuczyńska A, Stankiewicz B, Kuczyński W. Do morphokinetic data sets inform pregnancy potential? *J Assist Reprod Genet* 2016;33: 357-365.

Miller JG, Schultz GA. Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol Reprod* 1987;36: 125-129.

Minasi MG, Colasante A, Riccio T, Ruberti A, Casciani V, Scarselli F, Spinella F, Fiorentino F, Varricchio MT, Greco E. Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study. *Hum Reprod* 2016;31: 2245-2254.

Mio Y, Maeda K. Time-lapse cinematography of dynamic changes occurring during in vitro development of human embryos. *Am J Ostet and Gynecol* 2008;199: 660. e661-660. e665.

Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod* 2009;80: 295-301.

Miyagi Y, Habara T, Hirata R, Hayashi N. Feasibility of predicting live birth by combining conventional embryo evaluation with artificial intelligence applied to a blastocyst image in patients classified by age. *Reprod Med Biol* 2019;18: 344-356.

Mochizuki L, Gleicher N. The PGS/PGT-A controversy in IVF addressed as a formal conflict resolution analysis. *J Assist Reprod Genet* 2020: 1-11.

Montag M, Liebenthron J, Köster M. Which morphological scoring system is relevant in human embryo development? *Placenta* 2011;32: S252-S256.

Morales H, Tilquin P, Rees JF, Massip A, Dessy F, Van Langendonckt A. Pyruvate prevents peroxide-induced injury of in vitro preimplantation bovine embryos. *Mol Reprod Dev* 1999;52: 149-157.

Morgan MJ, Faik P. Carbohydrate metabolism in cultured animal cells. *Biosci Rep* 1981;1: 669-686.

Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohi J, Meseguer M. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril* 2016;105: 376-384 e379.

Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab* 2015;21: 392-402.

Mukerji S., Mukherjee S., S.K. B. The feasibility of long term cryogenic freezing of viable human embryos - a brief pilot study report. *Indian J Cryogenics* 1978;3: 80.

Munné S, Alikani M, Tomkin G, Grifo J, Cohen J. Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil Steril* 1995;64: 382-391.

Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, Silverberg K, Kalista T, Handyside AH, Katz-Jaffe M *et al.* Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. *Fertil Steril* 2019;112: 1071-1079.e1077.

Munné S, Lee A, Rosenwaks Z, Grifo J, Cohen J. Fertilization and early embryology: Diagnosis of major chromosome aneuploidies in human preimplantation embryos. *Hum Reprod* 1993;8: 2185-2191.

Murphy LA, Seidler EA, Vaughan DA, Resetkova N, Penzias AS, Toth TL, Thornton KL, Sakkas D. To test or not to test? A framework for counselling patients on preimplantation genetic testing for aneuploidy (PGT-A). *Hum Reprod* 2019;34: 268-275.

Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 2009;417: 1-13.

Nagy ZP, Shapiro D, Chang CC. Vitrification of the human embryo: a more efficient and safer in vitro fertilization treatment. *Fertil Steril* 2020;113: 241-247.

Nagy ZP, Varghese AC, Agarwal A. Cryopreservation of Mammalian Gametes and Embryos. *Methods Mol Biol* 2017;1568.

Newman DL, Thurgood LA, Gregory SL. The impact of aneuploidy on cellular homeostasis. *Free Radic Res* 2019;53: 705-713.

Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Hum Reprod Update* 2018;24: 15-34.

Niederberger C, Pellicer A, Simon C, Kathrins M, Goldstein M, Sigman M, Schlegel PN, Munné S, Gardner DK, Cobo A. 25 historic papers: an ASRM 75th birthday gift from Fertility and Sterility. *Fertil Steril* 2019;112: e2-e27.

Nishi M, Kumar NM, Gilula NB. Developmental regulation of gap junction gene expression during mouse embryonic development. *Dev Biol* 1991;146: 117-130.

Nogales MDC, Bronet F, Basile N, Martínez EM, Liñán A, Rodrigo L, Meseguer M. Type of chromosome abnormality affects embryo morphology dynamics. *Fertil Steril* 2017;107: 229-235. e222.

O'Fallon J, Wright R. Pyruvate revisited: a non-metabolic role for pyruvate in preimplantation embryo development. *Theriogenology* 1995;1: 288.

Olivius C, Friden B, Borg G, Bergh C. Why do couples discontinue in vitro fertilization treatment? A cohort study. *Fertil Steril* 2004;81: 258-261.

Ombelet W, Cooke I, Dyer S, Serour G, Devroey P. Infertility and the provision of infertility medical services in developing countries. *Hum Reprod Update* 2008;14: 605-621.

Ombelet W, De Sutter P, Van der Elst J, Martens G. Multiple gestation and infertility treatment: registration, reflection and reaction-the Belgian project. *Hum Reprod Update* 2005;11: 3-14.

Özgür K, Berkkanoğlu M, Bulut H, Isikli A, Coetzee K. Higher clinical pregnancy rates from frozen-thawed blastocyst transfers compared to fresh blastocyst transfers: a retrospective matched-cohort study. *J Assist Reprod Genet* 2015;32: 1483-1490.

Ozgur K, Berkkanoglu M, Bulut H, Yoruk GDA, Candurmaz NN, Coetzee K. Single best euploid versus single best unknown-ploidy blastocyst frozen embryo transfers: a randomized controlled trial. *J Assist Reprod Genet* 2019;36: 629-636.

Pabon Jr JE, Findley WE, Gibbons WE. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. *Fertil Steril* 1989;51: 896-900.

Packer L, Witt EH, Tritschler HJ. Alpha-lipoic acid as a biological antioxidant. *Free Radic Biol Med* 1995;19: 227-250.

Palaniappan A, Dai A. Mitochondrial ageing and the beneficial role of α-lipoic acid. *Neurochem Res* 2007;32: 1552-1558.

Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, Bulletti C. Genomic DNA in human blastocoele fluid. *Reprod Biomed Online* 2013;26: 603-610.

Partridge RJ, Leese HJ. Consumption of amino acids by bovine preimplantation embryos. *Reprod Fertil Dev* 1996;8: 945-950.

Patel DV, Shah PB, Kotdawala AP, Herrero J, Rubio I, Banker MR. Morphokinetic behavior of euploid and aneuploid embryos analyzed by time-lapse in embryoscope. *J Hum Reprod Sci* 2016;9: 112-118.

Paternot G, Debrock S, D'Hooghe T, Spiessens C. Computer-assisted embryo selection: a benefit in the evaluation of embryo quality? *Reprod Biomed Online* 2011;23: 347-354.

Paulson RJ. Hidden in plain sight: the overstated benefits and underestimated losses of potential implantations associated with advertised PGT-A success rates. *Hum Reprod* 2020;35: 490-493.

Penzias A, Bendikson K, Butts S, Coutifaris C, Fossum G, Falcone T, Gitlin S, Gracia C, Hansen K, La Barbera A. Guidance on the limits to the number of embryos to transfer: a committee opinion. *Fertil Steril* 2017;107: 901-903.

Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Hum Reprod* 2016;31: 2231-2244.

Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, Leese HJ, Harris SE. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Mol Hum Reprod* 2010;16: 557-569.

Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, Zachary M-D, Remacle J. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. *Mech Aging Dev* 1990;51: 283-297.

Pikó L, Clegg KB. Quantitative changes in total RNA, total poly (A), and ribosomes in early mouse embryos. *Dev Biol* 1982;89: 362-378.

Pribenszky C, Nilselid A-M, Montag M. Response: time-lapse systems for ART. *Reprod Biomed Online* 2018;36: 290.

Reignier A, Girard J-M, Lammers J, Chtourou S, Lefebvre T, Barriere P, Freour T. Performance of Day 5 KIDScore™ morphokinetic prediction models of implantation and live birth after single blastocyst transfer. *J Assist Reprod Genet* 2019;36: 2279-2285.

Reitzer LJ, Wice B, Kennell D. The pentose cycle. Control and essential function in HeLa cell nucleic acid synthesis. *J Biol Chem* 1980;255: 5616-5626.

Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. *J Reprod Fertil* 1980;58: 161-164.

Rezazadeh Valojerdi M, Eftekhari-Yazdi P, Karimian L, Hassani F, Movaghar B. Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. *J Assist Reprod Genet* 2009;26: 347-354.

Rieger D, Loskutoff N, Betteridge K. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. *Reprod Fertil Dev* 1992;4: 547-557.

Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. Effects of oxygen tension on gene expression in preimplantation mouse embryos. *Fertil Steril* 2006;86: 1252-1265, e1251-1236.

Rodriguez-Osorio N, Kim IJ, Wang H, Kaya A, Memili E. Melatonin increases cleavage rate of porcine preimplantation embryos in vitro. *J Pineal Res* 2007;43: 283-288.

Rogers P, Murphy C, Gannon B. Changes in the spatial organization of the uterine vasculature during implantation in the rat. *J Reprod Fertil* 1982;65: 211-214.

Roh M, van der Meer R, Abdulkadir SA. Tumorigenic polyploid cells contain elevated ROS and ARE selectively targeted by antioxidant treatment. *J Cell Physiol* 2012;227: 801-812.

Ros R, Graves C. O2 levels in female rabbit reproductive-tract. *J Anim Sci*. 1974; 39: 994.

Rougier N, Bourc'his D, Gomes DM, Niveleau A, Plachot M, Pàldi A, Viegas-Péquignot E. Chromosome methylation patterns during mammalian preimplantation development. *Genes Dev* 1998;12: 2108-2113.

Rubino P, Tapia L, de Assin Alonso RR, Mazmanian K, Guan L, Dearden L, Thiel A, Moon C, Kolb B, Norian JM. Trophectoderm biopsy protocols can affect clinical outcomes: time to focus on the blastocyst biopsy technique. *Fertil Steril* 2020;113: 981-989.

Rubio C, Navarro-Sánchez L, García-Pascual CM, Ocali O, Cimadomo D, Venier W, Barroso G, Kopcow L, Bahçeci M, Kulmann MIR *et al.* Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts. *Am J Obstet Gynecol* 2020.

Rubio C, Rienzi L, Navarro-Sánchez L, Cimadomo D, García-Pascual CM, Albricci L, Soscia D, Valbuena D, Capalbo A, Ubaldi F. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications. *Fertil Steril* 2019;112: 510-519.

Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. *Fertil Steril* 2014;102: 1287-1294 e1285.

Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ, Bellver J, Meseguer M. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. *Fertil Steril* 2012;98: 1458-1463.

Ryall JG, Dell'Orso S, Derfoul A, Juan A, Zare H, Feng X, Clermont D, Koulnis M, Gutierrez-Cruz G, Fulco M. The NAD+-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. *Cell Stem Cell* 2015;16: 171-183.

Sakkas D, Percival G, D'Arcy Y, Sharif K, Afnan M. Assessment of early cleaving in vitro fertilized human embryos at the 2-cell stage before transfer improves embryo selection. *Fertil Steril* 2001;76: 1150-1156.

Salamonsen LA, Evans J, Nguyen HP, Edgell TA. The Microenvironment of Human Implantation: Determinant of Reproductive Success. *Am J Reprod Immunol* 2016;75: 218-225.

Salumets A, Hydén-Granskog C, Mäkinen S, Suikkari AM, Tiitinen A, Tuuri T. Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Hum Reprod* 2003;18: 821-825.

Santos Filho E, Noble JA, Poli M, Griffiths T, Emerson G, Wells D. A method for semi-automatic grading of human blastocyst microscope images. *Hum Reprod* 2012;27: 2641-2648.

Sasaki H, Hamatani T, Kamijo S, Iwai M, Kobanawa M, Ogawa S, Miyado K, Tanaka M. Impact of Oxidative Stress on Age-Associated Decline in Oocyte Developmental Competence. *Front Endocrinol (Lausanne)* 2019;10: 811.

Schmid F, Hinz HJ, Jaenicke R. Thermodynamic studies of binary and ternary complexes of pig heart lactate dehydrogenase. *Biochemistry* 1976;15: 3052-3059.

Schon EA, Kim SH, Ferreira JC, Magalhães P, Grace M, Warburton D, Gross SJ. Chromosomal non-disjunction in human oocytes: is there a mitochondrial connection? *Hum Reprod* 2000;15 Suppl 2: 160-172.

Schoolcraft WB, Fragouli E, Stevens J, Munne S, Katz-Jaffe MG, Wells D. Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertil Steril* 2010;94: 1700-1706.

Schoolcraft WB, Gardner DK, Lane M, Schlenker T, Hamilton F, Meldrum DR. Blastocyst culture and transfer: analysis of results and parameters affecting outcome in two in vitro fertilization programs. *Fertil Steril* 1999;72: 604-609.

Schultz GA, Kaye PL, McKay DJ, Johnson MH. Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. *J Reprod Fertil* 1981;61: 387-393.

Scotchie JG, Fritz MA, Mocanu M, Lessey BA, Young SL. Proteomic analysis of the luteal endometrial secretome. *Reprod Sci* 2009;16: 883-893.

Scott Jr RT, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, Tao X, Treff NR. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. *Fertil Steril* 2013;100: 697-703.

Scott Jr RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertil Steril* 2013;100: 624-630.

Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Thomas S. Large blastocyst diameter, early blastulation, and low preovulatory serum progesterone are dominant predictors of clinical pregnancy in fresh autologous cycles. *Fertil Steril* 2008;90: 302-309.

Shapiro BS, Richter KS, Harris DC, Daneshmand ST. A comparison of day 5 and day 6 blastocyst transfers. *Fertil Steril* 2001;75: 1126-1130.

Shen W, Liu K, Tian C, Yang L, Li X, Ren J, Packer L, Cotman CW, Liu J. R-alpha-lipoic acid and acetyl-L-carnitine complementarily promote mitochondrial biogenesis in murine 3T3-L1 adipocytes. *Diabetologia* 2008;51: 165-174.

Showstack JA, Stone MH, Schroeder SA. The role of changing clinical practices in the rising costs of hospital care. *N Engl J Med* 1985;313: 1201-1207.

Silva E, Greene AF, Strauss K, Herrick JR, Schoolcraft WB, Krisher RL. Antioxidant supplementation during in vitro culture improves mitochondrial function and development of embryos from aged female mice. *Reprod Fertil Dev* 2015;27: 975-983.

Simon AL, Kiehl M, Fischer E, Proctor JG, Bush MR, Givens C, Rabinowitz M, Demko ZP. Pregnancy outcomes from more than 1,800 in vitro fertilization cycles with the use of 24-chromosome single-nucleotide polymorphism–based preimplantation genetic testing for aneuploidy. *Fertil Steril* 2018;110: 113-121.

