Mitochondrial Bioenergetics in Slow and Fast Growing Preimplantation Bovine Embryos

By

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ABSTRACT

MITOCHONDRIAL BIOENERGETICS IN SLOW AND FAST GROWING PREIMPLANTATION BOVINE EMBRYOS

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University of Guelph, 2015

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The early embryo primarily depends on mitochondria for energy production. We have previously shown that metabolite levels differ in slow and fast embryos. Our goal therefore is to observe mitochondrial function in early embryos. Slow growing embryos stained with TMRM (mitochondrial membrane potential) and CM-H$_2$DCFDA (ROS) displayed higher fluorescence than fast growing embryos. This indicates that slow embryos may be associated with overcompensation in the OXPHOS pathway, which subsequently produces high levels of ROS. We used qPCR to determine the expression of GLYCOX (GAPDH, HK) and OXPHOS (ATP5b, COX5a) genes. Slow 8-cell embryos expressed higher levels of ATP5b and both slow morula and blastocyst embryos had high expression of GAPDH. Treatment with CoQ10 improved cleavage rate. In the presence of CoQ10, fast 8-cell embryos expressed lower GAPDH and slow 8-cell embryos expressed higher ATP5b. These results show a possible over compensation of slow embryos in response to impaired mitochondrial function.
DECLARATION OF WORK PERFORMED

I declare that all work presented in this thesis was performed by me with the exception of the procedures mentioned below.

Steven Huang, Heather Smale and Stephen Botha collected cattle ovaries. Elizabeth St. John prepared media used for in vitro production of bovine embryos. Edgardo Reyes and Allison MacKay were responsible for all ordering of materials.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ΔΨm</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted reproductive technology</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATP5b</td>
<td>ATP synthase subunit beta</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CM-H₂DCFDA</td>
<td>5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester</td>
</tr>
<tr>
<td>COC</td>
<td>Cumulus oocyte complex</td>
</tr>
<tr>
<td>COX5a</td>
<td>Cytochrome c oxidase subunit VA</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FG</td>
<td>Fast growing</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GLUT</td>
<td>Na⁺-independent glucose transporter glycoprotein</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>¹H NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>H⁺</td>
<td>Proton</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HPI</td>
<td>Hours post insemination</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IVC</td>
<td>In vitro culture</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
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<tr>
<td>IVM</td>
<td>In vitro maturation</td>
</tr>
<tr>
<td>IVP</td>
<td>In vitro produced</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5’,6,6’-tetrachloro-1,1’,3,3’-tetramethyl-benzimidazol carbocyanine iodide</td>
</tr>
<tr>
<td>MII</td>
<td>Metaphase II</td>
</tr>
<tr>
<td>MET</td>
<td>Maternal to embryonic transition</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td>NAD⁺/NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide</td>
</tr>
<tr>
<td>PB</td>
<td>Polar body</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PGD</td>
<td>Preimplantation genetic diagnosis</td>
</tr>
<tr>
<td>PGS</td>
<td>Preimplantation genetic screening</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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SG    Slow growing
TCA   Tricarboxylic acid
TMRM  tetramethyl rhodamine methyl ester
ZP    Zona pellucida
INTRODUCTION AND RATIONALE

In recent decades, the use of assisted reproductive technologies has increased dramatically both in humans and the livestock industry. Of the many technologies currently in use, one of the most common is *in vitro* fertilization. Unfortunately, the efficiency of IVF is quite low, where only 30-50% of embryos produced develop to the blastocyst stage (Bayer et al. 2011; Rizos et al. 2002). The early embryo requires a substantial amount of energy to develop properly and overcome events such as cell cleavage divisions, embryonic genome activation and formation of the blastocoel. In order to produce enough ATP the preimplantation embryo primarily uses the OXPHOS and GLYCOX pathways. Before the embryonic genome is activated, the embryo primarily utilizes the OXPHOS pathway for energy production and therefore the early embryo is heavily reliant on mitochondrial activity (Gardner et al. 1993; Javed and Wright 1991; Rieger et al. 1992b; Thompson 2000; Thompson et al. 1996). Recent studies have shown a link between mitochondrial dysfunction and embryo developmental potential (Thouas et al. 2004; Thouas, Trounson, and Jones 2006; Wang et al. 2009).

Our lab has previously shown that embryos developing at abnormally slow rates have high levels of pyruvate uptake at the 4-cell stage (Perkel 2015). This indicates that slow growing early embryos have insufficient energy levels. Slow growing embryos at the 16-cell stage extruded high levels of acetate compared to embryos that develop at a normal timeline. Acetyl-CoA is converted into acetate, and therefore the substrate acetyl-CoA is not likely entering the TCA cycle and instead being converted to acetate, resulting in lower ATP production. These results suggest that embryos with delayed development
have metabolic perturbations and may be encountering mitochondrial distress. For this reason it is imperative to observe the function of mitochondria in the early embryo to elucidate potential mechanisms for diminished \textit{in vitro} embryo development.
LITERATURE REVIEW

Bovine preimplantation embryo development

Preimplantation development comprises the preliminary stages of mammalian embryonic development from the formation of the zygote created when the sperm fertilizing the oocyte up until the development into the blastocyst stage. This period of growth lasts 4 days for mice (Fujimori 2010) and 7 days for both human and cattle (Leidenfrost et al. 2011; Niakan et al. 2012). The similar timelines of preimplantation development make cattle an ideal model for human studies. Furthermore, cattle and humans share several physiological similarities. Both cattle and human have similar lipid content and embryo metabolism (Ledda et al. 2001; Leese 2012) and the regulation of intrinsic maternal and paternal signal in the early embryo has been found to be parallel in both cattle and human models (Ménézo and Hérubel 2002).

After fertilization of the oocyte, the newly formed zygote divides sequentially forming a 2-, 4- and 8-cell embryo (Niakan et al. 2012). Transcription starts around the 8-cell stage when the embryonic genome is activated. When the embryo reaches 16-cells the individual cells are called blastomeres and they begin to adhere to each other in the process known as compaction; the embryo is called the morula (Niakan et al. 2012). As divisions continue, a fluid-filled space is created called the blastocoele (blastocyst cavity) as well as the formation of the inner cell mass (ICM). The surrounding cells create the trophectoderm and the overall embryo is called the blastocyst. This is the final stage of preimplantation development (Niakan et al. 2012) after which the embryo hatches from
the surrounding zona pellucida (ZP) to further develop. This period of development has been well documented both in vitro and in vivo in humans (Niakan et al. 2012) and cattle (Enright et al. 2000; Rizos et al. 2002; Tervit, Whittingham and Rowson 1972).

**Embryo quality assessment**

Since 1978, assisted reproductive technologies (ARTs) have helped families around the world who have experienced some form of reproductive challenge (CDC 2012). Although there are several forms of ARTs currently in use, 99% of procedures taking place in the United States are in vitro fertilization (IVF), of which 68% use intracytoplasmic sperm injection (ICSI) (CDC 2012). Among those seeking to receive treatment via IVF and ICSI, the matter of selecting the healthiest embryo for transfer becomes paramount. It is estimated that 27% of IVF live births are high order pregnancies, with the chances of twins/triplets increasing with two or more embryos transferred (CDC 2012). In Canada, 16.5% of IVF/ICSI live births are higher order pregnancies (CFAS 2012). High order multiple gestations are associated with pre-term deliveries and low birth weights in newborns, which can result in further health complications (Blondel et al. 2002). There is thus a need for embryo assessment when dealing with large numbers of embryos in attempts to strive for single embryo transfer and the ultimate goal is to reduce multiple gestations without affecting birth rates. In the livestock industry, there is also a need for improved embryo assessment. A declining trend has been observed in the fertility of cattle which requires an improvement in IVF efficiency (Royal et al. 2000; Silke et al. 2002).
Invasive techniques

Invasive techniques used to determine embryo developmental potential obtain specific genetic information about the developing embryo. Preimplantation genetic diagnosis (PGD) is a common technique where the ZP is opened using chemical, mechanical or contact laser methods (Gianaroli 2000). Cells are removed from embryos at the zygote, cleavage embryo or blastocyst stages where a polar body biopsy, blastomere biopsy and trophectoderm biopsy are carried out respectively (Sermon, Van Steirteghem, and Liebaers 2004; Spits and Sermon 2009). These biopsies are often used for aneuploidy screening and to determine monogenic disease status by use of polymerase chain reaction (PCR) and fluorescence in-situ hybridization (FISH) procedures (Sermon et al. 2004). Preimplantation genetic screening (PGS) along with PGD utilizes FISH and PCR to determine chromosome number. Unlike PGD, PGS is not used for diagnostics but just to identify aneuploidy. An alternative to FISH, comparative genomic hybridization (CGH) can be used to determine chromosome copy number, which overcome the technical limitations associated with FISH (Wells and Delhanty 2000).

One cell type typically removed for PGD is the polar body (PB). In the mammalian oocyte, PBs are extruded first during the maturation of the germinal vesicle to meiosis-II stage of the oocyte and second after fertilization. The embryo is capable of successful development without the presence of the first and second polar body, therefore their removal can be used for PGD. However, PBs only provide information pertaining to
the maternal contributions (Gianaroli 2000; Verlinksy et al. 1996). Blastomere biopsies can be carried out to provide information from both maternal and paternal sides.

Studies have shown that when performing biopsies at the appropriate stages with optimal techniques, there is no negative effect on the viability of the embryo in humans (Hardy et al. 1990), mouse (Depypere et al. 1991; Gordon and Gang 1990; Krzyminska, Lutjen, and O’Neill 1990) and bovine (Herr and Reed 1991). In addition, Magli et al. (2004) found that when a blastomere biopsy was performed in addition to a polar body biopsy in human embryos there was no significant difference seen in implantation rates against those that just underwent one of the procedures. However, the invasive nature of these techniques proves to be a large disadvantage as they cause embryos potential distress (De Vos and Van Steirteghem 2001).

Noninvasive techniques

The foremost common noninvasive technique used to assess preimplantation embryos is through static assessment with light microscopy to assess oocyte and embryo morphology. Its time and cost efficient nature makes it one of easiest procedures for clinics to non-invasively determine embryonic health. Various characteristics are observed such as zygote pronuclear scoring (Scott and Smith 1998), cleavage-stage cell number, multinucleation, degree of fragmentation, timing of development and blastomere size and shape (Racowsky et al. 2010). Detailed cleavage-stage scoring systems have been published by the Society for Assisted Reproductive Technology (SART 2015) and the ESHRE Special Interest Group of Embryology (ESHRE 2015). Many studies suggest
morphological analysis is adequate in determining embryo developmental potential, linking these various parameters to blastocyst formation, implantation rates and pregnancy rates (Alikani et al. 1999; Giorgetti et al. 1995; Hardy, Winston, and Handyside 1993; Staessen et al. 1993; Ziebe et al. 1997).

