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Embryo metabolism: what does it really mean?

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Abstract

The study of early embryo metabolism has fascinated researchers in the field for nearly a century. Herein, we give a brief account of the general features of embryo metabolism and some consideration of the research performed to reach such conclusions. It is becoming increasingly obvious that metabolism informs many fate decisions and outcomes beyond ATP generation, such as DNA methylation, Reactive Oxygen Species generation and cell signaling. We discuss the reasons for studying metabolism in the face of our current knowledge of the effect that the culture environment on the developing embryo and the downstream effects that can cause. The study of *in vitro* embryo metabolism can also give us insight into developmental perturbations *in vivo*. The strengths and limitations of the methods we use to study metabolism are reviewed with reference to species-specific fundamental biology and plasticity and we discuss what the future holds for metabolic studies and the unanswered questions that remain.

Keywords: ATP generation, method evaluation, preimplantation development.

Introduction

The study of mammalian early embryo metabolism has a rich history (Leese, 2012). Whilst work in the period of the 1940s-1960s focused on the effect of adding energy substrates to embryos in culture, real progress in understanding embryo metabolism was made in the 1970s by the likes of Biggers and Stern (1973), Brinster (1973) and Gwatkin and Haidri (1974) who examined the fate of radiolabeled compounds added to the medium. From experiments such as these, a picture of early embryo metabolism began to emerge. Like so much of our knowledge of early mammalian embryo development, the first data came from the classical laboratory model species; mouse and rabbit, as well as the hamster. Interest grew, and embryo metabolism was soon examined in the large domestic animals; pigs, cattle, sheep and, to a lesser extent, the horse, dog and cat. Underpinning research were studies on early human development with the aim of clinical translation for the treatment of infertility; a feat first achieved in 1978 by Steptoe and Edwards. Alongside this feat was the development of assisted conception techniques for use in farm animals. It is not the intention of this article to re-describe the history of the research that lead to successful embryo culture or the contribution that studies on metabolism made. For expert insight, the reader is encouraged to read (Leese, 2012; Chronopoulou and Harper, 2014).

Embryo metabolism: what do we know?

The description of carbohydrate metabolism during preimplantation development is largely accepted and will be familiar to anyone who has an interest in the early embryo. In almost all species studied, the cleavage stage embryo, from fertilisation through to formation of the morula, is relatively metabolically quiescent. Oxygen consumption at this time remains comparatively low, and the dominant substrate depleted from the culture environment is pyruvate. Pyruvate is consumed at an almost steady rate during cleavage, with a proportion of the carbon (depending on the species) appearing in the medium as lactate with the generation of metabolic energy. The source of the pyruvate involved in such reactions is generally either glycolytic conversion of glucose or that taken up directly from the external environment. Pyruvate may also enter the Tricarboxylic Acid (TCA; Krebs) cycle, where it can be oxidised completely generating electron donors for the electron transport chain which occurs in the matrix of mitochondria and relies on oxygen acting as the terminal electron acceptor. For this reason, oxygen consumption provides a good marker of overall oxidative metabolic activity (for review, see Smith and Sturmey, 2013).

As the cleavage stage embryo progresses to a blastocyst, there is a sharp and characteristic rise in the amount of glucose consumed in all species studied, and a concomitant rise in lactate release into the medium. Coincident with this is a fall in pyruvate consumption. This general pattern of "blastocyst glycolysis" appears to be conserved across all species studied. There are a range of explanations for this, however, as glycolysis is a comparatively inefficient means of generating ATP therefore energy production is unlikely to be the prime reason. Moreover, as the blastocyst forms, oxygen consumption also rises (Fridhandler *et al.,* 1957; Houghton *et al.,* 1996; Thompson *et al.,* 1996; Trimarchi *et al.*, 2000; Sturmey and Leese, 2003)

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further supporting the notion that glycolytic production of lactate is of minor consequence in contributing ATP for the blastocyst. It is much more likely that glycolysis rises to meet the need for carbon for biosynthetic processes. A description of glycolysis in the early embryo can be found in Smith and Sturmey (2013). This general picture of embryo metabolism was summarized with great prescience by Brinster in 1973; in the intervening years many laboratories across the world have generated evidence to support such a description, illustrating the robustness with which these findings can be considered.