Simsek-Duran F, Li F, Ford W, Swanson RJ, Jones HW, Jr., Castora FJ. Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. *PLoS One* 2013;8: e64955.

Smith GD, Silva ESCA. Developmental consequences of cryopreservation of mammalian oocytes and embryos. *Reprod Biomed Online* 2004;9: 171-178.

Somfai T, Kaneda M, Akagi S, Watanabe S, Haraguchi S, Mizutani E, Dang-Nguyen TQ, Geshi M, Kikuchi K, Nagai T. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod Fertil Dev* 2011;23: 912-920.

Son WY, Chung JT, Gidoni Y, Holzer H, Levin D, Chian RC, Tan SL. Comparison of survival rate of cleavage stage embryos produced from in vitro maturation cycles after slow freezing and after vitrification. *Fertil Steril* 2009;92: 956-958.

Steeves TE, Gardner DK. Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol Reprod* 1999;61: 731-740.

Stehlik E, Stehlik J, Katayama KP, Kuwayama M, Jambor V, Brohammer R, Kato O. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reprod Biomed Online* 2005;11: 53-57.

Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet* 1978;2: 366.

Stigliani S, Anserini P, Venturini P, Scaruffi P. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. *Hum Reprod* 2013;28: 2652-2660.

Storr A, Venetis CA, Cooke S, Kilani S, Ledger W. Inter-observer and intra-observer agreement between embryologists during selection of a single Day 5 embryo for transfer: a multicenter study. *Hum Reprod* 2017;32: 307-314.

Sturmey R, Reis A, Leese H, McEvoy T. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod Domest Anim* 2009;44: 50-58.

Succu S, Gadau SD, Serra E, Zinellu A, Carru C, Porcu C, Naitana S, Berlinguer F, Leoni GG. A recovery time after warming restores mitochondrial function and improves developmental competence of vitrified ovine oocytes. *Theriogenology* 2018;110: 18-26.

Sun X-F, Zhang W-H, Chen X-J, Xiao G-H, Mai W-Y, Wang W-H. Spindle dynamics in living mouse oocytes during meiotic maturation, ageing, cooling and overheating: a study by polarized light microscopy. *Zygote* 2004;12: 241-249.

Sunderam S, Kissin DM, Zhang Y, Folger SG, Boulet SL, Warner L, Callaghan WM, Barfield WD. Assisted Reproductive Technology Surveillance - United States, 2016. *MMWR Surveill Summ* 2019;68: 1-23.

Takahashi M, Keicho K, Takahashi H, Ogawa H, Schulte R, Okano A. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology* 2000;54: 137-145.

Takahashi T, Takahashi E, Igarashi H, Tezuka N, Kurachi H. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Mol Reprod Dev* 2003;66: 143-152.

Takeshima K, Jwa SC, Saito H, Nakaza A, Kuwahara A, Ishihara O, Irahara M, Hirahara F, Yoshimura Y, Sakumoto T. Impact of single embryo transfer policy on perinatal outcomes in fresh and frozen cycles—analysis of the Japanese Assisted Reproduction Technology registry between 2007 and 2012. *Fertil Steril* 2016;105: 337-346. e333.

Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P, Focarelli R. Cellular and molecular aspects of ovarian follicle ageing. *Hum Reprod Update* 2008;14: 131-142.

Tatone C, Di Emidio G, Barbonetti A, Carta G, Luciano AM, Falone S, Amicarelli F. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. *Hum Reprod Update* 2018;24: 267-289.

Tatone C, Di Emidio G, Vento M, Ciriminna R, Artini PG. Cryopreservation and oxidative stress in reproductive cells. *Gynecol Endocrinol* 2010;26: 563-567.

Taylor KD, Pikó L. Patterns of mRNA prevalence and expression of B1 and B2 transcripts in early mouse embryos. *Development* 1987;101: 877-892.

Thirumalaraju P, Hsu J, Bormann C, Kanakasabapathy M, Souter I, Dimitriadis I, Dickinson K, Pooniwala R, Gupta R, Yogesh V. Deep learning-enabled blastocyst prediction system for cleavage stage embryo selection. *Fertil Steril* 2019;111: e29.

Thompson J, Partridge R, Houghton F, Cox C, Leese H. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. *J Reprod Fertil* 1996;106: 299-306.

Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *J Reprod Fertil* 1990;89: 573-578.

Thouas GA, Trounson AO, Jones GM. Effect of female age on mouse oocyte developmental competence following mitochondrial injury. *Biol Reprod* 2005;73: 366-373.

Tiitinen A, Hydén-Granskog C, Gissler M. What is the most relevant standard of success in assisted reproduction?: The value of cryopreservation on cumulative pregnancy rates per single oocyte retrieval should not be forgotten. *Hum Reprod* 2004;19: 2439-2441.

Tilia L, Chapman M, Kilani S, Cooke S, Venetis C. Oocyte meiotic spindle morphology is a predictive marker of blastocyst ploidy—a prospective cohort study. *Fertil Steril* 2020;113: 105-113. e101.

Tilia L, Venetis C, Kilani S, Cooke S, Chapman M. Is oocyte meiotic spindle morphology associated with embryo ploidy? A prospective cohort study. *Fertil Steril* 2016;105: 1085-1092. e1087.

Tran D, Cooke S, Illingworth P, Gardner D. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. *Hum Reprod* 2019;34: 1011-1018.

Treff NR, Su J, Tao X, Levy B, Scott Jr RT. Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. *Fertil Steril* 2010;94: 2017-2021.

Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 1983;305: 707-709.

Truong T, Gardner D. Antioxidants improve IVF outcome and subsequent embryo development in the mouse. *Hum Reprod* 2017;32: 2404-2413.

Truong TT, Gardner DK. Antioxidants increase blastocyst cryosurvival and viability post-vitrification. *Hum Reprod* 2020;35: 12-23.

Truong TT, Soh YM, Gardner DK. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod* 2016;31: 1445-1454.

Turner K, Martin KL, Woodward BJ, Lenton EA, Leese HJ. Comparison of pyruvate uptake by embryos derived from conception and non-conception natural cycles. *Hum Reprod* 1994;9: 2362-2366.

Umaoka Y, Noda Y, Narimoto K, Mori T. Effects of oxygen toxicity on early development of mouse embryos. *Mol Reprod Dev* 1992;31: 28-33.

Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod* 1995;10: 415-424.

Van Montfoort APA, Arts E, Wijnandts L, Sluijmer A, Pelinck MJ, Land JA, Van Echten-Arends J. Reduced oxygen concentration during human IVF culture improves embryo utilization and cumulative pregnancy rates per cycle. *Hum Reprod Open* 2020;1: hoz036.

van Noord-Zaadstra BM, Looman CW, Alsbach H, Habbema JD, te Velde ER, Karbaat J. Delaying childbearing: effect of age on fecundity and outcome of pregnancy. *BMJ* 1991;302: 1361-1365.

Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W, Gerris J. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod* 1999;14: 2345-2349.

Van Winkle LJ. Amino acid transport in developing animal oocytes and early conceptuses. *Biochim Biophys Acta* 1988;947: 173-208.

Van Winkle LJ, Campione AL. Amino acid transport regulation in preimplantation mouse embryos: effects on amino acid content and pre-and peri-implantation development. *Theriogenology* 1996;45: 69-80.

Van Winkle LJ, Haghighat N, Campione AL. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *J Exp Zool* 1990;253: 215-219.

Vega M, Breborowicz A, Moshier EL, McGovern PG, Keltz MD. Blastulation rates decline in a linear fashion from euploid to aneuploid embryos with single versus multiple chromosomal errors. *Fertil Steril* 2014;102: 394-398.

Vera-Rodriguez M, Chavez SL, Rubio C, Pera RAR, Simon C. Prediction model for aneuploidy in early human embryo development revealed by single-cell analysis. *Nat Commun* 2015;6: 1-14.

Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, Mercader A, Meseguer M, Blesa D, Moreno I. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod* 2018;33: 745-756.

VerMilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M, Boostanfar R, Baker VL, Suraj V, Chen AA. Computer-automated time-lapse analysis results correlate with embryo implantation and clinical pregnancy: a blinded, multi-centre study. *Reprod Biomed Online* 2014;29: 729-736.

Waldenström U, Engström AB, Hellberg D, Nilsson S. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril* 2009;91: 2461-2465.

Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod Biomed Online* 2010;21: 402-410.

Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod* 2012;87: 24, 21-28.

Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod* 2013;89: 75.

Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2016;22: 2-22.

Walker DL, Gardner DK, Lane M, Tummon IS, Session DR, Thornhill AR. Cryosystem assessment by glucose uptake of murine blastocysts. *Reprod Biomed Online* 2005;11: 601-607.

Walker KJ, Green MP, Gardner DK. Spatial asynchronous transfer of cleavage-stage mouse embryos to the uterus compromises fetal development. *Mol Reprod Dev* 2015;82: 80.

Wang A, Kort J, Behr B, Westphal LM. Euploidy in relation to blastocyst sex and morphology. *J Assist Reprod Genet* 2018;35: 1565-1572.

Wang W-H, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling–rewarming observed using polarized light microscopy. *Hum Reprod* 2001;16: 2374-2378.

Warburg O. On the origin of cancer cells. *Science* 1956;123: 309-314.

Watson A. Oocyte cytoplasmic maturation: a key mediator of oocyte and embryo developmental competence. *J Anim Sci* 2007;85: E1-E3.

Watson AJ, Damsky CH, Kidder GM. Differentiation of an epithelium: factors affecting the polarized distribution of Na+, K+-ATPase in mouse trophectoderm. *Dev Biol* 1990;141: 104-114.

Watson AJ, Kidder GM. Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K-ATPase during mouse embryogenesis. *Dev Biol* 1988;126: 80-90.

Watson AJ, Kidder GM, Schultz GA. How to make a blastocyst. *Biochem Cell Biol* 1992;70: 849-855.

Watson AJ, Pape C, Emanuel JR, Levenson R, Kidder GM. Expression of NA, K-ATpase α and β subunit genes during preimplantation development of the mouse. *Dev Genet* 1990;11: 41-48.

Wei D, Liu JY, Sun Y, Shi Y, Zhang B, Liu JQ, Tan J, Liang X, Cao Y, Wang Z *et al.* Frozen versus fresh single blastocyst transfer in ovulatory women: a multicentre, randomised controlled trial. *Lancet* 2019;393: 1310-1318.

Wells D, Kaur K, Grifo J, Glassner M, Taylor JC, Fragouli E, Munne S. Clinical utilisation of a rapid low-pass whole genome sequencing technique for the diagnosis of aneuploidy in human embryos prior to implantation. *J Med Genet* 2014;51: 553-562.

West JD, Flockhart JH. Genetic differences in glucose phosphate isomerase activity among mouse embryos. *Development* 1989;107: 465-472.

Whitaker BD, Casey SJ, Taupier R. The effects of N-acetyl-L-cysteine supplementation on in vitro porcine oocyte maturation and subsequent fertilisation and embryonic development. *Reprod Fertil Dev* 2012;24: 1048-1054.

Whitten W. The effect of oxygen on cleavage of mouse eggs in vitro. *Pro Soc Study Reprod* 1969;2: 29.

Whitten WK. The effect of progesterone on the development of mouse eggs in vitro. *J Endocrinol* 1957;16: 80-85.

Whitten WK, Biggers JD. Complete development in vitro of the pre-implantation stages of the mouse in a simple chemically defined medium. *J Reprod Fertil* 1968;17: 399-401.

Whittingham DG. The failure of lactate and phosphoenolpyruvate to support development of the mouse zygote in vitro. *Biol Reprod* 1969;1: 381-386.

Whittingham DG. Culture of mouse ova. *J Reprod Fertil Suppl* 1971;14: 7-21.

Whittingham DG, Biggers JD. Fallopian tube and early cleavage in the mouse. *Nature* 1967;213: 942-943.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Pera RAR. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010;28: 1115.

Wong KM, van Wely M, Mol F, Repping S, Mastenbroek S. Fresh versus frozen embryo transfers in assisted reproduction. *Cochrane Database Syst Rev* 2017;3: Cd011184.

Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336: 1-17.

Wu J, Huang B, Chen H, Yin Q, Liu Y, Xiang Y, Zhang B, Liu B, Wang Q, Xia W *et al.* The landscape of accessible chromatin in mammalian preimplantation embryos. *Nature* 2016;534: 652-657.

Xu J, Fang R, Chen L, Chen D, Xiao J-P, Yang W, Wang H, Song X, Ma T, Bo S. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci U S A* 2016;113: 11907-11912.

Xue Z, Huang K, Cai C, Cai L, Jiang CY, Feng Y, Liu Z, Zeng Q, Cheng L, Sun YE *et al.* Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. *Nature* 2013;500: 593-597.

Yamanaka M, Hashimoto S, Amo A, Ito-Sasaki T, Abe H, Morimoto Y. Developmental assessment of human vitrified-warmed blastocysts based on oxygen consumption. *Hum Reprod* 2011;26: 3366-3371.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Hum Reprod* 1998;13: 998-1002.

Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, Peck AC, Sills ES, Salem RD. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. *Mol Cytogenet* 2012;5: 24.

Yi X, Maeda N. Endogenous production of lipoic acid is essential for mouse development. *Mol Cell Biol* 2005;25: 8387-8392.

Yin H, Jiang H, He R, Wang C, Zhu J, Li Y. The effects of blastocyst morphological score and blastocoele re-expansion speed after warming on pregnancy outcomes. *Clin Exp Reprod Med* 2016;43: 31-37.

Zander-Fox DL, Mitchell M, Thompson JG, Lane M. Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. *Reprod Biomed Online* 2010;21: 219-229.

Zeilmaker GH, Alberda AT, van Gent I, Rijkmans CM, Drogendijk AC. Two pregnancies following transfer of intact frozen-thawed embryos. *Fertil Steril* 1984;42: 293-296.

Zhang D, Tang Z, Huang H, Zhou G, Cui C, Weng Y, Liu W, Kim S, Lee S, Perez-Neut M. Metabolic regulation of gene expression by histone lactylation. *Nature* 2019;574: 575-580.

Zhao XM, Fu XW, Hou YP, Yan CL, Suo L, Wang YP, Zhu HB, Dinnyés A, Zhu SE. Effect of vitrification on mitochondrial distribution and membrane potential in mouse two pronuclear (2-PN) embryos. *Mol Reprod Dev* 2009;76: 1056-1063.

Zheng H, Jin H, Liu L, Liu J, Wang W-H. Application of next-generation sequencing for 24-chromosome aneuploidy screening of human preimplantation embryos. *Mol Cytogenet* 2015;8: 38.