In spite of several advantages reported, studies have shown the inaccurate and subjective nature of these techniques. Balaban et al. (1998) observed the morphology of oocytes, elucidating that the implantation rates of oocytes deemed abnormal were similar to those classified as normal morphology. Guerif et al. (2007) used four morphological parameters to assess embryos and proved that these parameters have low predictive capability for blastocyst formation. Finally, studies have shown that newly cleaved blastomeres are capable of reabsorbing fragments, therefore using this parameter at these stages is not reliable to assess embryonic potential (Hardarson et al. 2002).

Morphokinetic assessment

The environment in which embryos are exposed to during in vitro development creates various stresses that impede development. Some of these stresses include fluctuations of pH and temperature (Swain 2010). The embryo therefore may experience alterations in its developmental rate. The timing of embryo cleavage is a potential predictor for embryo viability. Studies suggest that the timing of embryo growth is associated with pregnancy outcome. Bos-Mikich et al. (2001) found that early cleavage (between 25 and 29 hours post insemination) in humans had successful pregnancies opposed to those that cleaved later. Furthermore, various studies found that cleavage rate
is associated with blastocyst and pregnancy rates and thus a predictor for embryo developmental potential in humans (Fenwick et al. 2002; Fu et al. 2009; Lundin et al. 2001; Meseguer et al. 2011; Sakkas et al. 2001; Shoukir et al. 1997) and bovine (Lonergan et al. 1999, 2000; Plante et al. 1994; Van Soom et al. 1997; Yadav et al. 1993). Embryos that develop close to the rate of their in vivo counterparts are considered to be healthier whereas embryos growing at rates slower tend to have lower viability (La Salle 2012). Other studies have shown that slow growing embryos have negative intrinsic effects in bovine embryos. Brevini et al. (2002) found that embryos cleaving later than 27 hours post insemination (hpi) had abnormal mRNA polyadenylation. Therefore, these embryos that develop faster are generally considered to be more viable.

On the other side of the spectrum, embryos developing at rates faster than what are typically observed in vivo also indicate poor quality. Abnormally fast developing embryos are associated with chromosomal and metabolic marker aberrations in humans (Alikani et al. 2000; Cummins et al. 1986; Magli et al. 1998) and mice (Velker et al. 2012). The timing of first cleavage in bovine varies in the literature from 24 hpi to 48 hpi. Optimal first cleavage time has been found to be around 30 hpi (Dinnyés et al. 1999; Lonergan et al. 1999; Vandaele et al. 2006; Ward et al. 2001). Studies have also observed that the development rates of bovine embryos are associated with embryo sex. Xu et al. (1992) found that male bovine embryos tend to develop faster than female bovine embryos in vitro. In 1993, Yadav et al. supported these results as they determined that early cleaving embryos (24-28 hpi) were more likely to be male.
Producing embryos *in vitro* forces embryos to be exposed to suboptimal culture conditions, thereby exposing embryos to environments outside of the incubator in order to observe morphology and morphokinetic characteristics have detrimental effects. Alternatively, the use of time-lapse cinematography can capture images of developing embryos without removing embryos from the incubator. During imaging procedures, embryos are kept at constant temperature, carbon dioxide and oxygen levels (Payne et al. 1997). This technique has allowed for the visualization of early morphological and morphokinetic events such as polar body presence, cleavage rate and embryo morphology (Grisartet al. 1994; Holm et al. 1998; Sugimura et al. 2012). Sugimura et al. (2012) monitored the timing of first cleavage, blastomere number and fragmentation in bovine embryos to predict pregnancy with 78.9% accuracy. Time lapse imaging, when used with laser scanning confocal microscopy, can produce a 3D representation of the embryo using z-stack images. Furthermore, this imaging has been utilized to research molecular mechanisms of the embryo when combined with the use of live cell-permeable fluorescent probes in bovine (Komatsu et al. 2014). This study observed 1- and 2-cell stage mice embryos stained with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetramethyl-benzimidazol carbocyanine iodide (JC-1) which indicates the inner mitochondrial membrane potential (ΔΨm) and CellRox Deep Red Reagent which fluoresces in the presence of reactive oxygen species (ROS).

Embryo selection via morphological and morphokinetic examination proves to be a simple and inexpensive way to determine embryonic potential in the efforts toward single embryo transfer. These techniques, however, are still under investigation and a
clinical procedure with high accuracy and low invasiveness is still lacking. Recent research has been focusing on the use of metabolomics in embryo culture media.

The metabolism of an embryo is comprised of reactions involved in its growth and development. Analyzing the substances, called metabolites, that are utilized and created through these processes, can give instant insight into the health status of an embryo. This is known as the study of metabolomics. For human embryos undergoing IVF, studies have investigated various metabolic parameters including levels of pyruvate (Conaghan et al. 1993), lactate (Gott et al. 1990), glucose (Gardner et al. 2001) and amino acids (Brison et al. 2004) in the culture media, which is the immediate environment of the embryos in vitro. There are a variety of techniques that have been used to obtain metabolomics data, with proton nuclear magnetic resonance (H\textsuperscript{1} NMR) spectroscopy and mass spectrometry (MS) being the most common (Singh and Sinclair 2007).

**Metabolism of the bovine preimplantation embryo**

During the preimplantation stages, the embryo experiences drastic changes in biosynthesis, gene expression and metabolism. Cattle and humans have shown to have a similar pattern of metabolism (Thompson et al. 1996). Embryonic development relies on the ability to produce adequate levels of energy (adenosine triphosphate; ATP) via various metabolic pathways, where any disruptions lead to slow or arrested development (Gardner 1998; Lane and Gardner 1996). A comprehensive understanding of embryo metabolism in vitro is essential to ensure culture conditions are as optimal as possible.
Metabolic pathways are compartmentalized, occurring in either the cytoplasm or the mitochondria. These pathways utilize energy substrates glucose, pyruvate, lipids and amino acids to produce ATP. The maternal to embryonic transition (MET) that the embryo undergoes requires a significant amount of energy. This transition occurs between the 8-cell and 17-cell stages in bovine (Fair et al. 1995). Before the bovine embryonic genome is activated, transcription is limited to maternal contributions found in the oocyte. As a result, the early embryonic stages produce energy from oxidative phosphorylation, or the OXPHOS pathway. As the MET occurs, there is an increase in oxidation of glucose and the embryo begins to acquire most of its energy from the GLYCOX pathway (Gardner and Wale 2013; Gardner 1998; Khurana and Niemann 2000; Rieger et al. 1992b). Thompson et al. (1996) found that oxidative phosphorylation produces 93-96% of ATP in the embryo before compaction; afterwards it accounts for 82% as glucose is increasingly consumed. Khurana & Niemann (2000) mirrored these results, noticing an increase in the oxidation of glucose at the 12-cell and 16-cell stages. Thus pre-compaction embryos rely most heavily on the OXPHOS pathway (Gardner et al. 1993; Javed and Wright 1991; Rieger et al. 1992b; Thompson 2000; Thompson et al. 1996).

**Pre-compaction: OXPHOS pathway**

The early embryo relies heavily on aerobic respiration. Before compaction in cattle, sheep and humans the embryo’s preferred energy substrates are amino acids, pyruvate and lactate (Gardner et al. 1993; Hardy et al. 1989; Rieger et al. 1992a; Rieger
et al. 1992b; Thompson et al. 1993). These substrates contribute to energy production via oxidative phosphorylation. This process occurs in the mitochondria, where metabolites enter the tricarboxylic acid (TCA) cycle. This cycle allows for high levels of ATP production indirectly by reducing NAD+ (nicotinamide adenine dinucleotide) to NADH. This pyridine nucleotide is then re-oxidized as it donates electrons to the electron transport chain (ETC) found on the inner mitochondrial membrane. Through this process, protons are pumped across protein complexes creating a proton gradient, which then powers the ATP synthase protein.

Several transport mechanisms have been identified to allow amino acid uptake, most of which involve co-transport with Na⁺ (Van Winkle 2001). Glucogenic amino acids, as the name suggests, are precursors for gluconeogenesis (metabolic pathway generating glucose). They may also be catabolized in the TCA cycle to produce energy, or they can be converted to pyruvate or various intermediates of the TCA cycle through degradation of their carbon skeletons where they are able to produce ATP. Ketogenic amino acids enter the TCA cycle via degradation to acetyl coenzyme-A (acetyl-CoA). The addition of amino acids such as glutamine to synthetic oviductal fluid in IVF has been shown to increase the blastocyst rate in humans (Devreker et al. 1998), morula rate in cattle (Takahashi and First 1992) and cleavage rate in sheep (Gardner et al. 1994).

Pyruvate is a product of the glycolytic pathway and is shuttled into the mitochondria using specific transport proteins. It is then converted into acetyl-CoA to be further oxidized in the TCA cycle. Studies have demonstrated pyruvate’s key role in the
pre-compaction stage embryo. Khurana & Niemann (2000) used radioactive isotope [1-\textsuperscript{14}C]pyruvate to observe its function in the preimplantation bovine embryo. Labeled glucose and lactose were also used and they observed pyruvate being the major substrate utilized for energy.

Lactate, unlike pyruvate, is unable to be transported into the mitochondria. It must first be converted to pyruvate in the cytosol to be utilized in the TCA cycle. This is accomplished through the reduction of NAD\textsuperscript{+} to NADH in the cytosol. Adequate NAD\textsuperscript{+} must be present in the cytosol for lactate metabolism to occur. Proper NAD\textsuperscript{+}/NADH compartmentalization is maintained through mechanisms such as the malate-aspartate shuttle (MAS), which is necessary as NADH is not able to cross the mitochondrial membranes (Cooper et al. 1985). Lane & Gardner (2005) displayed the importance of the MAS in mouse embryos by supplementing media lacking pyruvate with high levels of aspartate. The embryos were able to develop to the blastocyst stage and carry out successful pregnancies.

Studies in the mouse have shown that pyruvate is necessary for the first cleavage event to occur. Although lactate is still utilized for energy in the mouse zygote, the substrate isn’t capable of providing adequate energy for this first division (Biggers et al. 1967; Lane and Gardner 2000; Wales and Whittingham 1973; Whittingham 1969). At the 2-cell stage however, lactate has shown to have the capability to support embryonic development (Brinster 1965).
Although not primarily metabolized, glucose still plays an important role for the pre-compaction embryo. Glucose enters the pentose phosphate pathway (PPP) where it produces ribose sugars, essential for the formation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), as well nicotinamide adenine dinucleotide phosphate (NADPH).

