REPRODUCTION
REVIEW

Mitochondria and human preimplantation embryo development

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Abstract

Human reproduction, like all biological systems, is characterised by a large level of variability. In this field, the variability is observed as a large difference in implantation potential of human embryos developing *in vitro*, despite similarities in observable parameters such as rate of development and morphology of these embryos. One of the underlying factors that determines developmental potential in these embryos is the availability of energy in the form of ATP for development. Here, we suggest that, despite the evidence suggesting that mitochondrial metabolism is relatively inactive during preimplantation embryo development, aerobic (mitochondrial) metabolism contributes a major role in the supply of ATP. A second pathway, anaerobic respiration, is also active and the two pathways work in synchrony to supply all the ATP necessary. We discuss the differences in the two forms of energy production and suggest that, although anaerobic respiration can supplement deficiencies in the energy supply in the short term, this is not sufficient to substitute for aerobic respiration over long periods. Therefore, we suggest that deficiencies in the levels of aerobic respiration can explain variability in the implantation potential of apparently equivalent embryos.

Reproduction (2009) **137** 619–624

Introduction

After the process of fertilisation, the development of a human embryo with a high potential to implant and form a viable foetus depends on several factors. Visible factors include the rate of cell division, fragmentation of blastomeres and the correct formation of the morula and blastocyst. Underlying these events is a complex series of processes involving the quality of the cytoplasm, levels of stored molecules and development of the cytoskeleton. Research has elucidated many of the mechanisms that assist in the formation of an embryo and foetus. However, still not entirely clear are the reasons why apparently equivalent embryos have drastically diverse developmental fates.

The human ovary is designed to produce a single oocyte per menstrual cycle. However, artificial stimulation of the human ovary, such as that which occurs in assisted reproduction, can cause the formation of multiple oocytes. Interestingly, in these cases, not all oocytes produced have the same developmental fate despite being produced at the same period of time and hence under similar conditions. When exposed to spermatozoa, the fertilisation rate of mature oocytes from a single cohort varies between 40 and 100% with an average fertilisation rate of 60–70% being achieved (CDC 2001, http://www.cdc.gov/ART/ART01/index.htm; HFEA 2001, http://www.hfea.gov.uk/en/406.html; FIVNAT-CH 2002, http://www.sgrm.org/wb/pages/fivnat/statistiken.php). These data already suggest that not all oocytes are of the same quality. Further evidence comes from developmental data. In fact, in a cohort of fertilised oocytes from the same stimulation cycle, the rate of development varies drastically. Many embryos arrest development before implantation. Usually, only 10–20% of embryos transferred into the human uterus implant to form a viable foetus (CDC 2001, http://www.cdc.gov/ART/ART01/index.htm; HFEA 2001, http://www.hfea.gov.uk/en/406.html; FIVNAT-CH 2002, http://www.sgrm.org/wb/pages/fivnat/statistiken.php), again suggesting that human embryo development is a vastly variable process despite the similarities in the environment to which a single cohort is exposed at the same period of time.

What causes this variability?

The variability of human oocytes and developing embryos probably forms part of the process of natural selection in that many oocytes are formed but only a few can reach the stage of producing a viable foetus. However, for the reproductive physiologist, this explanation is not sufficient and we would like to understand further the factors determining oocyte quality. Although

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/REP-08-0444
Online version via www.reproduction-online.org

many factors – both 'programmed' within the oocyte during oogenesis and casually occurring during development – can affect the viability of a single oocyte, one of the major factors underlying the development of a human embryo appears to be in the quality of the oocyte cytoplasm. One of the organelles present in major abundance in the oocyte cytoplasm is the mitochondria, and these have the vital role of producing energy through the formation of ATP. Since embryo development requires a huge amount of energy, the data suggest that the quality of mitochondria strongly influences the developmental potential of the oocyte.