Zhu J, Tsai H-J, Gordon MR, Li R. Cellular stress associated with aneuploidy. *Dev Cell* 2018;44: 420-431.

Zimmerman R, Tao X, Marin D, Werner M, Hong K, Lonczak A, Landis J, Taylor D, Zhan Y, Scott Jr R. Preclinical validation of a targeted next generation sequencing-based comprehensive chromosome screening methodology in human blastocysts. *Mol Hum Reprod* 2018;24: 37-45.

Ziomek C, Pratt H, Johnson M. The origins of cell diversity in the early mouse embryo *Functional integration of cells in animal tissues BSCB Symp*. 1981: 149-165.

Ziomek CA. Cell polarity in the preimplantation mouse embryo. *The Mammalian Preimplantation Embryo*. 1987: 23-41.

Zmuda J, Friedenson B. Changes in intracellular glutathione levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. *J Immunol* 1983;130: 362-364.

Abdelrazik H, Sharma R, Mahfouz R, Agarwal A. L-carnitine decreases DNA damage and improves the in vitro blastocyst development rate in mouse embryos. *Fertil Steril* 2009;91: 589-596.

Adashi EY, Barri PN, Berkowitz R, Braude P, Bryan E, Carr J, Cohen J, Collins J, Devroey P, Frydman R *et al.* Infertility therapy-associated multiple pregnancies (births): an ongoing epidemic. *Reprod Biomed Online* 2003;7: 515-542.

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, Gupta S. The effects of oxidative stress on female reproduction: a review. *Reproductive biology and endocrinology* 2012;10: 49.

Agarwal A, Durairajanayagam D, Du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. *Reprod Biol Endocrinol* 2014;12: 112.

Akarsu S, Gode F, Isik AZ, Dikmen ZG, Tekindal MA. The association between coenzyme Q10 concentrations in follicular fluid with embryo morphokinetics and pregnancy rate in assisted reproductive techniques. *J Assist Reprod Genet* 2017;34: 599-605.

Albrecht JL, Tomich PG. The maternal and neonatal outcome of triplet gestations. *Am J Obstet Gynecol* 1996;174: 1551-1556.

Alfarawati S, Fragouli E, Colls P, Stevens J, Gutiérrez-Mateo C, Schoolcraft WB, Katz-Jaffe MG, Wells D. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. *Fertil Steril* 2011;95: 520-524.

Ali A, Bilodeau J, Sirard M. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology* 2003;59: 939-949.

Almagor M, Harir Y, Fieldust S, Or Y, Shoham Z. Ratio between inner cell mass diameter and blastocyst diameter is correlated with successful pregnancy outcomes of single blastocyst transfers. *Fertil Steril* 2016;106: 1386-1391.

Anderson ME, Meister A. Transport and direct utilization of gamma-glutamylcyst (e) ine for glutathione synthesis. *Proc Natl Acad Sci U S A* 1983;80: 707-711.

Andriani GA, Almeida VP, Faggioli F, Mauro M, Tsai WL, Santambrogio L, Maslov A, Gadina M, Campisi J, Vijg J. Whole Chromosome Instability induces senescence and promotes SASP. *Sci Rep* 2016;6: 35218.

Armstrong S, Vail A, Mastenbroek S, Jordan V, Farquhar C. Time-lapse in the IVF-lab: how should we assess potential benefit? *Hum Reprod* 2015: deu250.

Baart EB, Martini E, van den Berg I, Macklon NS, Galjaard RJ, Fauser BC, Van Opstal D. Preimplantation genetic screening reveals a high incidence of aneuploidy and mosaicism in embryos from young women undergoing IVF. *Hum Reprod* 2006;21: 223-233.

Baird DT, Collins J, Egozcue J, Evers LH, Gianaroli L, Leridon H, Sunde A, Templeton A, Van Steirteghem A, Cohen J *et al.* Fertility and ageing. *Hum Reprod Update* 2005;11: 261-276.

Balaban B, Urman B, Ata B, Isiklar A, Larman MG, Hamilton R, Gardner DK. A randomized controlled study of human Day 3 embryo cryopreservation by slow freezing or vitrification: vitrification is associated with higher survival, metabolism and blastocyst formation. *Hum Reprod* 2008;23: 1976-1982.

Balakier H, Sojecki A, Motamedi G, Librach C. Impact of multinucleated blastomeres on embryo developmental competence, morphokinetics, and aneuploidy. *Fertil Steril* 2016;106: 608-614. e602.

Barbehenn EK, Wales RG, Lowry OH. The explanation for the blockade of glycolysis in early mouse embryos. *Proc Natl Acad Sci U S A* 1974;71: 1056-1060.

Barcroft LC, Offenberg H, Thomsen P, Watson AJ. Aquaporin proteins in murine trophectoderm mediate transepithelial water movements during cavitation. *Dev Biol* 2003;256: 342-354.

Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet* 1986;1: 1077-1081.

Barker DJ, Osmond C, Golding J, Kuh D, Wadsworth ME. Growth in utero, blood pressure in childhood and adult life, and mortality from cardiovascular disease. *BMJ* 1989;298: 564-567.

Barker DJP. The Welcome Foundation Lecture, 1994. The fetal origins of adult disease. *Proc Biol Sci* 1995;262: 37-43.

Barnes FL. The effects of the early uterine environment on the subsequent development of embryo and fetus. *Theriogenology* 2000;53: 649-658.

Barron DJ, Valdimarsson G, Paul DL, Kidder GM. Connexin32, a gap junction protein, is a persistent oogenetic product through preimplantation development of the mouse. *Genesis* 1989;10: 318-323.

Bartmann AK, Romão GS, Ramos Eda S, Ferriani RA. Why do older women have poor implantation rates? A possible role of the mitochondria. *J Assist Reprod Genet* 2004;21: 79-83.

Basile N, del Carmen Nogales M, Bronet F, Florensa M, Riqueiros M, Rodrigo L, García-Velasco J, Meseguer M. Increasing the probability of selecting chromosomally normal embryos by time-lapse morphokinetics analysis. *Fertil Steril* 2014;101: 699-704. e691.

Basile N, Vime P, Florensa M, Aparicio Ruiz B, Garcia Velasco JA, Remohi J, Meseguer M. The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. *Hum Reprod* 2015;30: 276-283.

Batt PA, Gardner DK, Cameron AW. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. *Reprod Fertil Dev* 1991;3: 601-607.

Bavister BD, Arlotto T. Influence of single amino acids on the development of hamster one-cell embryos in vitro. *Mol Reprod Dev* 1990;25: 45-51.

Becker M, Becker A, Miyara F, Han Z, Kihara M, Brown DT, Hager GL, Latham K, Adashi EY, Misteli T. Differential in vivo binding dynamics of somatic and oocyte-specific linker histones in oocytes and during ES cell nuclear transfer. *Mol Biol Cell* 2005;16: 3887-3895.

Bentov Y, Casper RF. The aging oocyte--can mitochondrial function be improved? *Fertil Steril* 2013;99: 18-22.

Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc Natl Acad Sci U S A* 2010;107: 3394-3399.

Berthelot F, Terqui M. Effects of oxygen, CO2/pH and medium on the in vitro development of individually cultured porcine one- and two-cell embryos. *Reprod Nutr Dev* 1996;36: 241-251.

Bhusane K, Bhutada S, Chaudhari U, Savardekar L, Katkam R, Sachdeva G. Secrets of Endometrial Receptivity: Some Are Hidden in Uterine Secretome. *Am J Reprod Immunol* 2016;75: 226-236.

Biggers J, Bell JE, Benos DJ. Mammalian blastocyst: transport functions in a developing epithelium. *Am J Physiol* 1988;255: C419-C432.

Biggers J, Bell JE, Benos DJ. Mammalian blastocyst: transport functions in a developing epithelium. *American Journal of Physiology-Cell Physiology* 1988;255: C419-C432.

Biggers JD, Racowsky C. The development of fertilized human ova to the blastocyst stage in KSOM(AA) medium: is a two-step protocol necessary? *Reprod Biomed Online* 2002;5: 133-140.

Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Natl Acad Sci U S A* 1967;58: 560-567.

Bilska A, Wlodek L. Lipoic acid-the drug of the future. *Pharmacol Rep* 2005;57: 570-577.

Binder NK, Hannan NJ, Gardner DK. In vitro embryo outgrowth is a bioassay of in vivo embryo implantation and development. *Asian Pacific Journal of Reproduction* 2015;4: 240-241.

Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Database Syst Rev* 2012: CD008950.

Boomsma CM, Kavelaars A, Eijkemans MJ, Amarouchi K, Teklenburg G, Gutknecht D, Fauser BJ, Heijnen CJ, Macklon NS. Cytokine profiling in endometrial secretions: a non-invasive window on endometrial receptivity. *Reprod Biomed Online* 2009;18: 85-94.

Bowman P, McLaren A. Cleavage rate of mouse embryos in vivo and in vitro. *J Embryol Exp Morphol* 1970;24: 203-207.

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four-and eight-cell stages of preimplantation development. *Nature* 1988;332: 459-461.

Brinster RL. Studies on the development of mouse embryo in vitro. II. The effect of energy source. *J Exp Zool* 1965;158: 59-68.

Brinster RL. Studies on the Development of Mouse Embryos in Vitro. I. The Effect of Osmolarity and Hydrogen Ion Concentration. *J Exp Zool* 1965;158: 49-57.

Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, Humpherson PG, Lieberman BA, Leese HJ. Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. *Hum Reprod* 2004;19: 2319-2324.

Bunton C. Oxidation of α-Diketones andα-Keto-Acids by Hydrogen Peroxide. *Nature* 1949;163: 444-444.

Burton GJ, Hempstock J, Jauniaux E. Oxygen, early embryonic metabolism and free radical-mediated embryopathies. *Reprod Biomed Online* 2003;6: 84-96.

Calhaz-Jorge C, De Geyter C, Kupka MS, de Mouzon J, Erb K, Mocanu E, Motrenko T, Scaravelli G, Wyns C, Goossens V. Assisted reproductive technology in Europe, 2013: results generated from European registers by ESHRE. *Hum Reprod* 2017;32: 1957-1973.

Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Hickman CFL. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. *Reprod Biomed Online* 2013;26: 477-485.

Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Thornton S. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. *Reprod Biomed Online* 2013;27: 140-146.

Campbell A, Fishel S, Laegdsmand M. Aneuploidy is a key causal factor of delays in blastulation: author response to 'A cautionary note against aneuploidy risk assessment using time-lapse imaging'. *Reprod Biomed Online* 2014;28: 279-283.

Canto´ C, Menzies KJ, Auwerx J. NAD+ metabolism and the control of energy homeostasis: a balancing act between mitochondria and the nucleus. *Cell Metab* 2015;22: 31-53.

Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, Nagy ZP, Ubaldi FM. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. *Hum Reprod* 2014;29: 1173-1181.

Casado-Vela J, Rodriguez-Suarez E, Iloro I, Ametzazurra A, Alkorta N, García-Velasco JA, Matorras R, Prieto B, González S, Nagore D *et al.* Comprehensive proteomic analysis of human endometrial fluid aspirate. *J Proteome Res* 2009;8: 4622-4632.

Casslén BG. Free amino acids in human uterine fluid. Possible role of high taurine concentration. *J Reprod Med* 1987;32: 181-184.

Castillo-Martín M, Bonet S, Morató R, Yeste M. Supplementing culture and vitrification-warming media with l-ascorbic acid enhances survival rates and redox status of IVP porcine blastocysts via induction of GPX1 and SOD1 expression. *Cryobiology* 2014;68: 451-458.

Chason RJ, Csokmay J, Segars JH, DeCherney AH, Armant DR. Environmental and epigenetic effects upon preimplantation embryo metabolism and development. *Trends Endocrinol Metab* 2011;22: 412-420.

Chavez SL, Loewke KE, Han J, Moussavi F, Colls P, Munne S, Behr B, Pera RAR. Dynamic blastomere behaviour reflects human embryo ploidy by the four-cell stage. *Nat Commun* 2012;3: 1-12.

Chawla M, Fakih M, Shunnar A, Bayram A, Hellani A, Perumal V, Divakaran J, Budak E. Morphokinetic analysis of cleavage stage embryos and its relationship to aneuploidy in a retrospective time-lapse imaging study. *J Assist Reprod Genet* 2015;32: 69-75.

Choe C, Shin Y-W, Kim E-J, Cho S-R, Kim H-J, Choi S-H, Han M-H, Han J, Son D-S, Kang D. Synergistic effects of glutathione and β-mercaptoethanol treatment during in vitro maturation of porcine oocytes on early embryonic development in a culture system supplemented with L-cysteine. *J Reprod Dev* 2010;56: 575-582

Christianson MS, Zhao Y, Shoham G, Granot I, Safran A, Khafagy A, Leong M, Shoham Z. Embryo catheter loading and embryo culture techniques: results of a worldwide Web-based survey. *J Assist Reprod Genet* 2014;31: 1029-1036.

Ciemerych MA, Sicinski P. Cell cycle in mouse development. *Oncogene* 2005;24: 2877-2898.

Clegg KB, Pikó L. Poly (A) length, cytoplasmic adenylation and synthesis of poly (A)+ RNA in early mouse embryos. *Dev Biol* 1983;95: 331-341.

Coello A, Meseguer M, Galán A, Alegre L, Remohí J, Cobo A. Analysis of the morphological dynamics of blastocysts after vitrification/warming: defining new predictive variables of implantation. *Fertil Steril* 2017;108: 659-666.e654.

Cole R. Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst. *J Embryol Exp Morphol* 1967;17: 481-490.

Conaghan J, Hardy K, Handyside AH, Winston RM, Leese HJ. Selection criteria for human embryo transfer: a comparison of pyruvate uptake and morphology. *J Assist Reprod Genet* 1993;10: 21-30.

Croxatto HB, Díaz S, Fuentealba B, Croxatto HD, Carrillo D, Fabres C. Studies on the duration of egg transport in the human oviduct. I. The time interval between ovulation and egg recovery from the uterus in normal women. *Fertil Steril* 1972;23: 447-458.

Cummins JM, Breen TM, Harrison KL, Shaw JM, Wilson LM, Hennessey JF. A formula for scoring human embryo growth rates in in vitro fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *J In Vitro Fert Embryo Transf* 1986;3: 284-295.

Curchoe CL, Bormann CL. Artificial intelligence and machine learning for human reproduction and embryology presented at ASRM and ESHRE 2018. *J Assist Reprod Genet* 2019;36: 591-600.

D'Autréaux B, Toledano MB. ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 2007;8: 813-824.

De Matos D, Furnus C. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: effect of β-mercaptoethanol, cysteine and cystine. *Theriogenology* 2000;53: 761-771.