Post compaction: GLYCOX pathway

Glucose (a monosaccharide) is one of the most important carbohydrates involved in mammalian metabolism. There are many ways glucose can be transported into the embryo. These mechanisms include passive transport via concentration gradient, facilitated transport via Na$^+$-independent glucose transporter glycoproteins (GLUTs) and active transport via Na$^+$-coupled carrier system (Leppens-Luisier et al. 2001; Scott and Whittingham 2002). Once inside the embryo, glucose can be metabolized in both aerobic and anaerobic conditions. Mammalian embryos have been shown to do so in both conditions (Gardner and Leese 1990; Wales 1969, 1986). Glucose is metabolized primarily through the glycolysis pathway occurring in the cytoplasm. Various enzymes in the pathway convert glucose ($C_6H_{12}O_6$) to pyruvate ($CH_3COCOO^-$), in the process phosphorylating ADP to ATP and reducing NAD$^+$ to NADH. The final product pyruvate moves on to the TCA cycle in the mitochondria to be metabolized in the OXPHOS pathway.

As the embryo continues to develop past the 8-cell stage, more energy is needed for the increased biosynthesis and events such as blastocoel formation. Khurana & Niemann (2000) showed that glucose consumption was 15-fold higher in blastocysts
compared to zygotes with radioactive labeling ([U-14C]glucose) and is essential for
development to the blastocyst stage. Studies have suggested that at compaction, only 10% of
-glucose has been oxidized and the rate of oxidation increases after the 16-cell stage (Bavister and Squirrel 2000; Wilding et al. 2009). We have established that pyruvate is the predominant substrate consumed for embryonic metabolism before compaction, but the big question is: Why is glucose utilization low in the early embryo? Adequate levels of glycolytic enzymes are present in the early embryo (Biggers and Stern 1973; Chi et al. 1988; Martin et al. 1993). Enzymes that catalyze essential irreversible reactions in metabolic pathways are potential sites for control. Phosphofructokinase (PFK) of glycolysis is one of these enzymes. Through the measurement of various critical glycolytic enzymes in early mouse embryos, Barbehenn et al. (1974) showed the block at the 6-PFK step of glycolysis in the pre-compaction embryo. The binding of allosteric effectors inhibits the ability for this enzyme to catalyze the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate halting the glycolytic pathway. The ratio of ATP to ADP in the early embryo decreases throughout development as the embryo requires energy for further cleavage divisions, increased biosynthesis, compaction and formation of the blastocoel. When the ratio is high, as seen before compaction, ATP allosterically inhibits PFK. As the demand for energy increases, as seen after compaction, this ratio lowers and allows PFK to function in the glycolytic pathway (Figure 1).
Embryos produced in vitro have been shown to exhibit high levels of aerobic glycolysis compared to their in vivo counterparts. In conditions with adequate oxygen, the embryo still demonstrates high glycolytic activity after compaction (Bavister 1995; Khurana and Niemann 2000). Embryos can experience arrest at the 8- to 16-cell stage when glycolysis is active during the pre-compaction stages (Gardner and Lane 1997; Thompson et al. 1992). This is a known as the “block stage” in bovine embryos (Thibault et al. 1966). Scott & Whittingham (2002) showed that when glucose uptake was inhibited, embryos were able to overcome the 2-cell developmental block seen in mice (Goddard and Pratt 1983).
Not only is glucose essential for development in the post-compaction embryo, but also higher consumption of glucose has shown to be beneficial. Studies have demonstrated that cattle and mouse blastocysts that uptake high levels of glucose had improved development in vitro and in utero. The optimal level of glucose uptake was found to be glucose levels >5 µg/h in cattle (Gardner and Leese 1987; Renard, Philippon, and Menezo 1980). Higher glucose uptake was found in healthy in vitro produced (IVP) embryos that were implanted than those that were unsuccessful in implantation.

**Spent media of slow and fast growing preimplantation embryos**

Embryos grown in vitro have been shown to have a delay in development compared to their in vivo counterparts. As previously described, fast growing embryos have been considered to be ‘healthier’ than slow growing embryos. Bovine embryos that develop in vivo first cleave 24-28 hpi (Thibault et al. 1987). Thus, in efforts to accurately simulate in vivo conditions, it is desirable for embryos to also cleave at this time. Embryos cleaving at these times are considered fast growing (FG) embryos, with slow growing (SG) embryos cleaving anytime after 40 hours post insemination. The time points for slow and fast embryos during all preimplantation stages are described in Table 1.

Distinct differences have been identified in the metabolomics profiles in the spent media of SG and FG bovine preimplantation embryos (Perkel 2015). This study observed energy metabolites (lactate, pyruvate, acetate) and amino acids (leucine, isoleucine, valine, threonine, alanine, methionine, glutamate, lysine, arginine, glycine, tyrosine,
histidine, tryptophan, phenylalanine) in the spent media of single embryo culture synthetic oviductal fluid (SOF) droplets.

At the 4-cell stage, SG embryo spent media had a reduction in pyruvate levels compared to FG embryo media, indicating higher consumption of pyruvate by the SG embryos. Pyruvate is an essential metabolite involved in energy production as it enters the TCA cycle in the mitochondria of the early embryo. As the embryo primarily utilizes the OXPHOS pathway in its early stages, this uptake of pyruvate at the 4-cell stage is potentially indicative of intrinsic stress in the SG embryo (Eguchi et al. 1997; Liu et al. 1996).

After embryonic genome activation, the spent media of SG 16-cell embryos had higher levels of acetate than the FG embryo media (Perkel 2015). Acetate typically enters the TCA cycle when converted into acetyl-CoA. The high levels of acetate indicate that acetyl-CoA may be converted back to acetate and that it is not entering the TCA cycle. This would result in lower ATP production and potentially contribute to the slow growth. Acetate is also associated with the production of hydrogen peroxide (H₂O₂), and thus the SG embryos may be exposed to higher levels of ROS (Guérin et al. 2001). Both of these observations indicate metabolic differences between slow and fast growing embryos in vitro.
Mitochondria and the early embryo

Mitochondria are double membrane bound intracellular organelles found in eukaryotic cells. The structure includes an outer membrane, permeable to molecules up to 10 kDa, and an impermeable inner membrane. Between the outer and inner mitochondrial membranes is the intermembrane space and the area enclosed by the inner mitochondrial membrane is known as the matrix. These structures are derived from prokaryotes through the process of endosymbiosis and serve essential metabolic functions in the eukaryotic cell. Mitochondria are best known for producing most of the energy in a cell in the form of ATP via the OXPHOS pathway. This is accomplished through the metabolism of carbohydrates, fats and amino acids.

This organelle also plays important roles in cell signaling, cellular differentiation, cell senescence and the regulation of the cell cycle. There are two ways in which mitochondria regulate cell signaling. The first being the regulation of calcium (Ca^{2+}) and ROS levels, which are molecules involved in intracellular signaling. Secondly, the mitochondrial structure itself serves as a location for protein signaling interactions (Tait and Green 2012).

The cytoplasm of the oocyte has a plethora of mitochondria available for energy production. Specific processes during the formation of primordial oocytes allow for the selection of mitochondria for transmission to the offspring (Hauswirth and Laipis 1982; Jansen 2000; Wai, Teoli, and Shoubridge 2008). Although the spermatozoon donates DNA and centrioles to the oocyte upon fertilization, the mitochondria are degraded
through ubiquitin-dependent proteolysis (Johns 2003; Schwartz and Vissing 2002; Sutovsky et al. 2004) and thus the embryo consists of maternally derived mitochondria (Cummins 2000; Giles et al. 1980; Hutchison et al. 1974; Al Rawi et al. 2011; Sato and Sato 2013).

Mitochondria are unique organelles as they contain their own mitochondrial DNA (mtDNA). Similar to nuclear DNA, mtDNA is also double-stranded, however it is organized as a circular, covalently closed, double-stranded structure. The sequences encode 37 genes, 13 of which are for proteins in the ETC, and the rest are genes for transfer RNA (tRNA) and ribosomal RNA (rRNA) in humans. Bovine mtDNA is 62-79% homologous to humans (Anderson et al. 1982). The copy number of mtDNA is useful in determining the amount of mitochondria present in a cell. A single mitochondrion can have up to eleven copies of mtDNA (Wiesner et al. 1992). Unfortunately, the location of mtDNA in the matrix makes the DNA vulnerable to degradation by free radicals produced via the OXPHOS pathway (Loeb et al. 2005; Shokolenko et al. 2009; Tarín 1995). Furthermore, mtDNA does not possess the same DNA defense and repair mechanisms as nuclear DNA (Linnane et al. 1989, 1992) and thus damage accumulated over time can disturb the production of ETC proteins involved in aerobic respiration and negatively affect cellular energy production (Van Blerkom 2004; Harman 1972; Wilding et al. 2005). This suggests that maternal age can affect oocyte health through mtDNA damage. Studies have shown that mtDNA mutations are related to a multitude of disorders including but not limited to myopathies, cardiomyopathies and encephalomyopathies (Schon et al. 1997). A new technique to prevent the transmission of
mtDNA diseases employs the transfer of pronuclei between a donor egg carrying mtDNA mutations and a healthy recipient egg, successfully performed by Craven et al. (2010) in the United Kingdom. In doing so it was found that less than 2% of the donor mtDNA is carried over, and thus proves to be a promising procedure to help minimize mitochondrial genetic disorders.

Aside from genetically transmitted diseases, mitochondrial dysfunction plays a role in early embryonic developmental mechanisms. Studies have shown that the absence of mitochondrial ATP is associated with a variety of developmental issues such as fertilization failure. Reynier et al. (2001) found that oocytes which failed to fertilize had a significantly lower copy number than those that were successfully fertilized. Mitochondrial dysfunction is also hypothesized to be associated to chromosomal abnormalities. As meiosis is an ATP dependent process, inadequate levels of ATP would negatively affect the successful segregation of chromosomes, as described by Schon et al. (2000). Other developmental defects include fragmentation and apoptosis, where Perez et al. (2000) found that microinjecting embryos with healthy mitochondria reduced these adverse effects. Conversely apoptosis was induced in oocytes with the microinjection of abnormal mitochondria (Perez et al. 2007). Finally, mitochondrial dysfunction has shown to contribute to delayed preimplantation development and embryonic arrest. Thouas et al. (2004) observed the effects of mitochondrial function of embryonic development by photosensitizing fluorescent dye rhodamine-123, a method that induces mitochondrial damage. Oocytes with impaired mitochondria had either development retardation or arrest. Furthermore, Van Blerkom et al. (1995) determined that although metaphase II
(MII) human oocytes had a wide range of ATP contents, oocytes with more than 2 pmol/oocyte of ATP had improved preimplantation development. Overall mitochondria play an essential role in the development of the early embryo and suboptimal function of mitochondria leads to development defects found in the preimplantation embryo.