Pathways to energy creation in mammalian cells

In all eukaryotic cells, energy in the form of ATP is created through two pathways (Fig. 1). In the first, glucose is metabolised to pyruvate, which can then be converted to lactic acid. The energy created by this mechanism does not involve oxygen (hence the term anaerobic respiration) and produces a low amount of energy due to the incomplete metabolism of glucose ($\Delta G = -47$ kcal/mol glucose). Anaerobic respiration is cytoplasmic and lactic acid is expelled from the cell in order to maintain the cytoplasmic pH. Lactic acid can also be reconverted to pyruvate through lactate

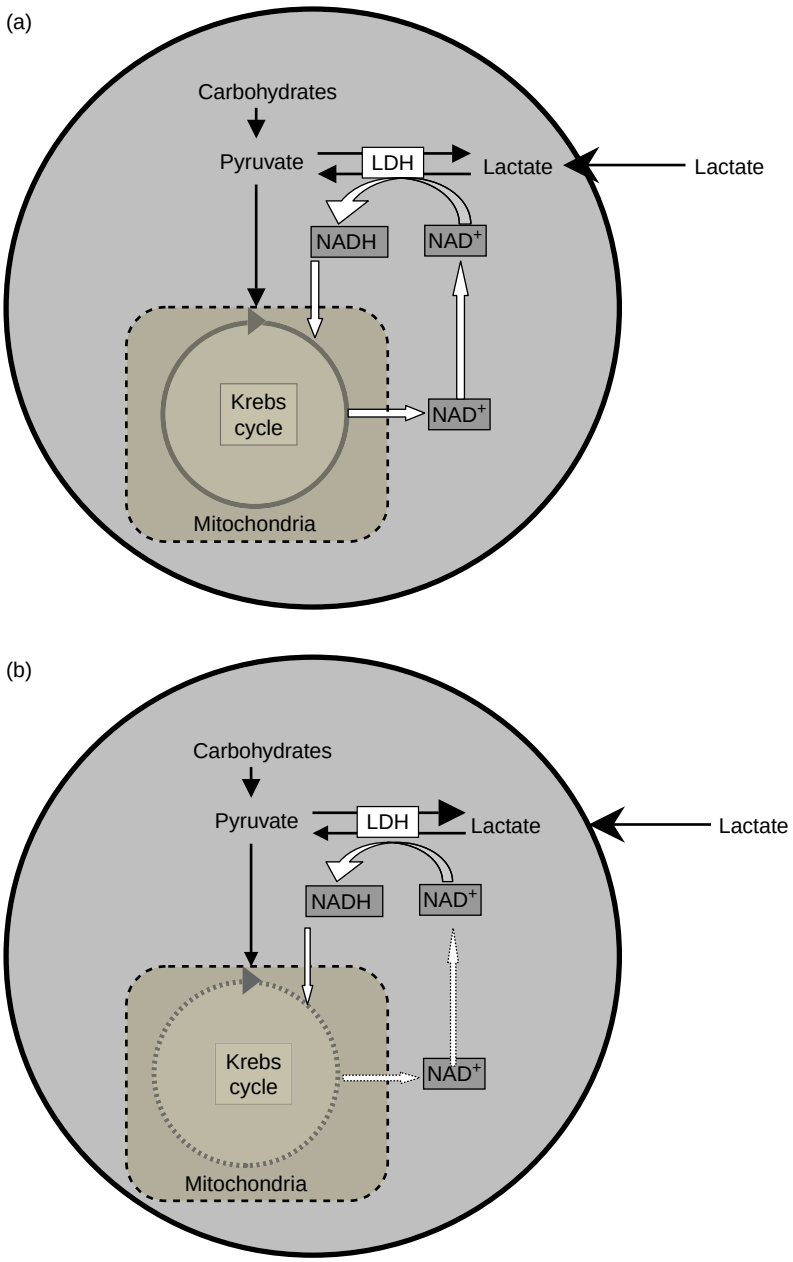

Figure 1 Association between aerobic and anaerobic respiration. The figure is representative of the metabolic state of a mammalian oocyte in two metabolic situations. (a) Normal metabolism including both aerobic and anaerobic components and (b) severe repression of aerobic repression. As demonstrated in the figures, lactic acid, present in the external medium, enters the oocyte and is also produced by anaerobic respiration in the oocyte cytoplasm. Conversion of lactate to pyruvate requires the cofactor NAD^+, which is reduced to NADH and then reoxidised to NAD^+ during Krebs cycle (Newsholme & Leech 1983). In the normal situation (a), NADH is adequately reoxidised and both aerobic and anaerobic respiration can continue. When aerobic respiration is repressed (panel b, which could represent oocytes from females of advanced age), two imbalances occur. First, excess lactic acid is produced, and leads to build-up of this product in the oocyte cytoplasm. Secondly, insufficient reoxidisation of NADH occurs. We hypothesise that this leads in the long term to the rundown of NAD^+ and consequently causes ATP insufficiency.

dehydrogenase, creating an equilibrium reaction. In consequence, if lactic acid builds up, for example when levels of anaerobic respiration are high, the mechanism will slow down. The second pathway to ATP formation, aerobic respiration, in fact starts again with the conversion of glucose to pyruvate in the cytoplasm. However, in this case, pyruvate enters the inner membrane of the mitochondria where it is completely metabolised to carbon dioxide and water. The energy released from this process ($\Delta G = -686$ kcal/mol glucose) is harnessed in a cyclical reaction called the Krebs' cycle and ATP is formed (Krebs 1965, Van Blerkom *et al*. 1997, Van Blerkom 2000, Liu *et al*. 2000, Pozzan *et al*. 2000, Mohamad *et al*. 2005, Wilding *et al*. 2005; Fig. 1). The end product of aerobic respiration is the complete breakdown of glucose to water and carbon dioxide. Because these products are easily eliminated from the mitochondria no end-product inhibition can occur, and the rate of aerobic respiration depends simply on the supply of oxygen and carbohydrate substrate. Contrary to popular belief, aerobic and anaerobic respiration are not mutually exclusive and, at any one time, a proportion of both aerobic and anaerobic metabolism contributes to the energy requirement of the mammalian cell.