While early work focused on carbohydrate metabolism, it is now clear that the metabolism of amino acids, lipids and vitamins such as folate all also act in an interdependent manner to produce a viable embryo. Amino acids are crucial components of the culture environment *in vitro* (reviewed by Sturmey *et al.,* 2010). Their addition to simple culture medium either singly (Rieger *et al.,* 1992a) or in combinations (Chatot *et al.,* 1989; Gardner and Lane, 1993) permitted mouse embryos to be cultured past the so-called 2-cell block (Chatot *et al.,* 1989) and their widespread inclusion lead to improved blastocyst rates in almost every species studied. The addition of amino acids has had such a positive effect on the efficacy of *in vitro* embryo culture, that their inclusion is often described as having a primary role in the formulation of "next generation medium" (Leese, 2012). The precise mechanism for the positive effect of amino acid provision is still to be defined, however it is well established that addition of amino acids to *in vitro* medium can alleviate culture associated stress in flushed murine embryos (Lane and Gardner, 1998). The contribution that amino acid metabolism makes to ATP production remains unclear, however the turnover of amino acids (that is, the sum of their depletion or accumulation into the culture droplet) has been linked to embryo blastocyst rates (Houghton *et al*., 2002), human embryo live birth rates (Brison *et al.,* 2004), DNA damage (Sturmey, 2009), aneuploidy (Picton *et al.,* 2010) embryo sex (Sturmey *et al.,* 2009a), maternal age (Picton *et al.,* 2010) and embryonic stress (Wale and Gardner, 2012).

When considering energy metabolism of early embryos, it is vital that the contribution made by endogenous triglyceride is not overlooked. Fatty acid β-oxidation was studied in detail in the 1970s by Kane and colleagues (Kane, 1979) but then largely ignored, with the notable exception of the work by Downs (see Downs, 2015). However, interest in fatty acid metabolism has re-awakened, partly in response to the report from Dunning *et al.* (2010) who elegantly demonstrated that mouse oocytes require fatty acid oxidation in order to develop. A similar conclusion was drawn by Sturmey and Leese (2003) in the pig, underlining the importance of fatty acid β-oxidation during oocyte maturation, development and in the preimplantation stages. Species differences in the importance of fatty acid oxidation during oocyte and embryo development have also been identified. For example, where a mouse zygote will arrest after 15 h in media lacking nutrients (cited in Leese, 2012) a rabbit embryo can complete up to 3 cleavage divisions in the absence of energy substrates (Kane, 1987) and sheep embryos can also develop to the blastocyst stage in the absence of glucose (Thompson *et al.,* 1992). This can be explained by the differences in intracellular triglyceride content, acting in a buffering capacity by providing an alternate energy source (Ferguson and Leese, 2006; Sturmey *et al.,* 2009b). Recently, a number of laboratories have described altered fatty acid metabolism by embryos from overweight and obese mice (Pantasri *et al.,* 2015; Reynolds *et al.,* 2015) and the human (Leary *et al.,* 2014). After receiving comparatively little attention since the work of Kane, interest in fatty acid metabolism by oocytes and embryos has been intense, and has been widely reviewed in recent years (Sturmey *et al.,* 2009b; Leroy *et al.,* 2012; McKeegan and Sturmey, 2011; Dunning *et al.,* 2014; Downs, 2015).

This very brief overview is intended to remind the reader of the basic features of early embryo energy metabolism. However, 'metabolism' refers to significantly more functions than ATP generation. For example, there is an extensive literature describing the role of the pentose phosphate pathway (Downs *et al.,* 1998; Sutton-McDowall *et al.,* 2010) in mammalian oocytes and early embryos. Moreover, metabolic processes link to signaling mechanisms (Manser and Houghton, 2006), generation of Reactive Oxygen Species (Agarwal *et al.,* 2005) and gene expression in terms of establishment of epigenetic marks such as methylation and acetylation and post-translational modifications of proteins (DeBerardinis and Thompson, 2012). For example, defects in folate metabolism have been linked to methylation and epigenetic modifications affecting developmental competence (Xu and Sinclair, 2015). However, reviewing all of the literature on embryo metabolism in its broadest sense would require several articles and so in the remainder of this article, we will consider some more fundamental aspects.

Why do we study embryo metabolism?