Mitochondria and reactive oxygen species

Among the roles of mitochondria, an important one is the production of ROS, which occurs in most mammalian cells (Andreyev et al. 2005; Balaban et al. 2005; Turrens 2003). The ETC carries out a series of redox reactions with enzymatic electron donors and acceptors, where each successive acceptor is more electronegative than the one previous. Electrons enter the chain from many sources such as NADH, succinate or electron transfer flavoprotein. As electrons are passed through the chain, energy is released which allows for the generation of the proton gradient. In 1979, Chance et al. demonstrated that isolated mitochondria produce H₂O₂. As electrons flow through the ETC, a small percentage leak out and produce the free radical superoxide (O₂⁻; an anion) through the reduction of O₂. The addition of another electron to O₂⁻ creates H₂O₂ (Forman and Kennedy 1974; Loschen et al. 1974). Leakage in the electron transport chain occurs primarily at complex I (NADH dehydrogenase) and complex III (Co-enzyme Q – cytochrome c reductase) (Jastroch et al. 2010). The majority of ROS found in cells originates from the mitochondria, where 0.2-2% of oxygen consumed by cells is converted to ROS (Harper et al. 2004; Orrenius et al. 2007).
The ROS produced in the mitochondria contributes to cell signaling for functions including erythropoietin production and the monitoring of oxygen tension and serves to benefit the embryo in many ways when at low levels. Mechanisms are in place to maintain “redox homeostasis”, keeping ROS at safe levels. However when in excess, ROS can be damaging to cells (Balaban et al. 2005; Dröge 2002). The presence of ROS in the mitochondria can create oxidative stress and damage the mtDNA (Shokolenko et al. 2009) and the plasma membrane via lipid peroxidation (Vladimirov et al. 1980; Wilhelm 1990).

Reactive oxygen species in the early embryo

Oxidative stress has shown to have implications for early embryos. When exposed to atmospheric oxygen levels, a rise in ROS can be seen in bovine and mouse preimplantation embryos (Goto et al. 1993; Nagao et al. 1994). When the oxygen concentration was reduced, embryo development was improved in both bovine and mouse. Pabon et al. (1989) observed that mouse embryos cultured under 5% O2 overcame the 2-cell block and had a higher survival rate than those exposed to 20% O2. This supports the claim that the ROS-induced lipid peroxidation contributes to the 2-cell block in mice (Nasr-Esfahani et al. 1990; Noda et al. 1991). Oxidative stress as a result of exposure to atmospheric oxygen, has been shown to have the most detrimental effects during the cleavage stages in mouse and human embryos (Kovacic, Sajko, and Vlaisavljević 2010; Kovacic and Vlaisavljević 2008; Wale and Gardner 2010).
High levels of free radicals are associated with cell death. Pierce et al. (1991) found that H$_2$O$_2$ facilitates apoptosis in blastocysts. This study is supported by the work of Yang et al. (1998), where fragmented human embryos had higher concentrations of H$_2$O$_2$ with apoptosis occurring only in the fragmented embryos. Yang & Rajamahendran (1999) also found that fragmented blastocysts were associated with apoptosis.

Embryos at various stages of preimplantation development respond differently to oxidative stress. Morales et al. (1999) observed in vitro development of bovine embryos using H$_2$O$_2$ as a stress inducer. It was found that embryos from the 5-8-cell stage and blastocysts had a dose-dependent survival when exposed to H$_2$O$_2$. Embryos at the 9-16-cell stages saw little difference in survival with H$_2$O$_2$ exposure, suggesting that embryos around the compaction stage are more resilient to oxidative stress. This corresponds to a study by Dalvit et al. (2005), where the physiological levels of ROS in IVP bovine embryos were observed for the first time. As the embryo developed to the late morula stage, ROS production significantly increased and at the blastocyst stage the ROS levels dropped back to concentrations found in the starting oocyte.

Embryos often need help to protect against oxidative damage, and various compounds exist to aid in this matter. Antioxidants such as vitamin E and A are present in bovine follicular fluid (Schweigert and Zucker 1988). Pyruvate has also shown to act as an antioxidant by neutralizing H$_2$O$_2$. Pyruvate is decarboxylized by H$_2$O$_2$, which produces acetate, carbon dioxide and water (O’Donnell-Tormey et al. 1987). Morales et al. (1999) found that pyruvate had high H$_2$O$_2$ degrading ability in the SOF media in
bovine embryos. Glutathione (GSH) is another essential antioxidant, which is oxidized to glutathione disulfide by the enzyme glutathione peroxidase (GPX) which converts $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$. Studies have shown its vital role reducing oxidative stress in bovine embryos (Gardner 1998; Takahashi et al. 1993). Embryos developed in vivo have the benefit of these defense mechanisms found in follicular and oviductal fluids. For IVP embryos, these protective molecules can be included in the SOF to help prevent oxidative damage.

**Mitochondrial Nutrients**

A series of nutrients have been identified that either directly or indirectly protect mitochondria from ROS-induced damage (Liu 2008). These nutrients act directly through the removal or neutralization of free radicals and preventing the generation of ROS. Indirect protection involves the repair of ROS-induced damage. Included in this list of nutrients is Co-enzyme Q10 (CoQ10).

*Co-enzyme Q10*

CoQ10, also known as ubiquinone, is a lipid-soluble component of cellular membranes. The compound consists of a quinone head which is a 1,4 benzoquinone group and a hydrophobic chain made up of isoprenoid subunits. The ‘10’ in CoQ10 indicates the isoprenoid subunits found in the side chain (Trumpower 1981). The empirical formula of CoQ10 is $\text{C}_{59}\text{H}_{90}\text{O}_4$ and has a molecular weight of 863.34 g/mol. The structure can be seen in Figure 2. The benzoquinone head has the ability to oxidize and reduce with the result that CoQ10 can exist in three different states: ubiquinol
(CoQH$_2$), semiquinone radical (CoQH•) and ubiquinone (CoQ). This characteristic makes CoQ10 an essential participant in the ETC.

The role of CoQ10 in oxidative phosphorylation was first proposed in 1957 by Crane et al. as an electron carrier between the NADH/succinate dehydrogenases (complexes I and II) and coenzyme Q – cytochrome c reductase (complex III). It was shown in 1969 that ubiquinone-depleted submitochondrial particles in the presence of NADH and succinate had declined respiration, and only when the ubiquinone was returned could the chain properly function (Ernster et al. 1969). Studies later showed the antioxidant properties of CoQ10 in its reduced form ubiquinol, as it inhibits lipid peroxidation of lipid membranes (Forsmark-Andrée et al. 1997; Lea and Kwietney 1962; Stocker, Bowry, and Frei 1991) and specifically mitochondrial membranes (Mellors and Tappel 1966). CoQ10 also possesses the ability to control cell signaling and gene expression (Crane 2001), however its role as a free radical scavenger makes it a very important component in mammalian membranes.

As mitochondrial dysfunction is believed to contribute to impaired embryonic development, the use of mitochondrial nutrients such as CoQ10, has been investigated as a potential supplement to improve embryo quality. CoQ10 has been previously shown to treat multiple ailments such as congestive heart failure (Langsjoen and Langsjoen 2008), hypertension (Rosenfeldt et al. 2003), macular degeneration (Feher et al. 2005) and much more (Bentov et al. 2010). Bentov et al. (2010) found that CoQ10 supplementation was associated with increased oocyte numbers and oocyte mitochondrial activity parameters.
Studies then started to explore the possibility of treating IVP embryos with CoQ10. Stojkovic et al. (1999) treated IVP bovine embryos with CoQ10 and found a dose dependent increase in early cleavage. Blastocysts that were treated with CoQ10 also hatched earlier and had a more abundant inner cell mass and higher ATP content compared to untreated blastocysts. Turi et al. (2012) then reported the CoQ10 content in follicular fluid. They found that follicular fluid with higher levels of CoQ10 were associated with mature oocytes and high-grade embryos. CoQ10’s crucial intrinsic functions in cellular metabolism and defense of oxidative damage make it a desirable candidate as a potential supplemented nutrient to improve IVP embryo quality and the overall efficiency of IVF.

**Figure 2.** The structure of CoQ10 composed of the benzoquinone group and isoprenoid chain.
HYPOTHESIS & OBJECTIVES

I hypothesize that slow growing preimplantation bovine embryos have impaired GLYCOX and/or OXPHOS pathways.

Three objectives will be covered in this study:

1. Ascertain the mitochondrial presence in slow and fast growing embryos at timed stages of development.
2. Elucidate the molecular mechanism for the differential metabolic profiles between slow and fast embryos of the same embryonic stage.
3. Determine the effects of augmenting metabolism with OXPHOS enhancers on embryo health.
MATERIALS AND METHODS

All chemicals used for experiments were procured from Sigma Aldrich (Oakville, ON, Canada) unless otherwise stated.

In vitro Production of Bovine Embryos

In vitro embryo production was adapted from work previously done by Favetta et al. (2004) and Favetta et al. (2007). Bovine ovaries were collected from a local Canadian Food Inspection Agency approved slaughterhouse (Guelph, ON, Canada) and transported to the laboratory in thermoflasks containing phosphate buffered saline (NaCl, 136.9mM; Na₂HPO₄, 8.1mM; KCl, 1.47 mM; KH₂PO₄, 1.19 mM; MgCl₂·6H₂O, 0.49mM) that was maintained at a temperature of approximately 35-36°C. Follicular aspiration was carried out from follicles ranging between 4 and 8 mm diameter using an 18G vacutainer needle and collected into HEPES-buffered Hams F-10, supplemented with 2% donor calf serum (PAA Laboratories Inc., Etobicoke, ON, Canada). Cumulus oocyte complexes (COC) were washed twice in maturation medium consisting of TCM-199 plus 2% donor calf serum and twice in maturation medium with the addition of 0.5 µg/mL of FSH, 1 µg/mL of LH and 1 µg/mL of estradiol. Groups of approximately 15-20 COCs with homogenous cytoplasms and at least 4-5 layers of compact cumulus were matured in 80 µL maturation medium drops under silicone oil for 22 to 24 h at 38.5°C under an atmosphere of 5% CO₂ in air with maximum humidity.

Following maturation, oocytes were washed two times in 1 mL of HEPES-buffered Tyrode’s albumin-lactate-pyruvate medium (HEPES/Sperm TALP)
supplemented with 15% BSA (0.0084 mg/mL final; fatty acid free) and two times in fertilization medium consisting of IVF-TALP (IVF-TALP consisting of Tyrode’s solution, supplemented with 15% BSA and 2 mg/mL heparin). Approximately 15-20 COCs were placed in 80 µL fertilization drops under silicone oil. Frozen-thawed bovine semen (Gencor, Guelph, ON, Canada) was prepared by swim-up technique. Thawed semen was added to HEPES/Sperm TALP medium supplemented with 15% BSA (0.0084 mg/mL final) for 1 h at 38.5°C under an atmosphere of 5% CO₂ in air with maximum humidity before centrifugation at 200 g for 10 min. The COCs and sperm were co-incubated at a final concentration of ~1 X 10⁶ motile sperm/mL at 38.5°C, under an atmosphere of 5% CO₂ in air with maximum humidity.