De Matos DG, Furnus CC, Moses DF, Baldassarre H. Effect of cysteamine on glutathione level and developmental capacity of bovine oocyte matured in vitro. *Mol Reprod Dev* 1995;42: 432-436.

De Sutter P, Gerris J, Dhont M. A health-economic decision-analytic model comparing double with single embryo transfer in IVF/ICSI. *Hum Reprod* 2002;17: 2891-2896.

Debrock S, Peeraer K, Fernandez Gallardo E, De Neubourg D, Spiessens C, D'Hooghe TM. Vitrification of cleavage stage day 3 embryos results in higher live birth rates than conventional slow freezing: a RCT. *Hum Reprod* 2015;30: 1820-1830.

Desai N, Goldberg JM, Austin C, Falcone T. Are cleavage anomalies, multinucleation, or specific cell cycle kinetics observed with time-lapse imaging predictive of embryo developmental capacity or ploidy? *Fertil Steril* 2018;109: 665-674.

Desai N, Ploskonka S, Goodman LR, Austin C, Goldberg J, Falcone T. Analysis of embryo morphokinetics, multinucleation and cleavage anomalies using continuous time-lapse monitoring in blastocyst transfer cycles. *Reprod Biol Endocrinol* 2014;12: 54.

Desai NN, Goldberg JM, Austin C, Falcone T. The new Rapid-i carrier is an effective system for human embryo vitrification at both the blastocyst and cleavage stage. *Reprod Biol Endocrinol* 2013;11: 41.

Desler C, Munch-Petersen B, Stevnsner T, Matsui S, Kulawiec M, Singh KK, Rasmussen LJ. Mitochondria as determinant of nucleotide pools and chromosomal stability. *Mutat Res* 2007;625: 112-124.

Diebold L, Chandel NS. Mitochondrial ROS regulation of proliferating cells. *Free Radic Biol Med* 2016;100: 86-93.

Donohoe DR, Bultman SJ. Metaboloepigenetics: interrelationships between energy metabolism and epigenetic control of gene expression. *J Cell Physiol* 2012;227: 3169-3177.

Dos Santos E, Varchetta R, de Lima C, Ispada J, Martinho H, Fontes P, Nogueira M, Gasparrini B, Milazzotto M. The effects of crocetin supplementation on the blastocyst outcome, transcriptomic and metabolic profile of in vitro produced bovine embryos. *Theriogenology* 2019;123: 30-36.

Dosouto C, Haahr T, Humaidan P. Gonadotropin-releasing hormone agonist (GnRHa) trigger - State of the art. *Reprod Biol* 2017;17: 1-8.

Du QY, Wang EY, Huang Y, Guo XY, Xiong YJ, Yu YP, Yao GD, Shi SL, Sun YP. Blastocoele expansion degree predicts live birth after single blastocyst transfer for fresh and vitrified/warmed single blastocyst transfer cycles. *Fertil Steril* 2016;105: 910-919.e911.

Ducibella T, Anderson E. Cell shape and membrane changes in the eight-cell mouse embryo: prerequisites for morphogenesis of the blastocyst. *Dev Biol* 1975;47: 45-58.

Dumoulin JC, Meijers CJ, Bras M, Coonen E, Geraedts JP, Evers JL. Effect of oxygen concentration on human in-vitro fertilization and embryo culture. *Hum Reprod* 1999;14: 465-469.

Duncan FE, Hornick JE, Lampson MA, Schultz RM, Shea LD, Woodruff TK. Chromosome cohesion decreases in human eggs with advanced maternal age. *Aging Cell* 2012;11: 1121-1124.

Eagle H. Amino acid metabolism in mammalian cell cultures. *Science* 1959;130: 432-437.

Ebner T, Vanderzwalmen P, Shebl O, Urdl W, Moser M, Zech NH, Tews G. Morphology of vitrified/warmed day-5 embryos predicts rates of implantation, pregnancy and live birth. *Reprod Biomed Online* 2009;19: 72-78.

Edwards LJ, Williams DA, Gardner DK. Intracellular pH of the mouse preimplantation embryo: amino acids act as buffers of intracellular pH. *Hum Reprod* 1998;13: 3441-3448.

Edwards RG, Bavister BD, Steptoe PC. Early stages of fertilization in vitro of human oocytes matured in vitro. *Nature* 1969;221: 632-635.

Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, Steptoe PC, Webster JM. Factors influencing the success of in vitro fertilization for alleviating human infertility. *J In Vitro Fert Embryo Transf* 1984;1: 3-23.

Edwards RG, Steptoe PC, Purdy JM. Fertilization and cleavage in vitro of preovulator human oocytes. *Nature* 1970;227: 1307-1309.

Eichenlaub-Ritter U, Vogt E, Yin H, Gosden R. Spindles, mitochondria and redox potential in ageing oocytes. *Reprod Biomed Online* 2004;8: 45-58.

El Mouatassim S, Guérin P, Ménézo Y. Mammalian oviduct and protection against free oxygen radicals: expression of genes encoding antioxidant enzymes in human and mouse. *Eur J Obstet Gynecol Reprod Biol* 2000;89: 1-6.

Enderasn A, Schlafke S. The fine structure of the blastocyst: some comparative studies *Ciba Foundation Symposium-Preimplantation Stages of Pregnancy*. 1965. Wiley Online Library, pp. 29-59.

Epstein CJ, Smith SA. Amino acid uptake and protein synthesis in preimplantation mouse embryos. *Dev Biol* 1973;33: 171-184.

Ferreux L, Bourdon M, Sallem A, Santulli P, Barraud-Lange V, Le Foll N, Maignien C, Chapron C, de Ziegler D, Wolf JP *et al.* Live birth rate following frozen-thawed blastocyst transfer is higher with blastocysts expanded on Day 5 than on Day 6. *Hum Reprod* 2018;33: 390-398.

Ferrick L, Lee YSL, Gardner DK. Reducing time to pregnancy and facilitating the birth of healthy children through functional analysis of embryo physiology. *Biol Reprod* 2019;101: 1124-1139.

Ferrick L, Lee YSL, Gardner DK. Metabolic activity of human preimplantation blastocysts correlates to their morphokinetics, morphological grade, KIDScore and artificial intelligence ranking. *Hum Reprod* 2020;In press.

Feuer S, Rinaudo P. Preimplantation stress and development. *Birth Defects Res C Embryo Today* 2012;96: 299-314.

Feuer S, Rinaudo P. From Embryos to Adults: A DOHaD Perspective on In Vitro Fertilization and Other Assisted Reproductive Technologies. *Healthcare (Basel)* 2016;4.

Feugang JM, de Roover R, Moens A, Léonard S, Dessy F, Donnay I. Addition of beta-mercaptoethanol or Trolox at the morula/blastocyst stage improves the quality of bovine blastocysts and prevents induction of apoptosis and degeneration by prooxidant agents. *Theriogenology* 2004;61: 71-90.

Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil* 1993;99: 673-679.

Fissore RA, Kurokawa M, Knott J, Zhang M, Smyth J. Mechanisms underlying oocyte activation and postovulatory ageing. *Reproduction* 2002;124: 745-754.

Fitzgerald O, Harris K, Paul RC, Chambers GM. Assisted reproductive technology in Australia and New Zealand 2015. *National Perinatal Epidemiology and Statistics Unit, the University of New South Wales Sydney* 2017.

Fleming T, Velazquez M, Eckert J. Embryos, DOHaD and david barker. *J Dev Orig Health Dis* 2015;6: 377-383.

Forman EJ, Hong KH, Ferry KM, Tao X, Taylor D, Levy B, Treff NR, Scott Jr RT. In vitro fertilization with single euploid blastocyst transfer: a randomized controlled trial. *Fertil Steril* 2013;100: 100-107. e101.

Franasiak JM, Forman EJ, Hong KH, Werner MD, Upham KM, Treff NR, Scott Jr RT. The nature of aneuploidy with increasing age of the female partner: a review of 15,169 consecutive trophectoderm biopsies evaluated with comprehensive chromosomal screening. *Fertil Steril* 2014;101: 656-663. e651.

Gangloff Y-G, Mueller M, Dann SG, Svoboda P, Sticker M, Spetz J-F, Um SH, Brown EJ, Cereghini S, Thomas G. Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. *Mol Cell Biol* 2004;24: 9508-9516.

Gardner D, Kelley R. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *Journal of developmental origins of health and disease* 2017;8: 418-435.

Gardner DK. Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int* 1994;18: 1163-1179.

Gardner DK. Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. *Theriogenology* 1998;49: 83-102.

Gardner DK. Noninvasive metabolic assessment of single cells. *Methods Mol Med* 2007;132: 1-9.

Gardner DK. Lactate production by the mammalian blastocyst: manipulating the microenvironment for uterine implantation and invasion? *Bioessays* 2015;37: 364-371.

Gardner DK. The impact of physiological oxygen during culture, and vitrification for cryopreservation, on the outcome of extended culture in human IVF. *Reprod Biomed Online* 2016;32: 137-141.

Gardner DK, Balaban B. Assessment of human embryo development using morphological criteria in an era of time-lapse, algorithms and 'OMICS': is looking good still important? *Mol Hum Reprod* 2016;22: 704-718.

Gardner DK, Clarke RN, Lechene CP, Biggers JD. Development of a noninvasive ultramicrofluorometric method for measuring net uptake of glutamine by single preimplantation mouse embryos. *Gamete Res* 1989;24: 427-438.

Gardner DK, Harvey AJ. Blastocyst metabolism. *Reprod Fertil Dev* 2015;27: 638-654.

Gardner DK, Kelley RL. Impact of the IVF laboratory environment on human preimplantation embryo phenotype. *J Dev Orig Health Dis* 2017;8: 418-435.

Gardner DK, Kuramoto T, Tanaka M, Mitzumoto S, Montag M, Yoshida A. Prospective randomized multicentre comparison on sibling oocytes comparing G-Series media system with antioxidants versus standard G-Series media system. *Reprod Biomed Online* 2020;40: 637-644.

Gardner DK, Lane M. Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 1993;48: 377-385.

Gardner DK, Lane M. Alleviation of the '2-cell block' and development to the blastocyst of CF1 mouse embryos: role of amino acids, EDTA and physical parameters. *Hum Reprod* 1996;11: 2703-2712.

Gardner DK, Lane M. Culture and selection of viable blastocysts: a feasible proposition for human IVF? *Hum Reprod Update* 1997;3: 367-382.

Gardner DK, Lane M. Ex vivo early embryo development and effects on gene expression and imprinting. *Reprod Fertil Dev* 2005;17: 361-370.

Gardner DK, Lane M. Mammalian preimplantation embryo culture. *Methods Mol Med* 2014: 167-182.

Gardner DK, Lane M. Mammalian preimplantation embryo culture. *Mouse Molecular Embryology: Methods and Protocols* 2014: 167-182.

Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril* 1996;65: 349-353.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril* 2000;73: 1155-1158.

Gardner DK, Lane M, Stevens J, Schoolcraft WB. Noninvasive assessment of human embryo nutrient consumption as a measure of developmental potential. *Fertil Steril* 2001;76: 1175-1180.

Gardner DK, Larman MG, Thouas GA. Sex-related physiology of the preimplantation embryo. *Mol Hum Reprod* 2010;16: 539-547.

Gardner DK, Leese HJ. Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. *Hum Reprod* 1986;1: 25-27.

Gardner DK, Leese HJ. Assessment of embryo viability prior to transfer by the noninvasive measurement of glucose uptake. *J Exp Zool* 1987;242: 103-105.

Gardner DK, Leese HJ. The role of glucose and pyruvate transport in regulating nutrient utilization by preimplantation mouse embryos. *Development* 1988;104: 423-429.

Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil* 1990;88: 361-368.

Gardner DK, Pawelczynski M, Trounson AO. Nutrient uptake and utilization can be used to select viable day 7 bovine blastocysts after cryopreservation. *Mol Reprod Dev* 1996;44: 472-475.

Gardner DK, Pool TB, Lane M. Embryo nutrition and energy metabolism and its relationship to embryo growth, differentiation, and viability. In Med SR (ed) *Semin Reprod Med*. 2000. Copyright© 2000 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New …, pp. 205-218.

Gardner DK, Reed L, Linck D, Sheehan C, Lane M. Quality control in human in vitro fertilization *Semin Reprod Med*. 2005. Copyright© 2005 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New …, pp. 319-324.

Gardner DK, Sakkas D. Mouse embryo cleavage, metabolism and viability: role of medium composition. *Hum Reprod* 1993;8: 288-295.

Gardner DK, Sakkas D. Assessment of embryo viability: the ability to select a single embryo for transfer--a review. *Placenta* 2003;24 Suppl B: S5-12.

Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J. A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. *Hum Reprod* 1998;13: 3434-3440.

Gardner DK, Surrey E, Minjarez D, Leitz A, Stevens J, Schoolcraft WB. Single blastocyst transfer: a prospective randomized trial. *Fertil Steril* 2004;81: 551-555.

Gardner DK, Truong TT. Culture of the Mouse Preimplantation Embryo. *Methods Mol Biol* 2019;2006: 13-32.

Gardner DK, Vella P, Lane M, Wagley L, Schlenker T, Schoolcraft WB. Culture and transfer of human blastocysts increases implantation rates and reduces the need for multiple embryo transfers. *Fertil Steril* 1998;69: 84-88.

Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril* 2013;99: 1062-1072.

Gardner DK, Wale PL, Collins R, Lane M. Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome. *Hum Reprod* 2011;26: 1981-1986.

Gazzo E, Peña F, Valdéz F, Chung A, Bonomini C, Ascenzo M, Velit M, Escudero E. The KidscoreTM D5 algorithm as an additional tool to morphological assessment and PGT-A in embryo selection: a time-lapse study. *JBRA Assist Reprod* 2020;24: 55-60.

Gerris J, De Neubourg D, Mangelschots K, Van Royen E, Van de Meerssche M, Valkenburg M. Prevention of twin pregnancy after in-vitro fertilization or intracytoplasmic sperm injection based on strict embryo criteria: a prospective randomized clinical trial. *Hum Reprod* 1999;14: 2581-2587.

Ghosh J, Coutifaris C, Sapienza C, Mainigi M. Global DNA methylation levels are altered by modifiable clinical manipulations in assisted reproductive technologies. *Clin Epigenetics* 2017;9: 14.

Golbus MS, Calarco PG, Epstein CJ. The effects of inhibitors of RNA synthesis (α-amanitin and actinomycin D) on preimplantation mouse embryogenesis. *J Exp Zool* 1973;186: 207-216.