Understanding the basic physiology and metabolism of the early embryo is a noble quest in itself that has fascinated researchers over the past decades. However, a major gap in our knowledge is the metabolism of the *in vivo* produced embryo, as well as the embryo *in situ*, which remain an elusive goal. We aim to gain information that can, and has been, translated into clinical practice in many ways; to design appropriate species specific culture media with the aim of producing viable healthy offspring; to design non-invasive methods for embryo selection for transfer and

shed light on metabolic perturbations occurring *in vivo*. Moreover, as our understanding of somatic cell nuclear transfer (SCNT; Wilmut *et al.,* 2002) grows and becomes linked inextricably to stem cell physiology and regenerative medicine, we must also accept that we know comparatively little about the impact of such techniques may have on embryo physiology. Furthermore, we are on the brink of many new and exciting developments in Assisted Conception, including mitochondrial transfer for the treatment of debilitating hereditary conditions as well as the replenishment of mitochondria in aged oocytes with the aim of improving pregnancy rates in older women (Craven *et al.,* 2010; Smeets, 2013). Such techniques may be considered 'beyond experimental'; mitochondrial transfer was licensed for treatment in the UK in 2014 and autologous mitochondrial transfer for infertility is already commercially available in some countries. However, since each of the approaches described above involve, in some way, altering the mitochondrial content of embryos, the need for detailed understanding of metabolic regulation of individual preimplantation mammalian embryo has never been greater.

A further drive to study embryo metabolism comes from the need to identify biomarkers of embryo health and viability. This relies on the inherent variability in metabolism between different embryos and has been used in an attempt to select viable embryos for transfer, with the end goal being clinical IVF in humans. There have been several observations that have yielded promising results. The 'quiet embryo hypothesis' proposed by Leese in 2002, stated that those embryos that are viable have a decreased metabolic rate; a proposition that has been supported by several studies showing embryos with an upregulated metabolism of both carbohydrates and amino acids to have decreased viability post transfer (Lane and Gardner, 1996; Sturmey *et al.,* 2009a; Guerif *et al.,* 2013). However, the notion is contested, and there are recent studies suggesting that elevated metabolism, particularly with respect to glucose consumption is associated with embryo viability (Gardner *et al.,* 2011). Clearly, this is an area in which more work is needed.

Since pioneering observations linking human birth weight to cardiovascular events in later life by David Barker *et al*. (1989) it has now been shown unequivocally in many species that the periconceptual environment can have downstream effects which can impact on the viability of the developing embryo and on the future health of the resulting offspring (Ceelen *et al.,* 2008; Watkins *et al.,* 2008; Leroy *et al.,* 2009; Fleming *et al.,* 2012; Frank *et al.,* 2014). It is also clear that certain embryonic stages are more susceptible to damage (Rieger *et al.*, 1992a), such as the early cleavage embryo during embryonic genome activation, suggesting that progeny may have a 'memory' of their origins.

With the rising obesity epidemic both in humans and companion animals, in addition to metabolic disease in farm animal species due to increased production pressures, the study of embryo metabolism *in vitro* can provide insight into the mechanisms of resultant suppressed fertility and potentially identify therapeutic interventions.

These are important reasons for studying embryo metabolism, and it is clear that metabolic processes can directly influence gene expression (Van Hoeck *et al.,* 2011, 2013), and patterning of the embryo (Leary *et al.,* 2014). However, it is also of fundamental importance to be aware of what is measured when studying embryo metabolism. In the final part of this review, we will describe the strengths and limitations of embryo metabolic studies.

What are we actually measuring?

The measurement of embryo metabolism is faced with many technical challenges. Critically, the *in vivo* environment is still largely unknown for most species, meaning that the extrapolation of knowledge to an embryo *in vivo* is of questionable validity. The data available on embryo metabolism inform us of the strategy of substrate depletion and appearance in a given milieu. *In vitro*, this milieu is constrained by the addition of a limited number of substrates at static levels; supply and ratio of substrates varies only in response to an embryo's own activity. This is in stark contrast to the situation *in vivo*, which is dynamic and responsive (Leese *et al.,* 2008). Even in species for which the *in vivo* embryo environment has been described, the method used to define it should be noted. Often *post mortem* changes and/or inflammatory changes due to catheterization can influence results thus making samples non-representative (Leese *et al.,* 2008). Moreover, the embryo *in situ* likely exists in a microenvironment within the oviduct, thus any subtle, specific composition features will be lost in flushing of the tube.