At approximately 18 h after insemination, presumptive zygotes were denuded by gentle vortexing for 90 sec, washed twice in HEPES/Sperm TALP and twice in in vitro culture (IVC) media, consisting of 10 mL synthetic oviductal fluid (SOF; CaCl₂*2H₂O, 1.17 mM; KCl, 7.16 mM; KH₂PO₄, 1.19 mM; MgCl₂*6H₂O, 0.49 mM; NaCl, 107.7 mM; NaHCO₃, 25.07 mM, Na Lactate (60% syrup), 3.3 mM; Chemicon-Millipore, Billerica, MA, USA) supplemented with 200 µL of non-essential amino acids (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine; all 0.2 mM final), 100 µL of essential amino acids (L-arginine hydrochloride, 0.6 mM final; L-cysteine, 0.1 mM final; L-histidine hydrochloride-H₂O, 0.2 mM final; L-isoleucine, 0.4 mM final; L-leucine, 0.4 mM final; L-lysine hydrochloride, 0.4 mM final; L-methionine, 0.1 mM final; L-phenylalanine, 0.2 mM final; L-threonine, 0.4 mM final; L-tyrosine, 0.2 mM final; L-tryptophan, 0.05 mM final; L-valine, 0.4 mM final), 50 µL of sodium
pyruvate (0.00886 mg/mL final), 5 µL of gentamicin (25 mg/mL final; all from Invitrogen, Burlington, ON, Canada), 560 µL of 15% BSA (0.0084 mg/mL final) in SOF and 200 µL of 2% donor calf serum (PAA Laboratories, Etobicoke, Ontario, Canada). Approximately 30 presumptive zygotes with homogenous cytoplasm were cultured in 30 µL culture drops under silicone oil at 38.5°C under an atmosphere of 5% CO₂, 5% O₂, 90% N₂ for up to 8 days.

Collection of Embryos for Live Cell Imaging

Embryos were classified as FG or SG based on their cleavage times in hours post insemination (hpi; Table 1) as previously described by Van Soom et al. (1997).

<table>
<thead>
<tr>
<th>Cell stage</th>
<th>Fast growing (in hpi)</th>
<th>Slow growing (in hpi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-cell</td>
<td>30</td>
<td>42</td>
</tr>
<tr>
<td>4-cell</td>
<td>42</td>
<td>54</td>
</tr>
<tr>
<td>8-cell</td>
<td>60</td>
<td>80</td>
</tr>
<tr>
<td>16-cell</td>
<td>120</td>
<td>144</td>
</tr>
<tr>
<td>Morula</td>
<td>144</td>
<td>168</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>168</td>
<td>192</td>
</tr>
</tbody>
</table>

The SG are collected 12-24 hours after their FG counterpart. Embryos at the zygote, 2-cell fast, 2-cell slow, 4-cell fast, 4-cell slow, 8-cell fast and 8-cell slow were collected. Embryos were morphologically graded as adapted from previous work (Farin et al. 1995; Puissant et al. 1987; Steer et al. 1992). A total of 30 embryos were collected for each stage to be stained.
Live Cell Staining with TMRM and CM-H$_2$DCFDA

The staining protocol for TMRM and CM-H$_2$DCFDA was adapted from Mitra & Lippincott-Schwartz (2010). The preparation of each stain took place with as little light as possible. Embryos were first stained with the live fluorescent probe TMRM (0.013µM) for mitochondrial membrane potential. Embryos were then washed twice in SOF media prepared in droplets under silicone oil followed by staining with CM-H$_2$DCFDA (1.25µM) for ROS (Life Sciences Inc., Burlington, ON). After 2 more washes in SOF droplets, embryos were placed in SOF droplets of glass bottom culture dishes.

Time-lapse Imaging and Analysis

All imaging took place using an OLYMPUS FV1200 Laser Scanning Microscope equipped with a stage-mounted incubator. The culture environment was maintained at 38.5°C and 5% CO$_2$. Embryos were placed in the incubator directly after staining at 21hpi. Time-lapse images were obtained at 30-minute intervals from 21hpi to 40hpi in order to determine the cleavage time of fast and slow growing zygotes. Fluorescent images of zygotes were then analyzed at 21hpi. Fast and slow growing 2-cell, 4-cell and 8-cell embryo images were taken directly after staining. Images were analyzed using Image J software. Corrected total cell fluorescence (CTCF) was calculated using the following equation:

\[
\text{CTCF} = \text{Integrated Density} - \left( \text{Area of selected cell} \times \text{Mean fluorescence of background readings} \right)
\]
Collection of Embryos for Gene Expression Analysis

Embryos were collected at two stages before embryonic genome activation and two stages after. Embryos were collected at the 4-cell, 8-cell, morula and blastocyst stages. The 4-cell and 8-cell stages occur before the MET and the morula and blastocyst occur after the MET. At the time of collection, embryos in groups of 10 were washed three times in PBS-PVA 0.1%, snap-frozen in liquid nitrogen and stored at -80°C. A total of 660 embryos were collected for analysis (Table 2). Embryos treated with CoQ10, vehicle (DMSO) and control were collected in the same manner as described. A total of 2,340 embryos were collected (Table 3).

<table>
<thead>
<tr>
<th>4-cell</th>
<th>8-cell</th>
<th>Morula</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>120</td>
<td>120</td>
<td>60</td>
</tr>
<tr>
<td>Fast</td>
<td>120</td>
<td>120</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 2, Number of embryos collected for gene expression analysis

<table>
<thead>
<tr>
<th>2-cell (fast)</th>
<th>2-cell (slow)</th>
<th>4-cell (fast)</th>
<th>4-cell (slow)</th>
<th>8-cell (fast)</th>
<th>8-cell (slow)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>150</td>
<td>150</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>DMSO</td>
<td>150</td>
<td>150</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>CoQ10</td>
<td>150</td>
<td>150</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>

Table 3, Number of embryos collected in treatment groups for gene expression analysis

RNA Extraction

RNA was isolated from pooled embryos using the PicoPure RNA Isolation Kit (Life Technologies Inc., Burlington, ON), following the manufacturer’s instructions. In brief, Extraction Buffer was added to lyse the cells. Samples were vortexed for homogenization and transferred to an RN purification column to bind genomic DNA. Flow through was mixed with 70% ethanol. The column was then washed and dried,
followed by RNA elution in the Elution Buffer. RNA samples were reverse transcribed immediately following extraction using the one-step protocol with qScript™ cDNA SuperMix (Quanta Biosciences, Canada) following the manufacturer’s instructions. cDNA samples were stored at -20°C until needed.

**Gene Expression Analysis**

Quantitative real-time PCR (qPCR) was used to measure mRNA expression profiles of selected genes in FG and SG embryos at the 2-cell, 4-cell ad 8-cell stages. Each analysis was performed on three biological replicates with three technical replicates each. Peptidylprolyl isomerase A (PPIA) and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta (YWHAZ) were used as reference genes for all qPCR analyses. Relative quantity of target genes was log-transformed and normalized to relative quantity of the reference genes, PPIA and YWHAZ, across samples (ΔΔCq) (Bio-Rad CFX Manger 3.1). Therefore values presented in figures in the current chapter are relative normalized gene expression levels. The primer sequences are listed in Table 4. qPCR was carried out using the Bio-Rad CFX96 Real-Time PCR System and products were detected with SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. Each reaction contained 5 µL of the SsoFastTM EvaGreen® Supermix reaction mix, 1 µL of a mix of the forward and reverse primers (at a 0.1 µM concentration for each set of primers) and 2 µL cDNA for all genes. The final volume was adjusted to 10 µL using RNase-free water (Ref Ambion PCR-grade water). A standard curve was established for each primer set using ovarian tissue cDNA template in six serial dilutions and primers efficiencies were calculated and
used in the analysis. The amplification program was as follows: preincubation for EvaGreen® Supermix polymerase activation at 95°C for 10 minutes, followed by 50 amplification cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 10 seconds, elongation at 72°C for 10 seconds, and acquisition of fluorescence for 10 seconds. After the last 100 cycle, fluorescence acquisition was begun at 72°C, and measurements were taken every 0.5°C until 95°C to generate the melting curve.

Table 4. List of primers used for qPCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genebank accession number</th>
<th>Primer sequence (5’-3’)</th>
<th>Primer Efficiency</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>NM_001034034.2</td>
<td>5’-TTATGACCACGTCCACGACC-3’ 5’-ACTTGGCAGGTATCCAGG-3’</td>
<td>102.0%</td>
<td>243</td>
</tr>
<tr>
<td>HK</td>
<td>NM_001012668.1</td>
<td>5’-CGCCAGAGGACCACGTAGG-3’ 5’-GGTCATTAGAAGCGCATG-3’</td>
<td>104.3%</td>
<td>184</td>
</tr>
<tr>
<td>ATP5b</td>
<td>NM_175796.3</td>
<td>5’-CGCTTTGTTACGGAATAAG-3’ 5’-GGCCAGGTAGGTAGCTAACC-3’</td>
<td>99.5%</td>
<td>211</td>
</tr>
<tr>
<td>COX5a</td>
<td>NM_001002891.1</td>
<td>5’-CATGGATGCTGTTTGGGCG-3’ 5’-CCCTCCCTGGATATCCTTTGTTCC-3’</td>
<td>101.0%</td>
<td>244</td>
</tr>
<tr>
<td>PPIA</td>
<td>NM_178320.2</td>
<td>5’-TCTGGACGCAAATGCTG-3’ 5’-TTTCATCTTGGCAAGTACC-3’</td>
<td>104.4%</td>
<td>111</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>NM_174814.2</td>
<td>5’-GCATCCCCACAGACTATTTCC-3’ 5’-GCAAAGACACATGACGACCA-3’</td>
<td>103.0%</td>
<td>102</td>
</tr>
</tbody>
</table>

**Treatment of Coenzyme Q10**

Oocyte maturation and fertilization were carried out as described above. At the time of culture, the embryos were randomly split into three groups; control, CoQ10 treatment and vehicle. The control group was cultured in SOF. For the treatment group, CoQ10 was added to SOF. CoQ10 is lipophilic and thus was dissolved in 100% DMSO. A working solution of 30mM was prepared with 0.025 g of CoQ10 in 1mL DMSO. From the working solution, 1µL was added to 999µL to make a final concentration of 30µM. The selection of this concentration is based on previous studies (Abdulhasan, Fakhrildin,
and Shudder 2015; Gendelman and Roth 2012; Stojkovic et al. 1999). The vehicle group was cultured in SOF with 0.1% DMSO. Embryos were not cultured under oil as lipophilic CoQ10 could be absorbed in it. Embryos were collected at the 2-cell, 4-cell and 8-cell stages in at FG and SG time points.

Statistics

The difference in CTCF between FG and SG embryos at the zygote, 2-cell, 4-cell and 8-cell was analyzed with t-test (Graphpad Prism 6.0). Expression levels were calculated relative to those of internal reference genes PPIA and YWHAZ in Bio-Rad CFX Manager 3.1. Differences in gene expression of FG and SG embryos at the 4-cell, 8-cell morula and blastocyst stage were analyzed with one-way analysis of variance (ANOVA), and when statistical significance was observed (p<0.05), Tukey’s multiple comparison tests were performed to evaluate differences between the FG and SG groups within a single gene (Graphpad Prism 6.0). Differences at p<0.05 were considered to be statistically significant.
RESULTS

Production of embryos

The average cleavage rate for embryo production in our study was 84%. When embryos were grown until day 8 blastocysts rates of total embryos were 32%.