González IM, Martin PM, Burdsal C, Sloan JL, Mager S, Harris T, Sutherland AE. Leucine and arginine regulate trophoblast motility through mTOR-dependent and independent pathways in the preimplantation mouse embryo. *Dev Biol* 2012;361: 286-300.

González R, Ferrín G, Hidalgo AB, Ranchal I, López-Cillero P, Santos-Gónzalez M, López-Lluch G, Briceño J, Gómez MA, Poyato A *et al.* N-acetylcysteine, coenzyme Q10 and superoxide dismutase mimetic prevent mitochondrial cell dysfunction and cell death induced by d-galactosamine in primary culture of human hepatocytes. *Chem Biol Interact* 2009;181: 95-106.

Gook DA, Choo B, Bourne H, Lewis K, Edgar DH. Closed vitrification of human oocytes and blastocysts: outcomes from a series of clinical cases. *J Assist Reprod Genet* 2016;33: 1247-1252.

Gott A, Hardy K, Winston R, Leese H. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod* 1990;5: 104-108.

Gott AL, Hardy K, Winston RM, Leese HJ. Non-invasive measurement of pyruvate and glucose uptake and lactate production by single human preimplantation embryos. *Hum Reprod* 1990;5: 104-108.

Grifo JA, Hodes-Wertz B, Lee H-L, Amperloquio E, Clarke-Williams M, Adler A. Single thawed euploid embryo transfer improves IVF pregnancy, miscarriage, and multiple gestation outcomes and has similar implantation rates as egg donation. *J Assist Reprod Genet* 2013;30: 259-264.

Guerif F, McKeegan P, Leese HJ, Sturmey RG. A simple approach for COnsumption and RElease (CORE) analysis of metabolic activity in single mammalian embryos. *PLoS One* 2013;8: e67834.

Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Hum Reprod Update* 2001;7: 175-189.

Guertin DA, Stevens DM, Thoreen CC, Burds AA, Kalaany NY, Moffat J, Brown M, Fitzgerald KJ, Sabatini DM. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCα, but not S6K1. *Dev Cell* 2006;11: 859-871.

Gülcin İ. Antioxidant and antiradical activities of L-carnitine. *Life Sci* 2006;78: 803-811.

Gupta S, Malhotra N, Sharma D, Chandra A, Ashok A. Oxidative stress and its role in female infertility and assisted reproduction: clinical implications. *Intl J Fertil Steril* 2009;2: 147-167.

Gutiérrez-Mateo C, Colls P, Sánchez-García J, Escudero T, Prates R, Ketterson K, Wells D, Munné S. Validation of microarray comparative genomic hybridization for comprehensive chromosome analysis of embryos. *Fertil Steril* 2011;95: 953-958.

Guzman L, Nuñez D, López R, Inoue N, Portella J, Vizcarra F, Noriega-Portella L, Noriega-Hoces L, Munné S. The number of biopsied trophectoderm cells may affect pregnancy outcomes. *J Assist Reprod Genet* 2019;36: 145-151.

Haas J, Meriano J, Laskin C, Bentov Y, Barzilay E, Casper RF, Cadesky K. Clinical pregnancy rate following frozen embryo transfer is higher with blastocysts vitrified on day 5 than on day 6. *J Assist Reprod Genet* 2016;33: 1553-1557.

Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. *Annu Rev Pathol* 2010;5: 253-295.

Hamanaka RB, Chandel NS. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. *Trends Biochem Sci* 2010;35: 505-513.

Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, Ko MS. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum Mol Genet* 2004;13: 2263-2278.

Hannan N, Paiva P, Meehan K, Rombauts L, Gardner D, Salamonsen L. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology* 2011;152: 4948-4956.

Hannan NJ, Paiva P, Meehan KL, Rombauts LJ, Gardner DK, Salamonsen LA. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. *Endocrinology* 2011;152: 4948-4956.

Hansen P. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. *Theriogenology* 2007;68: S242-S249.

Harbottle S, Hughes C, Cutting R, Roberts S, Brison D, Embryologists AOC, Society TBF. Elective single embryo transfer: an update to UK best practice guidelines. *Hum Fertil* 2015;18: 165-183.

Hardy K, Hooper M, Handyside A, Rutherford A, Winston R, Leese H. Non-invasive measurement of glucose and pyruvate uptake by individual human oocytes and preimplantation embryos. *Hum Reprod* 1989;4: 188-191.

Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology* 2005;64: 992-1006.

Harvey AJ. The role of oxygen in ruminant preimplantation embryo development and metabolism. *Anim Reprod Sci* 2007;98: 113-128.

Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG. Oxygen-regulated gene expression in bovine blastocysts. *Biol Reprod* 2004;71: 1108-1119.

Harvey AJ, Kind KL, Thompson JG. REDOX regulation of early embryo development. *Reproduction* 2002;123: 479-486.

Harvey AJ, Rathjen J, Gardner DK. Metaboloepigenetic Regulation of Pluripotent Stem Cells. *Stem Cells Int* 2016;2016: 1816525.

Hashimoto S, Morimoto N, Yamanaka M, Matsumoto H, Yamochi T, Goto H, Inoue M, Nakaoka Y, Shibahara H, Morimoto Y. Quantitative and qualitative changes of mitochondria in human preimplantation embryos. *J Assist Reprod Genet* 2017;34: 573-580.

Hassold T, Hunt P. To err (meiotically) is human: the genesis of human aneuploidy. *Nat Rev Genet* 2001;2: 280-291.

Hérubel F, El Mouatassim S, Guérin P, Frydman R, Ménézo Y. Genetic expression of monocarboxylate transporters during human and murine oocyte maturation and early embryonic development. *Zygote* 2002;10: 175-181.

Hewitson LC, Leese HJ. Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. *J Exp Zool* 1993;267: 337-343.

Heydarnejad A, Ostadhosseini S, Varnosfaderani SR, Jafarpour F, Moghimi A, Nasr-Esfahani MH. Supplementation of maturation medium with CoQ10 enhances developmental competence of ovine oocytes through improvement of mitochondrial function. *Mol Reprod Dev* 2019;86: 812-824.

Ho JR, Arrach N, Rhodes-Long K, Ahmady A, Ingles S, Chung K, Bendikson KA, Paulson RJ, McGinnis LK. Pushing the limits of detection: investigation of cell-free DNA for aneuploidy screening in embryos. *Fertil Steril* 2018;110: 467-475. e462.

Ho Y, Wigglesworth K, Eppig JJ, Schultz RM. Preimplantation development of mouse embryos in KSOM: augmentation by amino acids and analysis of gene expression. *Mol Reprod Dev* 1995;41: 232-238.

Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, Rutherford AJ, Leese HJ. Non-invasive amino acid turnover predicts human embryo developmental capacity. *Hum Reprod* 2002;17: 999-1005.

Houghton FD, Thompson JG, Kennedy CJ, Leese HJ. Oxygen consumption and energy metabolism of the early mouse embryo. *Mol Reprod Dev* 1996;44: 476-485.

Houtkooper RH, Cantó C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 2010;31: 194-223.

Huang L, Bogale B, Tang Y, Lu S, Xie XS, Racowsky C. Noninvasive preimplantation genetic testing for aneuploidy in spent medium may be more reliable than trophectoderm biopsy. *Proc Natl Acad Sci U S A* 2019;116: 14105-14112.

Huang TT, Huang DH, Ahn HJ, Arnett C, Huang CT. Early blastocyst expansion in euploid and aneuploid human embryos: evidence for a non-invasive and quantitative marker for embryo selection. *Reprod Biomed Online* 2019;39: 27-39.

Igarashi H, Takahashi T, Nagase S. Oocyte aging underlies female reproductive aging: biological mechanisms and therapeutic strategies. *Reprod Med Biol* 2015;14: 159-169.

Imai S-i, Guarente L. NAD+ and sirtuins in aging and disease. *Trends in cell biology* 2014;24: 464-471.

Janny L, Menezo YJ. Maternal age effect on early human embryonic development and blastocyst formation. *Mol Reprod Dev* 1996;45: 31-37.

Jiang W, Li Y, Zhao Y, Gao Q, Jin Q, Yan C, Xu Y. l-carnitine supplementation during in vitro culture regulates oxidative stress in embryos from bovine aged oocytes. *Theriogenology* 2020;143: 64-73.

Kaing A, Kroener LL, Tassin R, Li M, Liu L, Buyalos R, Hubert G, Shamonki M. Earlier day of blastocyst development is predictive of embryonic euploidy across all ages: essential data for physician decision-making and counseling patients. *J Assist Reprod Genet* 2018;35: 119-125.

Kang H-J, Melnick AP, Stewart JD, Xu K, Rosenwaks Z. Preimplantation genetic screening: who benefits? *Fertil Steril* 2016;106: 597-602.

Katz-Jaffe MG, Linck DW, Schoolcraft WB, Gardner DK. A proteomic analysis of mammalian preimplantation embryonic development. *Reproduction* 2005;130: 899-905.

Kelley RL, Gardner DK. Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. *Reproductive biomedicine online* 2016;33: 537-549.

Kelley RL, Gardner DK. Combined effects of individual culture and atmospheric oxygen on preimplantation mouse embryos in vitro. *Reprod Biomed Online* 2016;33: 537-549.

Kelley RL, Gardner DK. In vitro culture of individual mouse preimplantation embryos: the role of embryo density, microwells, oxygen, timing and conditioned media. *Reprod Biomed Online* 2017;34: 441-454.

Kelley RL, Gardner DK. Individual culture and atmospheric oxygen during culture affect mouse preimplantation embryo metabolism and post-implantation development. *Reprod Biomed Online* 2019;39: 3-18.

Khurana NK, Wales RG. Effects of oxygen concentration on the metabolism of [U-14C]glucose by mouse morulae and early blastocysts in vitro. *Reprod Fertil Dev* 1989;1: 99-106.

Kim MK, Park EA, Kim HJ, Choi WY, Cho JH, Lee WS, Cha KY, Kim YS, Lee DR, Yoon TK. Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS? *Reprod Biomed Online* 2013;26: 22-29.

Kim MK, Park JK, Paek SK, Kim JW, Kwak IP, Lee HJ, Lyu SW, Lee WS. Effects and pregnancy outcomes of L-carnitine supplementation in culture media for human embryo development from in vitro fertilization. *J Obstet Gynaecol Res* 2018;44: 2059-2066.

Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril* 2013;99: 738-744 e734.

Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. *Theriogenology* 2004;62: 1186-1197.

Kramer YG, Kofinas JD, Melzer K, Noyes N, McCaffrey C, Buldo-Licciardi J, McCulloh DH, Grifo JA. Assessing morphokinetic parameters via time lapse microscopy (TLM) to predict euploidy: are aneuploidy risk classification models universal? *J Assist Reprod Genet* 2014;31: 1231-1242.

Krisher RL, Prather RS. A role for the Warburg effect in preimplantation embryo development: metabolic modification to support rapid cell proliferation. *Mol Reprod Dev* 2012;79: 311-320.

Kupka M, D'Hooghe T, Ferraretti A, de Mouzon J, Erb K, Castilla J, Calhaz-Jorge C, De Geyter C. Assisted reproductive technology in Europe, 2011: results generated from European registers by ESHRE. *Hum Reprod* 2016;31: 233-248.

Lamb V, Leese H. Uptake of a mixture of amino acids by mouse blastocysts. *J Reprod Fertil* 1994;102: 169-175.

Lane M, Gardner DK. Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *J Reprod Fertil* 1994;102: 305-312.

Lane M, Gardner DK. Removal of embryo-toxic ammonium from the culture medium by in situ enzymatic conversion to glutamate. *J Exp Zool* 1995;271: 356-363.

Lane M, Gardner DK. Selection of viable mouse blastocysts prior to transfer using a metabolic criterion. *Hum Reprod* 1996;11: 1975-1978.

Lane M, Gardner DK. Differential regulation of mouse embryo development and viability by amino acids. *J Reprod Fertil* 1997;109: 153-164.

Lane M, Gardner DK. Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts. *Hum Reprod* 1998;13: 991-997.

Lane M, Gardner DK. Lactate regulates pyruvate uptake and metabolism in the preimplantation mouse embryo. *Biol Reprod* 2000;62: 16-22.

Lane M, Gardner DK. Ammonium induces aberrant blastocyst differentiation, metabolism, pH regulation, gene expression and subsequently alters fetal development in the mouse. *Biol Reprod* 2003;69: 1109-1117.

Lane M, Gardner DK. Mitochondrial malate-aspartate shuttle regulates mouse embryo nutrient consumption. *J Biol Chem* 2005;280: 18361-18367.

Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reproduction, Fertility and Development* 2005;17: 371-378.

Lane M, Gardner DK. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. *Reprod Fertil Dev* 2005;17: 371-378.

Lane M, Maybach JM, Gardner DK. Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Human reproduction* 2002;17: 2686-2693.

Lane M, Maybach JM, Gardner DK. Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Hum Reprod* 2002;17: 2686-2693.

Lane M, Schoolcraft WB, Gardner DK, Phil D. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. *Fertil Steril* 1999;72: 1073-1078.

Larman MG, Katz-Jaffe MG, McCallie B, Filipovits JA, Gardner DK. Analysis of global gene expression following mouse blastocyst cryopreservation. *Hum Reprod* 2011;26: 2672-2680.

Latham KE. Mechanisms and control of embryonic genome activation in mammalian embryos. *Int Rev Cytol* 1999;193: 71-124.

Latham T, Mackay L, Sproul D, Karim M, Culley J, Harrison DJ, Hayward L, Langridge-Smith P, Gilbert N, Ramsahoye BH. Lactate, a product of glycolytic metabolism, inhibits histone deacetylase activity and promotes changes in gene expression. *Nucleic Acids Res* 2012;40: 4794-4803.

Lawitts JA, Biggers JD. Culture of preimplantation embryos. *Methods Enzymol* 1993;225: 153-164.

Leary C, Sturmey R. Metabolic profile of in-vitro derived human embryos is not affected by the mode of fertilization. *Mol Hum Reprod* 2020.

Leaver M, Wells D. Non-invasive preimplantation genetic testing (niPGT): the next revolution in reproductive genetics? *Hum Reprod Update* 2020;26: 16-42.

Lee MT, Bonneau AR, Giraldez AJ. Zygotic genome activation during the maternal-to-zygotic transition. *Annu Rev Cell Dev Biol* 2014;30: 581-613.

Lee YS, Thouas GA, Gardner DK. Developmental kinetics of cleavage stage mouse embryos are related to their subsequent carbohydrate and amino acid utilization at the blastocyst stage. *Hum Reprod* 2015;30: 543-552.

Lees JG, Gardner DK, Harvey AJ. Pluripotent stem cell metabolism and mitochondria: beyond ATP. *Stem Cells International* 2017;2017.