Mitochondria

Mitochondria are membrane-bound intracellular organelles consisting of a permeable outer mitochondrial membrane through which molecules up to 10 kDa can pass and an impermeable inner (matrix) membrane that contains all enzymes involved in the respiratory chain. The energy produced through the respiratory chain is converted to ATP production through an indirect pathway. In fact, the respiratory chain produces H^+ ions that are expelled from the matrix. This process creates an electrophysiologically negatively charged environment within the matrix, causing the net attraction of the expelled H^+ ions towards the mitochondrial matrix. The H^+ ions return to the matrix through an ATPase, hence linking ATP production to the activity of the respiratory chain. Therefore, according to Mitchell's chemiosmotic hypothesis (Mitchell & Moyle 1967, Mitchell 1979), the H^+ potential ($\Delta\Psi$) of the inner mitochondrial membrane is highly correlated with the capacity of ATP production within individual mitochondria. Mitochondria are thought to be evolved from a bacterial endosymbiont that was established at an early phase in eukaryotic cell development (Gray *et al*. 1999). In fact, mitochondria have many similarities with α-proteobacteria (Gray *et al*. 1999). Mitochondria also contain unique DNA (mtDNA) that codes for many of the enzymes involved in the respiratory chain (Anderson *et al*. 1981). Other enzymes are coded within nuclear DNA and it is thought that an evolutionary process to transpose mtDNA into the nucleus is in place. The current consensus is that one copy of mtDNA is present for every single mitochondrion and therefore provides a useful means of estimating the density of mitochondria within cells (Cummins 2002). Because of the unique position of mtDNA within the mitochondrial matrix, mtDNA is unfortunately subject to degeneration from the free radicals produced within the mitochondrial matrix during oxidative phosphorylation (Wallace 1987, Tarin 1995). This process is exacerbated because mtDNA has little in the way of DNA repair or protection mechanisms (Linnane *et al*. 1989, 1992). These data suggest that the production of some of the proteins involved in the respiratory chain will be subject to damage through free radical attack, and over long time scales the physiology of mitochondria could result in a breakdown in the efficiency of the respiratory pathway (Harman 1972, Van Blerkom 2004, Wilding *et al*. 2005).