Time-lapse mitochondrial membrane potential ($\Delta \Psi m$) and ROS in FG and SG zygotes

Fast and slow growing zygotes were analyzed to determine $\Delta \Psi m$ and the presence of ROS. Our time-lapse results confirmed that the majority of fast growing zygotes first cleaved by 30hpi and slow growing zygotes cleaved by 40hpi. At 29hpi 30% of the fast growing zygotes cleaved and 39% cleaved at 30hpi. At 39hpi 30% of slow growing zygotes cleaved and 37% cleaved at 40hpi (Figure 3).

The fluorescence at 21hpi of TMRM in slow growing zygotes was higher than the fluorescence seen in fast growing zygotes, indicating higher $\Delta \Psi m$ in the slow growing zygotes (Figures 4 & 5). Slow growing zygotes also displayed higher fluorescence of CM-H$_2$DCFDA than fast growing zygotes, showing that the presence of ROS is higher in the slow growing group (Figures 4 & 5). After the initial measurement at 21hpi, the fluorescent signal diminishes due to bleaching, where the fluorophore is destroyed as a result of prolonged exposure to excitation light.
Figure 3. The proportion of embryos that cleaved at various times. Fast growing embryos (a) were observed from 27hpi to 31hpi and slow growing embryos (b) were observed from 36hpi to 40hpi.
Figure 4. Fast cleaving zygote embryos stained with TMRM and CM-H$_2$DCFDA observed over 40 hours with a confocal microscope. Panel a represents the embryo under the bright field view. Panel b and c represent the fluorescence of TMRM and CM-H$_2$DCFDA respectively.

Figure 5. Slow growing zygote embryos stained with TMRM and CM-H$_2$DCFDA observed over 40 hours with a confocal microscope. Panel a represents the embryo under the bright field view. Panel b and c represent the fluorescence of TMRM and CM-H$_2$DCFDA respectively.
**Static mitochondrial membrane potential (ΔΨm) and ROS in FG and SG zygotes**

Embryos collected at the 2-cell stage showed results similar to the zygotes. Fast growing 2-cell embryos expressed lower fluorescence for TMRM and CM-H$_2$DCFDA than the slow growing 2-cell embryos (Figure 6). Fast growing 4-cell embryos also displayed lower fluorescence for both stains than the slow growing 4-cell embryos (Figure 7). Embryos collected at the 8-cell stage showed the same pattern. The slow growing embryos showed higher fluorescence than the fast growing 8-cell embryos (Figure 8), suggesting that the slow growing embryos have higher ΔΨm and presence of ROS than the fast growing embryos.

The CTCF was calculated to quantify the fluorescent signal and correct for background noise. Slow growing embryos at the zygote, 2-cell and 4-cell stages had significantly higher CTCF of TMRM than fast growing embryos (P<0.05) (Figure 9). Slow growing zygotes, 2-cell and 4-cell embryos displayed 163%, 110% and 250% higher fluorescence respectively than the fast growing groups and thus higher ΔΨm. The slow 8-cell embryos expressed higher fluorescence, however the difference was not significant. Slow growing zygotes expressed significantly higher CTCF of CM-H$_2$DCFDA than the fast growing embryos (P<0.05). The slow zygotes displayed 161% higher fluorescence than the fast growing zygotes. Although not significant, the slow embryos at the 2-cell, 4-cell and 8-cell stage also displayed higher CTCF than their fast growing counterparts (Figure 10). This indicates a higher presence of ROS is slow developing embryos.
Figure 6. Fast and slow growing 2-cell embryos stained with TMRM and CM-H$_2$DCFDA taken with confocal microscope. Panel a represents fast growing 2-cell embryos and panel b represents slow growing 2-cell embryos.
Figure 7. Fast and slow growing 4-cell embryos stained with TMRM and CM-H$_2$DCFDA taken with confocal microscope. Panel a represents fast growing 4-cell embryos and panel b represents slow growing 4-cell embryos.
Figure 8. Fast and slow growing 8-cell embryos stained with TMRM and CM-H$_2$DCFDA taken with confocal microscope. Panel a represents fast growing 4-cell embryos and panel b represents slow growing 4-cell embryos.
Figure 9. The corrected total cell fluorescence of TMRM in zygote, 2-cell, 4-cell and 8-cell in fast and slow growing preimplantation embryos. The fluorescence represents the mitochondrial membrane potential. Values are expressed as mean ± SEM. For each mean n = 30. Bars marked with an asterisk are significantly different from each other (p < 0.05).

Figure 10. The corrected total cell fluorescence of CM-H$_2$DCFDA in zygote, 2-cell, 4-cell and 8-cell in fast and slow growing preimplantation embryos. The fluorescence represents the mitochondrial membrane potential. Values are expressed as mean ± SEM. For each mean n = 30. Bars marked with an asterisk are significantly different from each other (p < 0.05).
Expression of GLYCOX and OXPHOS genes in FG and SG embryos

In order to determine the molecular mechanism underlying the metabolic differences in slow growing embryos, the expression of metabolic genes was observed in FG and SG embryos. At the 4-cell stage, no significant differences were seen in the expression of any of the four genes between the slow and fast groups, although the slow group appears to have a slightly lower expression of the four genes (Figure 1a). Slow growing 8-cell embryos showed a low expression of GLYCOX genes compared to the fast growing embryos, but this difference was not significant. However, 8-cell slow embryos expressed significantly higher levels of the ATP5b gene (P<0.05) (Figure 1b).

After the embryonic genome is activated, embryos (morula and blastocyst) showed lower expression of GLYCOX genes compared to OXPHOS genes. Slow growing morula embryos showed slightly lower expression of OXPHOS genes and higher expression of GLYCOX than the fast embryos. The slow growing morula expressed significantly higher GAPDH than the fast growing morulas (P<0.05) (Figure 12a). At the blastocyst stage, no significant differences were seen in the OXPHOS gene expression. Similar to the results seen at the morula stage, the slow growing blastocyst showed significantly higher expression of GAPDH than the fast growing blastocysts (P<0.05) (Figure 12b).
**Figure 11.** The expression of GLYCOX (HK; GAPDH) and OXPHOS (COX5a; ATP5b) genes in fast and slow growing embryos at the pre-compaction stages. Graph a represents 4-cell embryos and graph b represents 8-cell embryos. Values are expressed as mean ± SEM. For each mean n = 120. Bars marked with an asterisk are significantly different from each other (p<0.05).

**Figure 12.** The expression of GLYCOX (HK; GAPDH) and OXPHOS (COX5a; ATP5b) genes in fast and slow growing embryos at the post-compaction stages. Graph a represents morula embryos and graph b represents blastocyst embryos. Values are expressed as mean ± SEM. For each morula mean n = 60 and for mean blastocyst mean n = 30. Bars marked with an asterisk are significantly different from each other (p<0.05).
Expression of GLYCOX and OXPHOS genes in embryos treated with CoQ10

To observe the effects of mitochondrial enhancers on embryonic development, embryos were treated with either treatment of CoQ10, vehicle (DMSO) or control (no treatment). Embryos treated with CoQ10 had a significantly higher cleavage rate than the one observed in the control or vehicle groups as the rate was increased by 8% (P<0.05) (Figure 13). When treated with CoQ10, a higher proportion of embryos developed to the 2-cell, 4-cell and 8-cell stages. At the final 8-cell stage 58%, 45% and 39% of embryos remained in the CoQ10, vehicle and control group (respectively) (Figure 14).

Fast growing 2-cell embryos showed lower expression of GLYCOX genes compared to slow growing embryos. No significant differences were seen between treatment groups in the expression of HK, although the CoQ10 treatment group appears to have lower expression in both slow and fast embryos (Figure 15a). Fast growing DMSO and CoQ10 2-cell groups had significantly lower expression of GAPDH than the control group (P<0.05) (Figure 15a). The slow growing 2-cell vehicle group expressed significantly lower expression of GAPDH compared to the control group (P<0.05) (Figure 15a). The expression of OXPHOS genes at the 2-cell stage did not significantly differ between treatment groups (Figure 15b).

At the 4-cell stage, no significant differences were seen in the expression of GLYCOX genes between treatment groups (Figure 16a). Slow growing 4-cell embryos treated with CoQ10 displayed significantly lower expression of the OXPHOS gene
COX5a than the control group (P<0.05) and no changes seen between the treatment groups of the fast growing 4-cell embryos (Figure 16b).

At the 8-cell stage, fast embryos treated with CoQ10 showed significantly lower expression of GAPDH than the control group (P<0.05) (Figure 17a). No significant difference were seen in the expression of HK between treatment groups, however the slow growing group appears to have lower expression the fast growing 8-cell embryos (Figure 17a). Slow growing 8-cell embryos treated with CoQ10 expressed significantly higher expression of ATP5b (P<0.05) (Figure 17b). Although not significant, CoQ10 treated fast and slow embryos at the 8-cell stage show lower expression of the COX5a gene (Figure 17b).
**Figure 13.** The percent of embryos that cleaved with no treatment (control), vehicle (DMSO) and treatment of CoQ10 at 30µM. Values are expressed as mean ± SEM. For each mean n = 2340. Bars marked with an asterisk are significantly different from each other (p<0.05).

![Bar chart showing percent cleavage](chart1.png)

**Figure 14.** The average percentage of embryos with no treatment (control), vehicle (DMSO) and treatment with CoQ10 present at the zygote, 2-cell, 4-cell and 8-cell stages. For each mean n = 2340.

![Graph showing percentage of embryos](chart2.png)
Figure 15. The expression of GLYCOX (HK; GAPDH) and OXPHOS (COX5a; ATP5b) genes in slow growing and fast growing groups of 2-cell embryos with no treatment (control), vehicle (DMSO) and treatment with CoQ10. Graph a represents GLYCOX genes and graph b represents OXPHOS genes. Values are expressed as mean ± SEM. For each 2-cell mean n = 150. Bars marked with an asterisk are significantly different from each other (p<0.05).