Given the heterogeneity in developmental potential, measures pertaining to single embryos are key and thus highly sensitive assays are needed. Both the use of radiolabelled substrates (Rieger *et al.,* 1992b) and enzyme-linked fluorescence assays to detect the appearance and disappearance of a substrate from culture media have been described (Leese and Barton, 1984; Guerif *et al.,* 2013). The relative metabolic quiescence of single embryos means that 'analysis media' (that is a medium in which the concentrations of substrates is reduced to enable measurement of change) is often used in order to permit detection of changes in substrate concentration (Hardy *et al.,* 1989; Sturmey and Leese, 2003). This 'analysis medium' is often different to the *in vitro* culture media known to support development for most species, which, in turn differs vastly to the *in vivo* environment. Of course, it also must

be realized that there are many complex cell transport and metabolic pathways involved, and notions of influx and efflux leads' us to make what are essentially educated guesses about what occurs in the cell. Despite these limitations, these assays have greatly advanced our knowledge of metabolic pathways involved and have yielded highly repeatable results across different laboratories. Further methods that have been used to detect metabolic activity of embryos include culturing individually in micro-droplets or in large groups of embryos. However, the resolution of data from group culture is reduced since individual embryo heterogeneity is lost by 'averaging'.

New promising studies using NMR metabolomic technology, where substrate flux can be measured *in situ* have been recently described (Krisher *et al.,* 2015), however the subsequent interpretation and analysis of the complex data acquired presents new challenges.

Inferences about the contribution of oxidative metabolism are usually derived from measuring oxygen consumption. Methods vary, the most widely used being pyrene fluorescence (Houghton *et al.,* 1996) and nanorespirometry (Lopes *et al.,* 2010). Again while allowing accurate measurement of oxygen depletion in single embryos and seemingly not affecting development (Lopes *et al.,* 2005), the methods represents a significant 'alien' environment for the embryo.

Studies involving metabolic inhibitors and enzymatic co-factors have also added to our knowledge of embryo metabolism and in some cases provided the initial proof of certain pathways occurring and either being essential or non-essential for development. Among these, Brison and Leese (1994) showed that oxidative phosphorylation was not an absolute requirement for blastocoele formation in the rat by culturing embryos in the presence of cyanide, while Macháty *et al*. (2001) indicated that suppression of oxidative phosphorylation at the morula stage improved development to the blastocyst in the pig. Moreover, Dunning *et al.* (2010) have shown that β-oxidation is essential for optimal development in the mouse by culturing in the presence of etomoxir. In some cases, inhibition of certain metabolic pathways has been shown to improve developmental potential; for example the addition of EDTA to embryo culture medium (Gardner *et al.,* 2000). Although the mechanism is not confirmed, one possible role of EDTA in embryo culture medium is the suppression of glycolysis (Gardner *et al.,* 2000). However, it is equally likely that EDTA acts as an antioxidant by sequesteration of metal ions which would otherwise catalyse the formation of Reactive Oxygen Species (Orsi and Leese, 2001). Studies such as these illustrate the importance of appropriate regulation of metabolic pathways during development and also indicate why it is necessary for pathways to be correctly orchestrated to match needs at a given stage of development.

It all depends on the environment

It could be argued that measuring embryo metabolism *in vitro* (by necessity) amounts to measuring a stress response. This issue must be considered given the extremely adaptable nature of embryos of all species. Metabolism is necessarily dynamic, enabling rapid changes in needs to be met to maintain development. However, such dynamism means that the metabolic profile of an embryo can respond quickly in response to a change in external environment, shown clearly in mice, where perturbations occur within 3 h of *in vitro* culture in flushed *in vivo* blastocysts (Lane and Gardner, 1998). Both the presence and relative quantities of metabolic substrates in the environment in which experiments are conducted will significantly affect the results. While not attempting to provide a detailed discussion on the controversial aspects of *in vitro* culture systems, which still vary widely across laboratories, this point can be further illustrated by the differential metabolism that results from the presence or absence of serum and the atmospheric oxygen concentration (Wale and Gardner, 2010).

While the human IVF industry has moved towards defined culture media using macromolecular sources such as recombinant albumin, serum is still used in many production animal systems. Culture with serum has been shown to increase blastocyst development rates in the horse (Choi *et al.,* 2004) and the kinetics of blastocyst development in the cow (Rizos *et al.,* 2003). However, its presence has also been associated with increased intracellular lipid content (Ferguson and Leese, 2006) and altered metabolism (Reis *et al.,* 2003), up-regulation of oxidative stress and inflammatory pathways (Cagnone and Sirard, 2014) and decreased survival after vitrification (Gómez *et al.,* 2008). In addition, the oxygen tension of the reproductive tract in all species studied has been found to be below 10% (Fischer and Bavister, 1993). In terms of the environmental gas profile, there is now unequivocal evidence to support the notion that 20% oxygen reduces embryo development (Thompson *et al.,* 1990; Wale and Gardner, 2010) and that culture in low oxygen (5%) results in metabolic and proteomic profiles more closely matching *in vivo* counterparts (Thompson *et al.,* 1990; Katz-Jaffe *et al.,* 2005). Clearly, these factors will influence the results of any metabolic study and must be kept in mind when comparing studies.