Lees JG, Gardner DK, Harvey AJ. Nicotinamide adenine dinucleotide induces a bivalent metabolism and maintains pluripotency in human embryonic stem cells. *Stem Cells* 2020;38: 624-638.

Leese H. The formation and function of oviduct fluid. *J Reprod Fertil* 1988;82: 843-856.

Leese H. Metabolism of the preimplantation mammalian embryo. *Oxf Rev Reprod Biol* 1991;13: 35.

Leese H, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *Reproduction* 1984;72: 9-13.

Leese HJ. Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. *Bioessays* 2002;24: 845-849.

Leese HJ, Barton AM. Pyruvate and glucose uptake by mouse ova and preimplantation embryos. *J Reprod Fertil* 1984;72: 9-13.

Leese HJ, Guerif F, Allgar V, Brison DR, Lundin K, Sturmey RG. Biological optimization, the Goldilocks principle, and how much is lagom in the preimplantation embryo. *Mol Reprod Dev* 2016;83: 748-754.

Leese HJ, Sturmey RG, Baumann CG, McEvoy TG. Embryo viability and metabolism: obeying the quiet rules. *Hum Reprod* 2007;22: 3047-3050.

Leonavicius K, Royer C, Preece C, Davies B, Biggins JS, Srinivas S. Mechanics of mouse blastocyst hatching revealed by a hydrogel-based microdeformation assay. *Proc Natl Acad Sci U S A* 2018;115: 10375-10380.

Lessey BA. Endometrial receptivity and the window of implantation. *Baillieres Best Pract Res Clin Obstet Gynaecol* 2000;14: 775-788.

Liang B, Gao Y, Xu J, Song Y, Xuan L, Shi T, Wang N, Hou Z, Zhao Y-L, Huang WE. Raman profiling of embryo culture medium to identify aneuploid and euploid embryos. *Fertil Steril* 2019;111: 753-762. e751.

Liang S, Niu YJ, Shin KT, Cui XS. Protective Effects of Coenzyme Q10 on Developmental Competence of Porcine Early Embryos. *Microsc Microanal* 2017;23: 849-858.

Limoli CL, Giedzinski E, Morgan WF, Swarts SG, Jones GD, Hyun W. Persistent oxidative stress in chromosomally unstable cells. *Cancer Res* 2003;63: 3107-3111.

Linck D, Larman M, Gardner D. α-Lipoic acid: an antioxidant that improves embryo development and protects against oxidative stress. *Fertil Steril* 2007;88: S36-S37.

Liu W, Liu J, Du H, Ling J, Sun X, Chen D. Non-invasive pre-implantation aneuploidy screening and diagnosis of beta thalassemia IVSII654 mutation using spent embryo culture medium. *Ann Med* 2017;49: 319-328.

Liu Y, Chapple V, Feenan K, Roberts P, Matson P. Time-lapse deselection model for human day 3 in vitro fertilization embryos: the combination of qualitative and quantitative measures of embryo growth. *Fertil Steril* 2016;105: 656-662 e651.

Liu Y, Chapple V, Roberts P, Matson P. Prevalence, consequence, and significance of reverse cleavage by human embryos viewed with the use of the Embryoscope time-lapse video system. *Fertil Steril* 2014;102: 1295-1300 e1292.

Liu Z, Foote RH. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O2. *Biol Reprod* 1995;53: 786-790.

Lord T, Aitken RJ. Oxidative stress and ageing of the post-ovulatory oocyte. *Reproduction* 2013;146: R217-227.

Luke B, Bigger HR, Leurgans S, Sietsema D. The cost of prematurity: a case-control study of twins vs singletons. *Am J Public Health* 1996;86: 809-814.

Lundin K, Bergh C, Hardarson T. Early embryo cleavage is a strong indicator of embryo quality in human IVF. *Hum Reprod* 2001;16: 2652-2657.

Magnusson C, Hillensjö T, Hamberger L, Nilsson L. Oxygen consumption by human oocytes and blastocysts grown in vitro. *Hum Reprod* 1986;1: 183-184.

Manjunatha B, Devaraj M, Gupta P, Ravindra J, Nandi S. Effect of taurine and melatonin in the culture medium on buffalo in vitro embryo development. *Reprod Domest Anim* 2009;44: 12-16.

Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol* 2001;240: 182-193.

Martin PM, Sutherland AE, Van Winkle LJ. Amino Acid Transport Regulates Blastocyst Implantation. *Biol Reprod* 2003;69: 1101-1108.

Martin PM, Sutherland AE, Van Winkle LJ. Amino Acid Transport Regulates Blastocyst Implantation 1. *Biology of reproduction* 2003;69: 1101-1108.

Martino NA, Dell'aquila ME, Cardone RA, Somoskoi B, Lacalandra GM, Cseh S. Vitrification preserves chromatin integrity, bioenergy potential and oxidative parameters in mouse embryos. *Reprod Biol Endocrinol* 2013;11: 27.

Mastroianna L, Jones R. Oxygen tension within the rabbit fallopian tube. *J Reprod Fertil* 1965;9: 99-102.

Mastroianna L, Jones R. Oxygen tension within the rabbit fallopian tube. *Journal of reproduction and fertility* 1965;9: 99-102.

Maxwell SM, Grifo JA. Should every embryo undergo preimplantation genetic testing for aneuploidy? A review of the modern approach to in vitro fertilization. *Best Pract Res Clin Obstet Gynaecol* 2018;53: 38-47.

McArthur SJ, Leigh D, Marshall JT, de Boer KA, Jansen RP. Pregnancies and live births after trophectoderm biopsy and preimplantation genetic testing of human blastocysts. *Fertil Steril* 2005;84: 1628-1636.

Mehta TS, Kiessling AA. Development potential of mouse embryos conceived in vitro and cultured in ethylenediaminetetraacetic acid with or without amino acids or serum. *Biol Reprod* 1990;43: 600-606.

Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, Barnett BD, Madden JD. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod* 2009;24: 300-307.

Menke TM, McLaren A. Mouse blastocysts grown in vivo and in vitro: carbon dioxide production and trophoblast outgrowth. *J Reprod Dev* 1970;23: 117-127.

Meseguer M, Herrero J, Tejera A, Hilligsøe KM, Ramsing NB, Remohí J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011;26: 2658-2671.

Milewski R, Kuc P, Kuczynska A, Stankiewicz B, Lukaszuk K, Kuczynski W. A predictive model for blastocyst formation based on morphokinetic parameters in time-lapse monitoring of embryo development. *J Assist Reprod Genet* 2015;32: 571-579.

Milewski R, Milewska AJ, Kuczyńska A, Stankiewicz B, Kuczyński W. Do morphokinetic data sets inform pregnancy potential? *J Assist Reprod Genet* 2016;33: 357-365.

Miller JG, Schultz GA. Amino acid content of preimplantation rabbit embryos and fluids of the reproductive tract. *Biol Reprod* 1987;36: 125-129.

Minasi MG, Colasante A, Riccio T, Ruberti A, Casciani V, Scarselli F, Spinella F, Fiorentino F, Varricchio MT, Greco E. Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study. *Hum Reprod* 2016;31: 2245-2254.

Mio Y, Maeda K. Time-lapse cinematography of dynamic changes occurring during in vitro development of human embryos. *Am J Ostet and Gynecol* 2008;199: 660. e661-660. e665.

Mitchell M, Cashman KS, Gardner DK, Thompson JG, Lane M. Disruption of mitochondrial malate-aspartate shuttle activity in mouse blastocysts impairs viability and fetal growth. *Biol Reprod* 2009;80: 295-301.

Miyagi Y, Habara T, Hirata R, Hayashi N. Feasibility of predicting live birth by combining conventional embryo evaluation with artificial intelligence applied to a blastocyst image in patients classified by age. *Reprod Med Biol* 2019;18: 344-356.

Mochizuki L, Gleicher N. The PGS/PGT-A controversy in IVF addressed as a formal conflict resolution analysis. *J Assist Reprod Genet* 2020: 1-11.

Montag M, Liebenthron J, Köster M. Which morphological scoring system is relevant in human embryo development? *Placenta* 2011;32: S252-S256.

Morales H, Tilquin P, Rees JF, Massip A, Dessy F, Van Langendonckt A. Pyruvate prevents peroxide-induced injury of in vitro preimplantation bovine embryos. *Mol Reprod Dev* 1999;52: 149-157.

Morgan MJ, Faik P. Carbohydrate metabolism in cultured animal cells. *Biosci Rep* 1981;1: 669-686.

Motato Y, de los Santos MJ, Escriba MJ, Ruiz BA, Remohi J, Meseguer M. Morphokinetic analysis and embryonic prediction for blastocyst formation through an integrated time-lapse system. *Fertil Steril* 2016;105: 376-384 e379.

Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. *Cell Metab* 2015;21: 392-402.

Mukerji S., Mukherjee S., S.K. B. The feasibility of long term cryogenic freezing of viable human embryos - a brief pilot study report. *Indian J Cryogenics* 1978;3: 80.

Munné S, Alikani M, Tomkin G, Grifo J, Cohen J. Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertil Steril* 1995;64: 382-391.

Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, Silverberg K, Kalista T, Handyside AH, Katz-Jaffe M. Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. *Fertil Steril* 2019;112: 1071-1079. e1077.

Munné S, Kaplan B, Frattarelli JL, Child T, Nakhuda G, Shamma FN, Silverberg K, Kalista T, Handyside AH, Katz-Jaffe M *et al.* Preimplantation genetic testing for aneuploidy versus morphology as selection criteria for single frozen-thawed embryo transfer in good-prognosis patients: a multicenter randomized clinical trial. *Fertil Steril* 2019;112: 1071-1079.e1077.

Munné S, Lee A, Rosenwaks Z, Grifo J, Cohen J. Fertilization and early embryology: Diagnosis of major chromosome aneuploidies in human preimplantation embryos. *Hum Reprod* 1993;8: 2185-2191.

Murphy LA, Seidler EA, Vaughan DA, Resetkova N, Penzias AS, Toth TL, Thornton KL, Sakkas D. To test or not to test? A framework for counselling patients on preimplantation genetic testing for aneuploidy (PGT-A). *Hum Reprod* 2019;34: 268-275.

Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J* 2009;417: 1-13.

Nagy ZP, Shapiro D, Chang CC. Vitrification of the human embryo: a more efficient and safer in vitro fertilization treatment. *Fertil Steril* 2020;113: 241-247.

Nagy ZP, Varghese AC, Agarwal A. Cryopreservation of Mammalian Gametes and Embryos. *Methods Mol Biol* 2017;1568.

Newman DL, Thurgood LA, Gregory SL. The impact of aneuploidy on cellular homeostasis. *Free Radic Res* 2019;53: 705-713.

Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Hum Reprod Update* 2018;24: 15-34.

Niederberger C, Pellicer A, Simon C, Kathrins M, Goldstein M, Sigman M, Schlegel PN, Munné S, Gardner DK, Cobo A. 25 historic papers: an ASRM 75th birthday gift from Fertility and Sterility. *Fertil Steril* 2019;112: e2-e27.

Nishi M, Kumar NM, Gilula NB. Developmental regulation of gap junction gene expression during mouse embryonic development. *Dev Biol* 1991;146: 117-130.

Nogales MDC, Bronet F, Basile N, Martínez EM, Liñán A, Rodrigo L, Meseguer M. Type of chromosome abnormality affects embryo morphology dynamics. *Fertil Steril* 2017;107: 229-235. e222.

O'Fallon J, Wright R. Pyruvate revisited: a non-metabolic role for pyruvate in preimplantation embryo development. *Theriogenology* 1995;1: 288.

Olivius C, Friden B, Borg G, Bergh C. Why do couples discontinue in vitro fertilization treatment? A cohort study. *Fertil Steril* 2004;81: 258-261.

Ombelet W, Cooke I, Dyer S, Serour G, Devroey P. Infertility and the provision of infertility medical services in developing countries. *Hum Reprod Update* 2008;14: 605-621.

Ombelet W, De Sutter P, Van der Elst J, Martens G. Multiple gestation and infertility treatment: registration, reflection and reaction-the Belgian project. *Hum Reprod Update* 2005;11: 3-14.

Özgür K, Berkkanoğlu M, Bulut H, Isikli A, Coetzee K. Higher clinical pregnancy rates from frozen-thawed blastocyst transfers compared to fresh blastocyst transfers: a retrospective matched-cohort study. *J Assist Reprod Genet* 2015;32: 1483-1490.

Ozgur K, Berkkanoglu M, Bulut H, Yoruk GDA, Candurmaz NN, Coetzee K. Single best euploid versus single best unknown-ploidy blastocyst frozen embryo transfers: a randomized controlled trial. *J Assist Reprod Genet* 2019;36: 629-636.

Pabon Jr JE, Findley WE, Gibbons WE. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. *Fertil Steril* 1989;51: 896-900.

Packer L, Witt EH, Tritschler HJ. Alpha-lipoic acid as a biological antioxidant. *Free Radic Biol Med* 1995;19: 227-250.

Palaniappan A, Dai A. Mitochondrial ageing and the beneficial role of α-lipoic acid. *Neurochem Res* 2007;32: 1552-1558.

Palini S, Galluzzi L, De Stefani S, Bianchi M, Wells D, Magnani M, Bulletti C. Genomic DNA in human blastocoele fluid. *Reprod Biomed Online* 2013;26: 603-610.

Partridge RJ, Leese HJ. Consumption of amino acids by bovine preimplantation embryos. *Reprod Fertil Dev* 1996;8: 945-950.

Patel DV, Shah PB, Kotdawala AP, Herrero J, Rubio I, Banker MR. Morphokinetic behavior of euploid and aneuploid embryos analyzed by time-lapse in embryoscope. *J Hum Reprod Sci* 2016;9: 112-118.

Paternot G, Debrock S, D'Hooghe T, Spiessens C. Computer-assisted embryo selection: a benefit in the evaluation of embryo quality? *Reprod Biomed Online* 2011;23: 347-354.

Paulson RJ. Hidden in plain sight: the overstated benefits and underestimated losses of potential implantations associated with advertised PGT-A success rates. *Hum Reprod* 2020;35: 490-493.

Penzias A, Bendikson K, Butts S, Coutifaris C, Fossum G, Falcone T, Gitlin S, Gracia C, Hansen K, La Barbera A. Guidance on the limits to the number of embryos to transfer: a committee opinion. *Fertil Steril* 2017;107: 901-903.

Petersen BM, Boel M, Montag M, Gardner DK. Development of a generally applicable morphokinetic algorithm capable of predicting the implantation potential of embryos transferred on Day 3. *Hum Reprod* 2016;31: 2231-2244.

Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, Leese HJ, Harris SE. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Mol Hum Reprod* 2010;16: 557-569.

Picton HM, Elder K, Houghton FD, Hawkhead JA, Rutherford AJ, Hogg JE, Leese HJ, Harris SE. Association between amino acid turnover and chromosome aneuploidy during human preimplantation embryo development in vitro. *Molecular human reproduction* 2010;16: 557-569.

Pigeolet E, Corbisier P, Houbion A, Lambert D, Michiels C, Raes M, Zachary M-D, Remacle J. Glutathione peroxidase, superoxide dismutase, and catalase inactivation by peroxides and oxygen derived free radicals. *Mech Aging Dev* 1990;51: 283-297.

Pikó L, Clegg KB. Quantitative changes in total RNA, total poly (A), and ribosomes in early mouse embryos. *Dev Biol* 1982;89: 362-378.

Pribenszky C, Nilselid A-M, Montag M. Response: time-lapse systems for ART. *Reprod Biomed Online* 2018;36: 290.

Reignier A, Girard J-M, Lammers J, Chtourou S, Lefebvre T, Barriere P, Freour T. Performance of Day 5 KIDScore™ morphokinetic prediction models of implantation and live birth after single blastocyst transfer. *J Assist Reprod Genet* 2019;36: 2279-2285.

Reitzer LJ, Wice B, Kennell D. The pentose cycle. Control and essential function in HeLa cell nucleic acid synthesis. *J Biol Chem* 1980;255: 5616-5626.

Reitzer LJ, Wice B, Kennell D. The pentose cycle. Control and essential function in HeLa cell nucleic acid synthesis. *Journal of Biological Chemistry* 1980;255: 5616-5626.

Renard JP, Philippon A, Menezo Y. In-vitro uptake of glucose by bovine blastocysts. *J Reprod Fertil* 1980;58: 161-164.

Rezazadeh Valojerdi M, Eftekhari-Yazdi P, Karimian L, Hassani F, Movaghar B. Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. *J Assist Reprod Genet* 2009;26: 347-354.

Rieger D, Loskutoff N, Betteridge K. Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. *Reprod Fertil Dev* 1992;4: 547-557.

Rinaudo PF, Giritharan G, Talbi S, Dobson AT, Schultz RM. Effects of oxygen tension on gene expression in preimplantation mouse embryos. *Fertil Steril* 2006;86: 1252-1265, 1265 e1251-1236.

Rodriguez-Osorio N, Kim IJ, Wang H, Kaya A, Memili E. Melatonin increases cleavage rate of porcine preimplantation embryos in vitro. *J Pineal Res* 2007;43: 283-288.

Rogers P, Murphy C, Gannon B. Changes in the spatial organization of the uterine vasculature during implantation in the rat. *J Reprod Fertil* 1982;65: 211-214.

Roh M, van der Meer R, Abdulkadir SA. Tumorigenic polyploid cells contain elevated ROS and ARE selectively targeted by antioxidant treatment. *J Cell Physiol* 2012;227: 801-812.

Ros R, Graves C. O2 levels in female rabbit reproductive-tract *Journal of Animal Science*. 1974. AMER SOC ANIMAL SCIENCE 1111 NORTH DUNLAP AVE, SAVOY, IL 61874, pp. 994-994.

Rougier N, Bourc'his D, Gomes DM, Niveleau A, Plachot M, Pàldi A, Viegas-Péquignot E. Chromosome methylation patterns during mammalian preimplantation development. *Genes Dev* 1998;12: 2108-2113.

Rubino P, Tapia L, de Assin Alonso RR, Mazmanian K, Guan L, Dearden L, Thiel A, Moon C, Kolb B, Norian JM. Trophectoderm biopsy protocols can affect clinical outcomes: time to focus on the blastocyst biopsy technique. *Fertil Steril* 2020;113: 981-989.

Rubio C, Navarro-Sánchez L, García-Pascual CM, Ocali O, Cimadomo D, Venier W, Barroso G, Kopcow L, Bahçeci M, Kulmann MIR *et al.* Multicenter prospective study of concordance between embryonic cell-free DNA and trophectoderm biopsies from 1301 human blastocysts. *Am J Obstet Gynecol* 2020.

Rubio C, Rienzi L, Navarro-Sánchez L, Cimadomo D, García-Pascual CM, Albricci L, Soscia D, Valbuena D, Capalbo A, Ubaldi F. Embryonic cell-free DNA versus trophectoderm biopsy for aneuploidy testing: concordance rate and clinical implications. *Fertil Steril* 2019;112: 510-519.

Rubio I, Galan A, Larreategui Z, Ayerdi F, Bellver J, Herrero J, Meseguer M. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomized, controlled trial of the EmbryoScope. *Fertil Steril* 2014;102: 1287-1294 e1285.

Rubio I, Kuhlmann R, Agerholm I, Kirk J, Herrero J, Escriba MJ, Bellver J, Meseguer M. Limited implantation success of direct-cleaved human zygotes: a time-lapse study. *Fertil Steril* 2012;98: 1458-1463.

Ryall JG, Dell'Orso S, Derfoul A, Juan A, Zare H, Feng X, Clermont D, Koulnis M, Gutierrez-Cruz G, Fulco M. The NAD+-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. *Cell Stem Cell* 2015;16: 171-183.

Sakkas D, Percival G, D'Arcy Y, Sharif K, Afnan M. Assessment of early cleaving in vitro fertilized human embryos at the 2-cell stage before transfer improves embryo selection. *Fertil Steril* 2001;76: 1150-1156.

Salamonsen LA, Evans J, Nguyen HP, Edgell TA. The Microenvironment of Human Implantation: Determinant of Reproductive Success. *Am J Reprod Immunol* 2016;75: 218-225.

Salumets A, Hydén-Granskog C, Mäkinen S, Suikkari AM, Tiitinen A, Tuuri T. Early cleavage predicts the viability of human embryos in elective single embryo transfer procedures. *Hum Reprod* 2003;18: 821-825.

Santos Filho E, Noble JA, Poli M, Griffiths T, Emerson G, Wells D. A method for semi-automatic grading of human blastocyst microscope images. *Hum Reprod* 2012;27: 2641-2648.

Sasaki H, Hamatani T, Kamijo S, Iwai M, Kobanawa M, Ogawa S, Miyado K, Tanaka M. Impact of Oxidative Stress on Age-Associated Decline in Oocyte Developmental Competence. *Front Endocrinol (Lausanne)* 2019;10: 811.

Schmid F, Hinz HJ, Jaenicke R. Thermodynamic studies of binary and ternary complexes of pig heart lactate dehydrogenase. *Biochemistry* 1976;15: 3052-3059.

Schon EA, Kim SH, Ferreira JC, Magalhães P, Grace M, Warburton D, Gross SJ. Chromosomal non-disjunction in human oocytes: is there a mitochondrial connection? *Hum Reprod* 2000;15 Suppl 2: 160-172.

Schoolcraft WB, Fragouli E, Stevens J, Munne S, Katz-Jaffe MG, Wells D. Clinical application of comprehensive chromosomal screening at the blastocyst stage. *Fertil Steril* 2010;94: 1700-1706.

Schoolcraft WB, Gardner DK, Lane M, Schlenker T, Hamilton F, Meldrum DR. Blastocyst culture and transfer: analysis of results and parameters affecting outcome in two in vitro fertilization programs. *Fertil Steril* 1999;72: 604-609.

Schultz GA, Kaye PL, McKay DJ, Johnson MH. Endogenous amino acid pool sizes in mouse eggs and preimplantation embryos. *J Reprod Fertil* 1981;61: 387-393.

Scotchie JG, Fritz MA, Mocanu M, Lessey BA, Young SL. Proteomic analysis of the luteal endometrial secretome. *Reprod Sci* 2009;16: 883-893.

Scott Jr RT, Upham KM, Forman EJ, Hong KH, Scott KL, Taylor D, Tao X, Treff NR. Blastocyst biopsy with comprehensive chromosome screening and fresh embryo transfer significantly increases in vitro fertilization implantation and delivery rates: a randomized controlled trial. *Fertil Steril* 2013;100: 697-703.

Scott Jr RT, Upham KM, Forman EJ, Zhao T, Treff NR. Cleavage-stage biopsy significantly impairs human embryonic implantation potential while blastocyst biopsy does not: a randomized and paired clinical trial. *Fertil Steril* 2013;100: 624-630.

Shapiro BS, Daneshmand ST, Garner FC, Aguirre M, Thomas S. Large blastocyst diameter, early blastulation, and low preovulatory serum progesterone are dominant predictors of clinical pregnancy in fresh autologous cycles. *Fertil Steril* 2008;90: 302-309.

Shapiro BS, Richter KS, Harris DC, Daneshmand ST. A comparison of day 5 and day 6 blastocyst transfers. *Fertil Steril* 2001;75: 1126-1130.

Shen W, Liu K, Tian C, Yang L, Li X, Ren J, Packer L, Cotman CW, Liu J. R-alpha-lipoic acid and acetyl-L-carnitine complementarily promote mitochondrial biogenesis in murine 3T3-L1 adipocytes. *Diabetologia* 2008;51: 165-174.

Showstack JA, Stone MH, Schroeder SA. The role of changing clinical practices in the rising costs of hospital care. *N Engl J Med* 1985;313: 1201-1207.

Silva E, Greene AF, Strauss K, Herrick JR, Schoolcraft WB, Krisher RL. Antioxidant supplementation during in vitro culture improves mitochondrial function and development of embryos from aged female mice. *Reprod Fertil Dev* 2015;27: 975-983.

Simon AL, Kiehl M, Fischer E, Proctor JG, Bush MR, Givens C, Rabinowitz M, Demko ZP. Pregnancy outcomes from more than 1,800 in vitro fertilization cycles with the use of 24-chromosome single-nucleotide polymorphism–based preimplantation genetic testing for aneuploidy. *Fertil Steril* 2018;110: 113-121.

Simsek-Duran F, Li F, Ford W, Swanson RJ, Jones HW, Jr., Castora FJ. Age-associated metabolic and morphologic changes in mitochondria of individual mouse and hamster oocytes. *PLoS One* 2013;8: e64955.

Smith GD, Silva ESCA. Developmental consequences of cryopreservation of mammalian oocytes and embryos. *Reprod Biomed Online* 2004;9: 171-178.

Somfai T, Kaneda M, Akagi S, Watanabe S, Haraguchi S, Mizutani E, Dang-Nguyen TQ, Geshi M, Kikuchi K, Nagai T. Enhancement of lipid metabolism with L-carnitine during in vitro maturation improves nuclear maturation and cleavage ability of follicular porcine oocytes. *Reprod Fertil Dev* 2011;23: 912-920.

Son WY, Chung JT, Gidoni Y, Holzer H, Levin D, Chian RC, Tan SL. Comparison of survival rate of cleavage stage embryos produced from in vitro maturation cycles after slow freezing and after vitrification. *Fertil Steril* 2009;92: 956-958.

Steeves TE, Gardner DK. Temporal and differential effects of amino acids on bovine embryo development in culture. *Biol Reprod* 1999;61: 731-740.

Stehlik E, Stehlik J, Katayama KP, Kuwayama M, Jambor V, Brohammer R, Kato O. Vitrification demonstrates significant improvement versus slow freezing of human blastocysts. *Reprod Biomed Online* 2005;11: 53-57.

Steptoe PC, Edwards RG. Birth after the reimplantation of a human embryo. *Lancet* 1978;2: 366.

Stigliani S, Anserini P, Venturini P, Scaruffi P. Mitochondrial DNA content in embryo culture medium is significantly associated with human embryo fragmentation. *Hum Reprod* 2013;28: 2652-2660.

Storr A, Venetis CA, Cooke S, Kilani S, Ledger W. Inter-observer and intra-observer agreement between embryologists during selection of a single Day 5 embryo for transfer: a multicenter study. *Hum Reprod* 2017;32: 307-314.

Sturmey R, Reis A, Leese H, McEvoy T. Role of fatty acids in energy provision during oocyte maturation and early embryo development. *Reprod Domest Anim* 2009;44: 50-58.

Succu S, Gadau SD, Serra E, Zinellu A, Carru C, Porcu C, Naitana S, Berlinguer F, Leoni GG. A recovery time after warming restores mitochondrial function and improves developmental competence of vitrified ovine oocytes. *Theriogenology* 2018;110: 18-26.

Sun X-F, Zhang W-H, Chen X-J, Xiao G-H, Mai W-Y, Wang W-H. Spindle dynamics in living mouse oocytes during meiotic maturation, ageing, cooling and overheating: a study by polarized light microscopy. *Zygote* 2004;12: 241-249.

Sunderam S, Kissin DM, Zhang Y, Folger SG, Boulet SL, Warner L, Callaghan WM, Barfield WD. Assisted Reproductive Technology Surveillance - United States, 2016. *MMWR Surveill Summ* 2019;68: 1-23.

Takahashi M, Keicho K, Takahashi H, Ogawa H, Schulte R, Okano A. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. *Theriogenology* 2000;54: 137-145.

Takahashi T, Takahashi E, Igarashi H, Tezuka N, Kurachi H. Impact of oxidative stress in aged mouse oocytes on calcium oscillations at fertilization. *Mol Reprod Dev* 2003;66: 143-152.

Takeshima K, Jwa SC, Saito H, Nakaza A, Kuwahara A, Ishihara O, Irahara M, Hirahara F, Yoshimura Y, Sakumoto T. Impact of single embryo transfer policy on perinatal outcomes in fresh and frozen cycles—analysis of the Japanese Assisted Reproduction Technology registry between 2007 and 2012. *Fertil Steril* 2016;105: 337-346. e333.

Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P, Focarelli R. Cellular and molecular aspects of ovarian follicle ageing. *Hum Reprod Update* 2008;14: 131-142.

Tatone C, Di Emidio G, Barbonetti A, Carta G, Luciano AM, Falone S, Amicarelli F. Sirtuins in gamete biology and reproductive physiology: emerging roles and therapeutic potential in female and male infertility. *Hum Reprod Update* 2018;24: 267-289.

Tatone C, Di Emidio G, Vento M, Ciriminna R, Artini PG. Cryopreservation and oxidative stress in reproductive cells. *Gynecol Endocrinol* 2010;26: 563-567.

Taylor KD, Pikó L. Patterns of mRNA prevalence and expression of B1 and B2 transcripts in early mouse embryos. *Development* 1987;101: 877-892.

Thirumalaraju P, Hsu J, Bormann C, Kanakasabapathy M, Souter I, Dimitriadis I, Dickinson K, Pooniwala R, Gupta R, Yogesh V. Deep learning-enabled blastocyst prediction system for cleavage stage embryo selection. *Fertil Steril* 2019;111: e29.