Figure 16. The expression of GLYCOX (HK; GAPDH) and OXPHOS (COX5a; ATP5b) genes in slow growing and fast growing groups of 4-cell embryos with no treatment (control), vehicle (DMSO) and treatment with CoQ10. Graph a represents GLYCOX genes and graph b represents OXPHOS genes. Values are expressed as mean ± SEM. For each 2-cell mean n = 120. Bars marked with an asterisk are significantly different from each other (p<0.05).
Figure 17. The expression of GLYCOX (HK; GAPDH) and OXPHOS (COX5a; ATP5b) genes in slow growing and fast growing groups of 8-cell embryos with no treatment (control), vehicle (DMSO) and treatment with CoQ10. Graph a represents GLYCOX genes and graph b represents OXPHOS genes. Values are expressed as mean ± SEM. For each 8-cell mean n = 120. Bars marked with an asterisk are significantly different from each other (p<0.05).
DISCUSSION

Since 1978 the use of ARTs, such as IVF, has allowed for improved reproduction in both animals and humans. Although large strides have been made to improve the technique, the efficiency and success of IVF remains an issue with blastocyst rates at 30-40% in cattle (Rizos et al. 2002) and 30-50% in humans (Bayer et al. 2011). The mammalian embryo requires a generous amount of energy to successfully develop through its early stages. Sufficient energy must be present to undergo significant events such as cell cleavage divisions, compaction, embryonic genome activation and the formation of the blastocoel. To accomplish this goal, the embryo utilizes a combination of OXPHOS and GLYCOX pathways to produce ATP. Embryos that have inadequate levels of energy to support these processes will likely fail to develop properly. It is thought that dysfunction of the mitochondria plays a role in the quality and competency of IVP oocytes and embryos (Thouas et al. 2004, 2006; Wang et al. 2009). However the mechanism underlying mitochondrial dysfunction in IVP embryos is poorly understood. Therefore in this study we sought to evaluate mitochondrial activity in developmentally delayed embryos.

There were three goals for this study: i) to observe mitochondrial activity in slow and fast growing preimplantation bovine embryos; ii) to determine the expression of OXPHOS and GLYCOX genes in slow and fast growing preimplantation bovine embryos; iii) to observe the effects of mitochondrial enhancer CoQ10 on the development of preimplantation bovine embryos. Studies have looked at the effects of impaired
mitochondria on the oocyte and developing embryo where factors such as ATP content and mtDNA copy number have been shown to be associated with embryo competence (Van Blerkom et al. 1995; Reynier et al. 2001). Thouas et al. (2004) induced mitochondrial damage in mouse oocytes and determined that oocytes with diminished mitochondrial function had delayed and sometimes arrested development.

Research has shown that embryos developing at slow rates (SG embryos) result in lower implantation and pregnancy rates. This has been seen in both bovine (Lonergan et al. 1999; Van Soom, Boerjan, et al. 1997) and human (Meseguer et al. 2011; Sakkas et al. 2001; Shoukir et al. 1997). Although studies have shown mitochondrial impairment can result in developmental delay of embryos, they have not looked specifically at the activity of mitochondria in FG and SG embryos. Determining the activity of mitochondria in normal and developmentally delayed embryos will improve our understanding of the mechanisms underlying impaired embryonic development involved in IVF.

A common technique to visualize mitochondria in embryos is the use of live cell stains. The use of cell-permeable probes allows for the analysis of mitochondrial number, distribution and redox potential. Mitochondrial labels such as rhodamine 123, TMRE, TMRM are typically used for live cell imaging (Mitra and Lippincott-Schwartz 2010; Scaduto and Grotyohann 1999). These probes are lipophilic cations that become sequestered by the mitochondria due to their negative mitochondrial membrane potential ($\Delta\Psi_m$). When accumulated in mitochondria, the dyes display a red shift in both their absorption and fluorescence emission spectra. TMRM was found to have lower inhibition
of respiration than TMRE and rhodamine 123 and was thus utilized for this experiment (Joshi and Bakowska 2011; Scaduto and Grotyohann 1999). Recent studies have shown the successful use of TMRM to visualize mitochondria membrane potential in mouse oocytes and embryos (Basu et al. 2008; Igosheva et al. 2010; Zhang et al. 2006). Along with the live cell imaging of mitochondria, the presence of ROS can be imaged as well. The cell-permeant CM-H$_2$DCFDA is commonly used to detect species such as H$_2$O$_2$, hydroxyl radicals and peroxynitrite. It is the chloromethyl derivative of H$_2$DCFDA, however CM-H$_2$DCFDA has an additional thiol chloromethyl group which aids in its ability to be retained in cells by binding to intracellular components. When diffused into the cell, the acetate group is cleaved exposing a thiol-reactive chloromethyl group. When this group is oxidized by ROS, producing 2’,7’-dichlorofluorescein (DCF), it fluoresces green.

The first objective of this study aimed to determine the relative mitochondrial membrane potential between FG and SG embryos. The key finding of this experiment was the significantly lower $\Delta \Psi_m$ in pre-compaction embryos that developed fast compared to the $\Delta \Psi_m$ found in embryos that had delayed development (Figures 3-10). With the use of time-lapse cinematography, we were able to corroborate previous findings of the timing of cleavage for slow and fast growing embryos. We found that the majority of fast growing embryos cleaved at 30hpi and slow growing embryos cleaved by 40hpi (Figure 3). Van Soom et al. (1997) also concluded that fast and slow growing embryos cleaved at these times.
ΔΨm is a good indicator for mitochondrial health as it is a measure of ion transport and reflects metabolic activity and integrity of the mitochondrial membranes. The negative ΔΨm is created by protons (H+) being pumped out of the matrix into the intermembrane space that produces the H+-gradient necessary for ATP production. This suggests that high ΔΨm indicates poor embryonic health. These results are in agreement with several other studies (Acton et al. 2004; Igosheva et al. 2010; Schieke et al. 2008). Acton et al. (2004) found that arrested 2-cell mouse embryos displayed higher ΔΨm than those embryos that continued to develop normally. Furthermore, they found that a higher ΔΨm in 8-cell human embryos was associated with increased fragmentation. Igosheva et al. (2010) observed that compromised oocytes and embryos as a result of maternal obesity are associated with altered ΔΨm. Oocytes and embryos of obese mothers exhibited higher ΔΨm than the proposed healthier embryos. They also found that impaired embryos had increased oxidation of reducing equivalents NAD(P)H and FADH₂, which is a result of higher mitochondrial respiratory activity. Finally, Schieke et al. (2008) found that in mouse embryonic stem cells an increased ΔΨm was correlated with a reduced potential to differentiate.

However, there is a study that found different results that suggested higher ΔΨm indicates improved developmental potential in IVF (Komatsu et al. 2014). Komatsu et al. (2014) found that in vivo-fertilized mouse zygotes and 2-cell embryos displayed higher ΔΨm than in vitro-fertilized embryos. Furthermore, they found that low-ΔΨm 2-cell embryos had a tendency to arrest. However, we see a relative increase in the ΔΨm of SG embryos compared to FG embryos. There are a few possible reasons we are seeing this.
First, embryos that develop fast are considered to be more developmentally competent (La Salle 2012). On the other hand, embryos that develop excessively fast have been found to be associated with poor embryonic health (Alikani et al. 2000; Cummins et al. 1986; Magli et al. 1998). Therefore it would not be surprising for very fast growing embryos to have lower $\Delta \Psi_m$ and poor mitochondrial function. However this is likely not the case because although these embryos are termed “fast”, they are just relatively faster compared to the SG embryos, but in fact just follow the developmental timeline of in vivo produced embryos. These timings have been described by various studies, observing how bovine embryos that reach the 2-cell stage at 30hpi and 8-cell stage at 48hpi were considered fast developing. These fast embryos were correlated with higher total cell number and ICM cell numbers and higher blastocyst rates compared to embryos that reached these stages at later timings (Grisart et al., 1994; Van Soom et al., 1997).

Igosheva et al. (2010) suggested that the increased hyperpolarization of the mitochondrial membrane seen in impaired embryos is a result of increased presence of carbohydrates and fatty acid and thus an increased substrate influx through the OXPHOS pathway. However, why there would be an increased presence of these substrates is currently unknown. Studies have shown that mitochondrial hyperpolarization is an early indicator of apoptotic death (Giovannini et al. 2002; Sánchez-Alcázar et al. 2000). Acton et al. (2004) suggested that embryos exhibiting $\Delta \Psi_m$ outside of a proposed acceptable range could disrupt development. Another possible explanation for the high $\Delta \Psi_m$ seen in SG embryos is a possible overcompensation in the OXPHOS pathway. In this case, the $\Delta \Psi_m$ seen in FG embryos is a more normal level and the $\Delta \Psi_m$ seen in SG embryos is
excessively high. This could indicate that pre-compaction embryos have an insufficient amount of energy and are using the OXPHOS pathway to accommodate for this issue. If an early embryo was trying to compensate for lack of energy, the OXPHOS pathway is likely the way in which it would do so because this is the predominant pathway of the pre-compaction embryo. Embryos before compaction and genome activation have little to no oxidation of glucose via the GLYCOX pathway and primarily oxidize pyruvate through the TCA cycle and the OXPHOS pathway in the mitochondria (Gardner et al. 1993; Gardner and Wale 2013; Gardner 1998; Javed and Wright 1991; Khurana and Niemann 2000; Rieger et al. 1992b; Thompson et al. 1996, 2000).

These results support the “quiet embryo hypothesis” proposed by Leese (2002). Leese (2002) suggests that embryos with the lowest overall metabolism are the most viable preimplantation embryos. This hypothesis has been demonstrated in studies showing low glycolytic rates and low amino acid turnover resulting in embryos with higher developmental potential (Houghton et al. 2003; Lane and Gardner 1996). Our work is the first example of data supporting the hypothesis pertaining to the OXPHOS pathway.

It is important to note that the metabolism of the early embryo has been found to differ between sexes (Gardner, Larman, and Thouas 2010; Gardner and Leese 1987). Female mouse embryos were shown to have higher metabolic activity than male embryos. Tiffin et al. (1991) found that male bovine embryos exhibited higher total glucose metabolism, however female bovine embryos had increased PPP activity. As we
did not determine the sex of the embryos in this experiment, we are unable to determine if this was a factor.