Mitochondria in oocytes and embryos

The selection of mitochondria for transmission to offspring appears to occur through a genetic 'bottleneck' (Hauswirth & Laipis 1982, Jansen 2000, Shoubridge & Wai 2007, Wai *et al*. 2008). This is thought to occur during post-natal folliculogenesis, or during the formation of primordial oocytes (Hauswirth & Laipis 1982, Jansen 2000, Wai *et al*. 2008). Theories suggest either that the effective number of mitochondria is reduced (Hauswirth & Laipis 1982, Jansen 2000, Shoubridge & Wai 2007, Wai *et al*. 2008) or that the number does not change, but the number of segregation units is small (Cao *et al*. 2007). Once oogenesis occurs, this small pool of mitochondria then forms the cytoplasmic pool of mitochondria in the mature oocyte (Hauswirth & Laipis 1982, Jansen 2000, Cao *et al*. 2007, Shoubridge & Wai 2007, Wai *et al*. 2008). Although this appears an elegant evolutionary mechanism to eliminate poor-quality mitochondria from being passed to offspring, the mechanism causes a certain amount of 'programming' of each individual oocyte dependent on the quality of the mitochondrial precursor, which may partly explain the differences between oocytes within a single cohort. In fact, since paternal (sperm-derived) mitochondria do not survive after fertilisation (Cummins 2000), the entire mitochondrial content of the developing foetus is maternal. By measuring the mtDNA content of individual oocytes, it has been estimated that mature oocytes contain between 20 000 and 800 000 mitochondria (Reynier *et al*. 2001, Van Blerkom 2004, Brenner *et al*. 2004, Aiken *et al*. 2008). Failures in mitochondrial replication during oogenesis may be responsible for the failure of mature oocytes to fertilise. In fact, unfertilised oocytes were found to contain significantly fewer copies of mtDNA than the range described above, suggesting that mtDNA copy number, and by supposition number of mitochondria, is indicative of fertilisation potential (Reynier

et al. 2001). The data suggest that the rate of mitochondrial replication as well as the activity of the mitochondria can influence the quality of individual oocytes.

Relationship between mitochondrial activity and human embryo development

Mitochondria are probably the major energy-producing systems in the eukaryotic cell. Although anaerobic respiration is also a major energy-creating system in these cells, the efficiency of aerobic (mitochondrial) respiration is ~14-fold greater per molecule of glucose. Therefore, anaerobic respiration would have to operate at an extremely high level of activity to exceed mitochondrial respiration in the production of ATP. This stated, there is still a high level of discussion as to whether the human oocyte and developing embryo rely on aerobic or anaerobic respiration prior to implantation.

The evidence for the use of aerobic respiration suggests that this mechanism is active through oogenesis, fertilisation and preimplantation development. During oogenesis, correlations between the state of perifollicular vascularity of developing follicles, the dissolved oxygen content of follicular fluid and the quality of oocytes retrieved from these follicles suggest that oxidative phosphorylation plays an important role in oogenesis (Van Blerkom *et al*. 1997, Van Blerkom 2000). After the LH surge, the available evidence also suggests a fundamental role of mitochondrial metabolism in the maturation of the formed oocyte. The rate of oxygen consumption, a measure of mitochondrial metabolism, increases dramatically in the isolated oocyte after the LH surge (see Wilding *et al*. 2005). Furthermore, although an increase in lactic acid is observed after the LH surge, suggesting anaerobic respiration (see Wilding *et al*. 2005), the use of iodoacetate to block anaerobic respiration does not inhibit oocyte maturation, suggesting that anaerobic respiration is not necessary for oocyte maturation (Tsafriri *et al*. 1976). By contrast, oocyte maturation is blocked when aerobic respiration is suppressed (see Wilding *et al*. 2005). However, it must be stated here that oogenesis is a multicellular process in which a large component of maturation is determined by the granulosa cells themselves and measurements of respiration in the forming oocyte are near impossible. Therefore, the extent of respiration within the oocyte itself is difficult to estimate and may anyway be irrelevant to oocyte maturation *per se*.