Thompson J, Partridge R, Houghton F, Cox C, Leese H. Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. *J Reprod Fertil* 1996;106: 299-306.

Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *J Reprod Fertil* 1990;89: 573-578.

Thouas GA, Trounson AO, Jones GM. Effect of female age on mouse oocyte developmental competence following mitochondrial injury. *Biol Reprod* 2005;73: 366-373.

Tiitinen A, Hydén-Granskog C, Gissler M. What is the most relevant standard of success in assisted reproduction?: The value of cryopreservation on cumulative pregnancy rates per single oocyte retrieval should not be forgotten. *Hum Reprod* 2004;19: 2439-2441.

Tilia L, Chapman M, Kilani S, Cooke S, Venetis C. Oocyte meiotic spindle morphology is a predictive marker of blastocyst ploidy—a prospective cohort study. *Fertil Steril* 2020;113: 105-113. e101.

Tilia L, Venetis C, Kilani S, Cooke S, Chapman M. Is oocyte meiotic spindle morphology associated with embryo ploidy? A prospective cohort study. *Fertil Steril* 2016;105: 1085-1092. e1087.

Tran D, Cooke S, Illingworth P, Gardner D. Deep learning as a predictive tool for fetal heart pregnancy following time-lapse incubation and blastocyst transfer. *Hum Reprod* 2019;34: 1011-1018.

Treff NR, Su J, Tao X, Levy B, Scott Jr RT. Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. *Fertil Steril* 2010;94: 2017-2021.

Trounson A, Mohr L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 1983;305: 707-709.

Truong T, Gardner D. Antioxidants improve IVF outcome and subsequent embryo development in the mouse. *Hum Reprod* 2017;32: 2404-2413.

Truong TT, Gardner DK. Antioxidants increase blastocyst cryosurvival and viability post-vitrification. *Hum Reprod* 2020;35: 12-23.

Truong TT, Soh YM, Gardner DK. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod* 2016;31: 1445-1454.

Truong TT, Soh YM, Gardner DK. Antioxidants improve mouse preimplantation embryo development and viability. *Hum Reprod* 2016;31: 1445-1454.

Turner K, Martin KL, Woodward BJ, Lenton EA, Leese HJ. Comparison of pyruvate uptake by embryos derived from conception and non-conception natural cycles. *Hum Reprod* 1994;9: 2362-2366.

Umaoka Y, Noda Y, Narimoto K, Mori T. Effects of oxygen toxicity on early development of mouse embryos. *Mol Reprod Dev* 1992;31: 28-33.

Van Blerkom J, Davis PW, Lee J. ATP content of human oocytes and developmental potential and outcome after in-vitro fertilization and embryo transfer. *Hum Reprod* 1995;10: 415-424.

Van Montfoort APA, Arts E, Wijnandts L, Sluijmer A, Pelinck MJ, Land JA, Van Echten-Arends J. Reduced oxygen concentration during human IVF culture improves embryo utilization and cumulative pregnancy rates per cycle. *Hum Reprod Open* 2020;1: hoz036.

van Noord-Zaadstra BM, Looman CW, Alsbach H, Habbema JD, te Velde ER, Karbaat J. Delaying childbearing: effect of age on fecundity and outcome of pregnancy. *BMJ* 1991;302: 1361-1365.

Van Royen E, Mangelschots K, De Neubourg D, Valkenburg M, Van de Meerssche M, Ryckaert G, Eestermans W, Gerris J. Characterization of a top quality embryo, a step towards single-embryo transfer. *Hum Reprod* 1999;14: 2345-2349.

Van Winkle LJ. Amino acid transport in developing animal oocytes and early conceptuses. *Biochim Biophys Acta* 1988;947: 173-208.

Van Winkle LJ, Campione AL. Amino acid transport regulation in preimplantation mouse embryos: effects on amino acid content and pre-and peri-implantation development. *Theriogenology* 1996;45: 69-80.

Van Winkle LJ, Haghighat N, Campione AL. Glycine protects preimplantation mouse conceptuses from a detrimental effect on development of the inorganic ions in oviductal fluid. *J Exp Zool* 1990;253: 215-219.

Vega M, Breborowicz A, Moshier EL, McGovern PG, Keltz MD. Blastulation rates decline in a linear fashion from euploid to aneuploid embryos with single versus multiple chromosomal errors. *Fertil Steril* 2014;102: 394-398.

Vera-Rodriguez M, Chavez SL, Rubio C, Pera RAR, Simon C. Prediction model for aneuploidy in early human embryo development revealed by single-cell analysis. *Nat Commun* 2015;6: 1-14.

Vera-Rodriguez M, Diez-Juan A, Jimenez-Almazan J, Martinez S, Navarro R, Peinado V, Mercader A, Meseguer M, Blesa D, Moreno I. Origin and composition of cell-free DNA in spent medium from human embryo culture during preimplantation development. *Hum Reprod* 2018;33: 745-756.

VerMilyea MD, Tan L, Anthony JT, Conaghan J, Ivani K, Gvakharia M, Boostanfar R, Baker VL, Suraj V, Chen AA. Computer-automated time-lapse analysis results correlate with embryo implantation and clinical pregnancy: a blinded, multi-centre study. *Reprod Biomed Online* 2014;29: 729-736.

Waldenström U, Engström AB, Hellberg D, Nilsson S. Low-oxygen compared with high-oxygen atmosphere in blastocyst culture, a prospective randomized study. *Fertil Steril* 2009;91: 2461-2465.

Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod Biomed Online* 2010;21: 402-410.

Wale PL, Gardner DK. Oxygen regulates amino acid turnover and carbohydrate uptake during the preimplantation period of mouse embryo development. *Biol Reprod* 2012;87: 24, 21-28.

Wale PL, Gardner DK. Oxygen affects the ability of mouse blastocysts to regulate ammonium. *Biol Reprod* 2013;89: 75.

Wale PL, Gardner DK. The effects of chemical and physical factors on mammalian embryo culture and their importance for the practice of assisted human reproduction. *Hum Reprod Update* 2016;22: 2-22.

Walker DL, Gardner DK, Lane M, Tummon IS, Session DR, Thornhill AR. Cryosystem assessment by glucose uptake of murine blastocysts. *Reprod Biomed Online* 2005;11: 601-607.

Walker KJ, Green MP, Gardner DK. Spatial asynchronous transfer of cleavage-stage mouse embryos to the uterus compromises fetal development. *Mol Reprod Dev* 2015;82: 80.

Wang A, Kort J, Behr B, Westphal LM. Euploidy in relation to blastocyst sex and morphology. *J Assist Reprod Genet* 2018;35: 1565-1572.

Wang W-H, Meng L, Hackett RJ, Odenbourg R, Keefe DL. Limited recovery of meiotic spindles in living human oocytes after cooling–rewarming observed using polarized light microscopy. *Hum Reprod* 2001;16: 2374-2378.

Warburg O. On the origin of cancer cells. *Science* 1956;123: 309-314.

Watson A. Oocyte cytoplasmic maturation: a key mediator of oocyte and embryo developmental competence. *J Anim Sci* 2007;85: E1-E3.

Watson AJ, Damsky CH, Kidder GM. Differentiation of an epithelium: factors affecting the polarized distribution of Na+, K+-ATPase in mouse trophectoderm. *Dev Biol* 1990;141: 104-114.

Watson AJ, Kidder GM. Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K-ATPase during mouse embryogenesis. *Dev Biol* 1988;126: 80-90.

Watson AJ, Kidder GM, Schultz GA. How to make a blastocyst. *Biochem Cell Biol* 1992;70: 849-855.

Watson AJ, Kidder GM, Schultz GA. How to make a blastocyst. *Biochemistry and Cell Biology* 1992;70: 849-855.

Watson AJ, Pape C, Emanuel JR, Levenson R, Kidder GM. Expression of NA, K-ATpase α and β subunit genes during preimplantation development of the mouse. *Dev Genet* 1990;11: 41-48.

Watson AJ, Pape C, Emanuel JR, Levenson R, Kidder GM. Expression of NA, K-ATpase α and β subunit genes during preimplantation development of the mouse. *genesis* 1990;11: 41-48.

Wei D, Liu JY, Sun Y, Shi Y, Zhang B, Liu JQ, Tan J, Liang X, Cao Y, Wang Z *et al.* Frozen versus fresh single blastocyst transfer in ovulatory women: a multicentre, randomised controlled trial. *Lancet* 2019;393: 1310-1318.

Wells D, Kaur K, Grifo J, Glassner M, Taylor JC, Fragouli E, Munne S. Clinical utilisation of a rapid low-pass whole genome sequencing technique for the diagnosis of aneuploidy in human embryos prior to implantation. *J Med Genet* 2014;51: 553-562.

West JD, Flockhart JH. Genetic differences in glucose phosphate isomerase activity among mouse embryos. *Development* 1989;107: 465-472.

Whitaker BD, Casey SJ, Taupier R. The effects of N-acetyl-L-cysteine supplementation on in vitro porcine oocyte maturation and subsequent fertilisation and embryonic development. *Reprod Fertil Dev* 2012;24: 1048-1054.

Whitten W. The effect of oxygen on cleavage of mouse eggs in vitro. *Pro Soc Study Reprod* 1969;2: 29.

Whitten WK. The effect of progesterone on the development of mouse eggs in vitro. *J Endocrinol* 1957;16: 80-85.

Whitten WK, Biggers JD. Complete development in vitro of the pre-implantation stages of the mouse in a simple chemically defined medium. *J Reprod Fertil* 1968;17: 399-401.

Whittingham DG. The failure of lactate and phosphoenolpyruvate to support development of the mouse zygote in vitro. *Biol Reprod* 1969;1: 381-386.

Whittingham DG. Culture of mouse ova. *J Reprod Fertil Suppl* 1971;14: 7-21.

Whittingham DG, Biggers JD. Fallopian tube and early cleavage in the mouse. *Nature* 1967;213: 942-943.

Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Pera RAR. Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010;28: 1115.

Wong KM, van Wely M, Mol F, Repping S, Mastenbroek S. Fresh versus frozen embryo transfers in assisted reproduction. *Cochrane Database Syst Rev* 2017;3: Cd011184.

Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. *Biochem J* 1998;336 (Pt 1): 1-17.

Wu J, Huang B, Chen H, Yin Q, Liu Y, Xiang Y, Zhang B, Liu B, Wang Q, Xia W *et al.* The landscape of accessible chromatin in mammalian preimplantation embryos. *Nature* 2016;534: 652-657.

Xu J, Fang R, Chen L, Chen D, Xiao J-P, Yang W, Wang H, Song X, Ma T, Bo S. Noninvasive chromosome screening of human embryos by genome sequencing of embryo culture medium for in vitro fertilization. *Proc Natl Acad Sci U S A* 2016;113: 11907-11912.

Xue Z, Huang K, Cai C, Cai L, Jiang CY, Feng Y, Liu Z, Zeng Q, Cheng L, Sun YE *et al.* Genetic programs in human and mouse early embryos revealed by single-cell RNA sequencing. *Nature* 2013;500: 593-597.

Yamanaka M, Hashimoto S, Amo A, Ito-Sasaki T, Abe H, Morimoto Y. Developmental assessment of human vitrified-warmed blastocysts based on oxygen consumption. *Hum Reprod* 2011;26: 3366-3371.

Yang HW, Hwang KJ, Kwon HC, Kim HS, Choi KW, Oh KS. Detection of reactive oxygen species (ROS) and apoptosis in human fragmented embryos. *Hum Reprod* 1998;13: 998-1002.

Yang Z, Liu J, Collins GS, Salem SA, Liu X, Lyle SS, Peck AC, Sills ES, Salem RD. Selection of single blastocysts for fresh transfer via standard morphology assessment alone and with array CGH for good prognosis IVF patients: results from a randomized pilot study. *Mol Cytogenet* 2012;5: 24.

Yi X, Maeda N. Endogenous production of lipoic acid is essential for mouse development. *Mol Cell Biol* 2005;25: 8387-8392.

Yin H, Jiang H, He R, Wang C, Zhu J, Li Y. The effects of blastocyst morphological score and blastocoele re-expansion speed after warming on pregnancy outcomes. *Clin Exp Reprod Med* 2016;43: 31-37.

Zander-Fox DL, Mitchell M, Thompson JG, Lane M. Alterations in mouse embryo intracellular pH by DMO during culture impair implantation and fetal growth. *Reprod Biomed Online* 2010;21: 219-229.

Zeilmaker GH, Alberda AT, van Gent I, Rijkmans CM, Drogendijk AC. Two pregnancies following transfer of intact frozen-thawed embryos. *Fertil Steril* 1984;42: 293-296.

Zhang D, Tang Z, Huang H, Zhou G, Cui C, Weng Y, Liu W, Kim S, Lee S, Perez-Neut M. Metabolic regulation of gene expression by histone lactylation. *Nature* 2019;574: 575-580.

Zhao XM, Fu XW, Hou YP, Yan CL, Suo L, Wang YP, Zhu HB, Dinnyés A, Zhu SE. Effect of vitrification on mitochondrial distribution and membrane potential in mouse two pronuclear (2-PN) embryos. *Mol Reprod Dev* 2009;76: 1056-1063.

Zheng H, Jin H, Liu L, Liu J, Wang W-H. Application of next-generation sequencing for 24-chromosome aneuploidy screening of human preimplantation embryos. *Mol Cytogenet* 2015;8: 38.

Zhu J, Tsai H-J, Gordon MR, Li R. Cellular stress associated with aneuploidy. *Dev Cell* 2018;44: 420-431.

Zimmerman R, Tao X, Marin D, Werner M, Hong K, Lonczak A, Landis J, Taylor D, Zhan Y, Scott Jr R. Preclinical validation of a targeted next generation sequencing-based comprehensive chromosome screening methodology in human blastocysts. *Mol Hum Reprod* 2018;24: 37-45.

Ziomek C, Pratt H, Johnson M. The origins of cell diversity in the early mouse embryo *Functional integration of cells in animal tissues BSCB Symp*. 1981, pp. 149-165.

Ziomek CA. Cell polarity in the preimplantation mouse embryo *The Mammalian Preimplantation Embryo*. 1987. Springer, pp. 23-41.

Ziomek CA. Cell polarity in the preimplantation mouse embryo. *The Mammalian Preimplantation Embryo*. 1987. Plenum Publishing, New York, pp. 23-41.

Zmuda J, Friedenson B. Changes in intracellular glutathione levels in stimulated and unstimulated lymphocytes in the presence of 2-mercaptoethanol or cysteine. *J Immunol* 1983;130: 362-364.