In addition to the embryo adapting to its environment, the culture environment itself is not static. Depletion and accumulation of excreted of substrates such as lactate and amino acids will change the local environment. Spontaneous de-amination will occur at 37°C, especially of glutamine, resulting in ammonium build up (Gardner and Lane, 1993), lactate build up may

overwhelm pH buffering system of the media and depletion of energy substrates can lead to alternative ATP generating pathways being used (Kane, 1987).

It is also important to note that the manner in which an embryo responds to its environment is species specific. This can be seen in differences in response to hyperglycaemia. While species such as rodents and humans, will have significant diminished development in the presence of high glucose (Moley *et al.,* 1998; Frank *et al.,* 2014), others such as the horse and pig are apparently unaffected (Sturmey and Leese, 2003; Choi *et al.,* 2015). Qualitative testing of equine embryos produced in hyperglycaemic conditions however, highlights subtle differences not reflected in the blastocyst development rate such as a decrease in ICM cell number allocation (also observed in the rat) and known to be mediated through apoptosis (Moley *et al.,* 1998; Choi *et al.,* 2015).

It is thus vital to consider that studies on embryo metabolism provide us a snapshot of physiology in a given set of conditions. Whilst such data are of fundamental importance, care must be taken when extrapolating and comparing such information. It is thus much more desirable that studies on the depletion and appearance of embryo metabolism are reinforced by consideration of mechanisms of metabolic regulation of early development.

Embryo metabolism: some unanswered questions

As the emphasis in human IVF is increasingly on single embryo transfer, the identification of reliable non-invasive methods of determining embryo quality to maximize pregnancy rate per transfer remains the Holy Grail. Moreover, in species such as the horse where *in vitro* embryo production is rapidly generating interest, a specific tailored culture media has yet to be formulated. Whilst acceptable blastocyst rates (41%) and pregnancy rates after transfer (66%) can be achieved by some laboratories in the horse using cell culture media such as DMEM-F12, (Jacobson *et al.,* 2010; Hinrichs *et al.,* 2014) the more subtle effects of potentially inappropriate culture conditions leading to decreased viability remain to be seen. Identifying optimal species-specific culture systems presents an exciting challenge for those involved in studying embryo metabolism.

Sex selection is another lively area of embryo metabolism. Ethical considerations preclude the implementation of sex selection in the human, but in the production animal industry, and in dairy cattle in particular, appropriate non-invasive identification of sex before transfer would be an application with many uses. Promising results have been presented so far showing that both glucose metabolism and amino acid metabolism varies with sex (Sturmey *et al.,* 2010; for review see Gardner *et al.,* 2010), however more work will need performed to increase specificity in order for the technology to make the transition to commercial practice.

New information is emerging all the time on the far-reaching downstream effects of aberrations in early embryo metabolism (Harrison and Langley-Evans, 2009). Given the clear links between the periconceptual environment and sub-optimal health outcomes in the human (Barker *et al.,* 2002) and production species such as the bovine (for example, the so-called Large Offspring Syndrome; Young *et al.,* 1998), understanding and attempting to mitigate the negative effects on suboptimal embryo development and life-long health of the offspring is an important area for future study (Leese, 2014).

Conclusions

It is acknowledged"that metabolism pervades every aspect of cell physiology" (DeBerardinis and Thompson, 2012) and this is especially pertinent to the developmentally plastic early mammalian embryo. As genomic, transcriptomic and imaging techniques advance we will be able to expand our understanding of embryo metabolism and how it links inextricably with developmental pathways through subsequent stages of gestation leading to the birth of a healthy offspring. It is the responsibility of us all working in the earliest stages of this process to understand the periconceptual environmental challenges faced by the embryo and to optimize the conditions under which it is grown to ensure the best start in life. Metabolic studies allow us to gain vital information on the requirements of a competent embryo and identify when things go wrong, but the reader is cautioned towards careful interpretation of measures of metabolism especially between laboratories and to consider the environment as a whole under which they have been taken.

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