Once the mature oocyte is formed, the role of granulosa cells diminishes until these are detached from the oocyte after fertilisation. Therefore, measurements and analysis of the role of aerobic and anaerobic respiration in the zygote and developing embryo become possible. The role of aerobic respiration in human preimplantation embryo development, although clearly present, is controversial. Mitochondria within human oocytes and pre-blastocyst stage embryos have few matrix membrane foldings and appear relatively inactive (Bavister & Squirrell 2000, Motta *et al*. 2000). In fact, although oxygen is consumed during preimplantation embryo development (Houghton *et al*. 1996), estimates of the contribution of mitochondrial respiration to the energetic requirement of mammalian embryo development suggest that as little as 10% of glucose is metabolised through aerobic respiration in the early stages of development, although this rises to 85% in the blastocyst (Bavister & Squirrell 2000). However, since aerobic respiration is 14-fold more efficient than anaerobic respiration in theory, the 10% of glucose passing through aerobic respiration probably still produces more energy in the form of ATP than the 90% of total glucose metabolised without mitochondria. Mammalian oocytes and preimplantation embryos prefer pyruvate as an energy source (Butcher *et al*. 1998, Bavister & Squirrell 2000). The preference for pyruvate, however, eliminates neither aerobic nor anaerobic respiration as a possible route to ATP production. Lactic acid is present in fluid sampled from human tubules (Beier 1974), and is also produced during mammalian embryo development (Bavister & Squirrell 2000), suggesting a component of anaerobic respiration in the ATP-generating mechanism. Aerobic respiration appears to be upregulated at the blastocyst stage of development because the mitochondrial cristae become more compact, mitochondrial replication is initiated and the utilisation of glucose as a carbohydrate substrate increases at this stage (Houghton & Leese 2004, Aiken *et al*. 2008). These data suggest that both aerobic and anaerobic respiration pathways are active during both oocyte maturation and embryo preimplantation development, but aerobic respiration is upregulated during blastocyst development and implantation. Elimination of pyruvate drastically reduces the rate of development in human embryos, whereas elimination of glucose and lactate had little effect (Wilding *et al*. 2002). Evidence from mice also suggests that embryos can develop and even implant adequately in the absence of mitochondrial respiration, although development does not continue until birth (Piko & Chase 1973, Larsson *et al*. 1998, Li *et al*. 2000). These data suggest that, although aerobic respiration is not necessary for embryo development, anaerobic respiration is not sufficient, again suggesting that both pathways are required for optimal development (see Wilding *et al*. 2005).

Can the respiratory theory explain oocyte variability and the 'maternal age' effect in humans?

The above data then suggest that aerobic and anaerobic respiration are neither necessary or sufficient alone for optimal embryo development, implantation and birth. Our current hypothesis is that both mechanisms work in

synchrony to guarantee the level of ATP required for embryo development, implantation and birth. We (Wilding *et al*. 2005; Fig. 1) suggest that the interaction between aerobic and anaerobic respiration to provide the optimal ATP level for development explains why embryos can develop in a wide range of culture systems, and may help to explain the 'maternal age' effect in human reproduction.

The above data then suggest that aerobic respiration is a constant during human embryo development and that excesses in the requirement for energy are met through anaerobic mechanisms. This theory then may help to explain both why individual oocytes are variable in terms of developmental potential and why the developmental potential of oocytes from an individual changes over time. Individual oocytes have an individual mitochondrial content since this is determined at the time of the mitochondrial 'bottleneck' that selects the future pool of mitochondria in the oocyte. Although the 'bottleneck' is designed to eliminate poor-quality mitochondria, individual oocytes probably still have widely variable content in terms of mitochondrial quality. Although anaerobic respiration can of course supplement any deficits in the energy supply, this is probably not sufficient in all cases, especially since anaerobic respiration has its own limit through end-product inhibition. In fact, measurements of individual oocytes from a cohort demonstrate variability in the ATP content (Van Blerkom *et al*. 1995), suggesting that differences in oocytes exposed to similar conditions do occur. Therefore, where mitochondrial respiration is insufficient, the oocytes' implantation potential is low or zero even though optimal development may appear to occur for a period.

Apart from this variability in the quality of individual human oocytes at a single point in time, variations in the quality of human oocytes occur also in correlation with the age of the individual. The initial mechanism is the same in that mitochondria in human oocytes replicate from a small initial pool of organelles residing in primordial oocytes created during foetal development, and at birth the entire pool of human oocytes is already formed. Therefore, it follows that the age of the oocytes is equal to the age of the female subject. The mitochondria in primordial oocytes are therefore subject to the degeneration of the mtDNA through free radical attack, and although this continues at a low level over 40 years the small amount of degenerative activity could cause a detrimental effect. We therefore suggest that the reduction in fertility associated with advanced maternal age is caused by the slow loss in the capacity of these organelles to produce a viable mitochondrial content (Wilding *et al*. 2005). Some molecular data supports this hypothesis, although a precise relationship is yet to be defined (Barritt *et al*. 1999, Seifer *et al*. 2002). However, physiological data have suggested a relationship between mitochondrial activity and maternal age. Recent data have suggested that the level of aerobic respiration may be directly correlated with maternal age and strongly influence embryo quality. Mitochondrial activity decreases linearly with increasing age and influences both embryo development and IVF outcome (Wilding *et al*. 2001, 2003, 2005, Van Blerkom 2004). In the human, the elimination of energy substrates in the culture medium lowers the mitochondrial membrane potential and slows, but does not block development (Wilding *et al*. 2002). It must be assumed that the lack of aerobic respiration is compensated, at least in the short term, by anaerobic respiration, enabling sufficient energy production to permit the completion of the cell cycle. Our present hypothesis therefore suggests that aerobic and anaerobic respiration are coupled, and that a minimum of aerobic respiration is required to permit implantation, despite the fact that a level of embryo development can persist in the absence of aerobic respiration (Wilding *et al*. 2005).

Conclusions

Mitochondria are the powerhouse of eukaryotic cells, responsible for a large proportion of the production of energy in these cells. Mitochondria are also abundant in human and mammalian oocytes and preimplantation embryos. Despite this abundance, controversy exists as to the role of mitochondria in supplying energy for preimplantation embryo development, since these organelles appear to have little activity during this stage. We suggest that, despite the apparent lack of activity of mitochondria during preimplantation embryo development, aerobic respiration makes an important contribution to the supply of energy. Anaerobic respiration probably acts as the 'topping up' mechanism – complementing the energy supply where needed in the presence of a saturated aerobic pathway. We suggest that variability between individual oocytes and over time in oocytes from a single individual may be explained through the interaction between aerobic and anaerobic mechanisms. Our hypothesis is that anaerobic respiration can compensate in the short term for deficiencies in the supply of energy, but the properties of the pathway itself do not permit a long-term, gross compensation of the energy supply. Therefore, deficiencies in mitochondrial respiration may help explain why oocyte–oocyte variability occurs both within a time period and over long periods of time.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

Funding

This review did not receive any specific grant from any funding agency in the public, commercial or not-for-profit sector.

Acknowledgements

We thank the Fondazione Nuovi Orizzonti, Naples, Italy, and Vincenzo Monfrecola for their technical support.

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Received 22 October 2008
First decision 5 January 2009
Accepted 17 January 2009