In our first objective we also observed the presence of ROS in SG and FG zygotes, 2-cell, 4-cell and 8-cell embryos. We found that zygote embryos developing at slow rates had significantly higher fluorescence of CM-H$_2$DCFDA and thus higher abundance of ROS (Figures 3-10). These results are reflected in previous studies where high levels of ROS were associated with poor embryonic development (Goto et al. 1993; Igosheva et al. 2010; Kovacic et al. 2010; Kovacic and Vlaisavljević 2008; Nagao et al. 1994; Pabon et al. 1989; Takahashi 2012; Wale and Gardner 2010). The exposure of IVP embryos to high oxygen tension creates a stressful oxidative environment to the preimplantation embryo. Therefore the high levels of ROS we are seeing are likely contributing to the developmental delay of the SG embryos. The production of ROS is a byproduct of the OXPHOS pathway. Complexes I and III in the ETC release O$_2^-$ into the matrix of the mitochondria. The unusually high levels of ΔΨm seen in the SG embryos would thus produce high levels of ROS. Although mitochondrial-produced ROS serves important functions in cell signaling, when present in increased concentrations ROS can considerably damage cells (Balaban et al. 2005; Dröge 2002). These results show us that embryos developing at rates slower than in vivo-produced embryos have higher ΔΨm and levels of ROS compared to IVP embryos developing at normal rates. The molecular mechanism governing this difference is still unknown, which leads to our second objective.
The second objective of this study was to elucidate the molecular mechanism for the differential metabolic profiles between FG and SG embryos at the same embryonic stage. To accomplish this, we sought to determine the expression of GLYCOX (HK; GAPDH) and OXPHOS (COX5a; ATP5b) genes. Hexokinase (HK) is an enzyme that catalyzes the first step of glycolysis. Its function is to phosphorylate glucose to glucose 6-phosphate (Gibbs and Turner 1964). The sixth step of glycolysis is catalyzed by the enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH converts glyceraldehyde 3-phosphate is to 1,3-bisphosphoglycerate (Gibbs and Turner 1964). In the OXPHOS pathway, the ETC is made of five enzyme complexes. Cytochrome c oxidase (COX) is the fourth enzyme complex (Complex IV) in the ETC. Its role is to couple the transfer of electrons from cytochrome c to molecular oxygen and contributes to the mitochondrial electrochemical gradient (Barrientos 2002). Cytochrome c oxidase subunit VA (COX5a) is the heme A-containing chain of COX, which is the terminal oxidase in the ETC (Cui et al. 2006). Finally, ATP synthase (F1F0 ATP synthase or Complex V) is the final complex of the ETC (Barrientos 2002). In the presence of a proton gradient it functions to produce ATP from ADP. ATP synthase consists of a soluble catalytic F1 component and membrane-spanning F0 component. The catalytic component is made up 5 subunits: alpha, beta, gamma, delta and epsilon. ATP synthase subunit beta (ATP5b) is one of these subunits (Peddinti et al. 2008).

At the 4-cell stage we do not see any difference in the expression of GLYCOX and OXPHOS genes between FG and SG groups (Figure 11a). This is not entirely surprising as the embryonic genome has not yet been fully activated and minimal
transcription is occurring in the early embryo. Moving to the 8-cell stage, the embryonic genome is starting to become activated and transcription will be more active at this stage (Fair et al., 1995). At the 8-cell stage we saw no difference in the expression of GLYCOX genes (Figure 11b). As there is still little utilization of the GLYCOX pathway at this time due to the low ATP to ADP ratio, it makes sense that the embryo does not need to alter the levels of these genes (Barbehenn et al. 1974). However, SG embryos expressed significantly higher levels of ATP5b. It is possible that we see this increase in gene expression because the embryo has insufficient energy. The embryo at this time primarily uses the OXPHOS pathway to produce ATP. In an attempt to produce more energy, SG 8-cell embryos may be up-regulating ATP5b to aid in ATP production in the OXPHOS pathway. Furthermore, the SOF used in this experiment is supplemented with pyruvate, which reduces the significance of cytoplasmic metabolic enzymes in the supply of metabolic precursors. Consequently the major factor involved in energy production lies in the efficiency of mitochondrial respiration.

In the post-compaction embryos, we see a drastic increase of OXPHOS gene expression (Figure 12). Embryos at these stages would typically have high oxidation of glucose through the GLYCOX pathway, however we see very low levels of GLYCOX genes and high levels of OXPHOS genes. The SOF culture media we use for in vitro embryo production is devoid of exogenous glucose. The only glucose present is that in which is in the bovine serum albumin (BSA) added to the SOF. Thus, the embryos continue to use the OXPHOS pathway for the rest of their preimplantation development. Takahashi & First (1992) tested the developmental effects of culturing bovine embryos in
media containing energy substrates lactate and pyruvate but without glucose. This study demonstrated that the addition of glucose was not necessary for embryos to reach the blastocyst stage. They also indicated that embryo development to the morula stage was negatively impacted by addition of glucose to the SOF media. Edwards et al. (1997) mirrored these results, finding that SOF media with reduced glucose and increased pyruvate and lactate resulted in a higher blastocyst rate in bovine embryos. Our study has shown that the addition of exogenous glucose is not necessary for the embryo to develop to the blastocyst stage.

In both the morula and blastocyst, no differences were seen in the OXPHOS gene expression between FG and SG embryos (Figure 12). As the embryos at both of these stages are not able to primarily use the GLYCOX pathway as a result of low glucose levels, it isn’t surprising that both increase the expression of OXPHOS genes in the same way. However, both stages see a significant increase in the expression of GAPDH in their SG groups (Figure 12). We may be seeing this as a result of impaired mitochondrial function. If embryos aren’t acquiring enough energy from the OXPHOS pathway, they may be up-regulating GLYCOX genes in an attempt to produce more ATP even though little glucose is present. Studies have shown that GLYCOX genes typically increase transcription after the compaction stages. Lequarre et al. (1997) analyzed the expression of GLYCOX genes G6PDH, GPI and HK in bovine embryos at various stages of development. The expression of G6PDH and HK increased after compaction, with a large jump in HK seen at the 16-cell stage. This is contradictory to our results, where we have relatively low levels of HK expression. However as our media is lacking in exogenous
glucose it makes sense that HK expression would not necessarily be high. The information gained from the use of qPCR to determine gene expression is valuable. However it is important to understand that transcription of specific genes does not necessarily indicate translation into proteins. In the future, these results can be corroborated with the use of more protein/functional experiments.

The results from our second objective suggest that there is mitochondrial dysfunction at the 8-cell, morula and blastocyst stages. This supports the findings from our first objective. Therefore providing embryos with nutrients that enhance the mitochondria should improve preimplantation development, which leads to our third objective.

The third and final objective of this study was to determine the effects of augmenting metabolism with OXPHOS enhancers on embryo health. The poor quality of oocyte mitochondria remains one of the most challenging obstacles in achieving successful IVF. Therefore improving mitochondrial function will hopefully improve the efficiency of IVF. Pre-compaction stage embryos were split into three groups: control, CoQ10 treatment and DMSO vehicle. The supplementation of CoQ10 in the SOF media significantly improved the percent of cleaved embryos compared to the control group (Figures 13 & 14). As the CoQ10 both acts as electron acceptor/donor in the ETC and an antioxidant, it can assist in providing the embryo with more energy and reduce oxidative stress. This increase in cleavage rate supports previous studies. Stojkovic et al. (1999) treated embryos with CoQ10 and found that at 30µM, CoQ10 significantly increased
blastocyst rate, hatching rate, ATP content and inner cell mass. These observations suggest that CoQ10 is a promising treatment for supplementing IVF media.

The gene expression profiles provided some surprising and unexpected results. At the 2-cell stage, GAPDH had significantly lower expression in the vehicle and CoQ10 treatments of FG embryos (Figure 15a). The SG embryos similarly saw a significant decrease but only in the vehicle group. This suggests that the DMSO vehicle was affecting the expression of GAPDH. Although the concentration of DMSO was at 0.1% (v/v), which is considered safe for most cells, the early embryo appears to be more sensitive to its effects (Chaloupka, Krishnan, and Safe 1992; Chen et al. 1998; Qi, Ding, and Salvi 2008). Therefore we were not able to elucidate any important differences in the expression of GLYCOX and OXPHOS genes at the 2-cell stage.

Embryos at the 4-cell stage saw no significant difference in the expression of GLYCOX genes between FG and SG embryos (Figure 16a). We saw similar results in our second objective and therefore, this outcome is not surprising as the GLYCOX pathway is not heavily utilized at this time. However with the addition of an OXPHOS enhancer it is more likely to see a change seen in OXPHOS gene expression. At the same stage, we don’t see any difference in the expression of ATP5b, but we see significantly lower expression of COX5a in the SG embryo group. With the addition of CoQ10, this may be providing enough assistance to the SG embryos so they do not need to transcribe as much COX5a. It is also possible that complexes I, II and III would show different results than complex IV (COX5a) in response to CoQ10 treatment. In the ETC, CoQ10
transfers electrons from complexes I/II to complex III and therefore a greater effect may be seen at these locations. Shults et al. (1997) found that the activity of complexes I, II and III were increased in subjects treated with CoQ10. This activity difference was not seen in complex IV. In the future, observing the expression in earlier complexes of the chain may provide valuable insight into the effects of CoQ10.

At the 8-cell stage we found that FG embryos treated with CoQ10 had significantly lower expression of GAPDH compared to the control group, and no difference seen for GLYCOX genes in the SG group (Figure 17a). This is interesting because it could possibly suggest that FG embryos are down-regulating GAPDH in the presence of CoQ10. After the 8-cell stage the embryo begins to switch to the embryonic genome, and the embryo is typically still relying solely on the OXPHOS pathway. Even though the GLYCOX pathway is not heavily used, there are still levels of GAPDH transcription found in the early embryo. However, it is possible that with the addition of CoQ10 the embryo has a lower requirement for the transcription of GLYCOX genes. In the SG embryos at the 8-cell stage we do not see any significant differences in the HK gene in either FG or SG embryos. As CoQ10 assists in the functions of the OXPHOS pathway and the GLYCOX pathway is still down regulated, this is not surprising.

SG embryos at the 8-cell stage saw a significant increase in the expression of ATP5b in the CoQ10 treated group compared to the control group. This could possibly indicate that the SG embryos in the presence of CoQ10 are able to better use the OXPHOS pathway and are up-regulating ATP5b in response to provide adequate energy
to the embryo. In our second objective, SG embryos also displayed higher expression of ATP5b, and it appears the effects of CoQ10 may be enhancing this expression. In a study by Gendelman & Roth (2012) shows that maturations of bovine oocytes in CoQ10 supplemented media not only had an increased blastocyst rate, but increased the expression of the OXPHOS genes COXII, CYTB and ATP5b. This suggests that the embryos with impaired development may have impaired mitochondrial function. Furthermore we can speculate that, when augmented with OXPHOS enhancer CoQ10 the embryo responds with increased expression of ATP5b.

The treatment of early embryos with CoQ10 appears to improve embryonic development when vehicle concentrations are below cytotoxic levels. Stojkovic et al. (1999) treated bovine embryos using a specialized technology where the CoQ10 was supplied in submicron-sized drops. This allowed the lipophilic CoQ10 to be added to media without the use of solvents, such as DMSO or ethanol. The combination of this technique with CoQ10 treatment has the potential to be effective in improving blastocyst rate and the overall success of IVF. Studies have also found several benefits of the dietary supplementation of CoQ10. The administration of CoQ10 to patients with a primary or secondary CoQ10 deficiency showed improved mitochondrial dependent functions (Balercia et al. 2004; Chinnery et al. 2006; Quinzii, Hirano, and DiMauro 2007). A study by Ben-Meir et al. (2015) found that aged mice supplied with CoQ10 had improved mitochondrial activity, implicating insufficient CoQ10 availability with age-associated oocyte damage. Overall these findings suggest that CoQ10 is a promising treatment to help restore mitochondrial function in embryos with delayed development.
SUMMARY AND CONCLUSIONS

The significant observations of this study include:

- Our study assesses a time range for first cleavage times for slow and fast growing embryos.
- Early embryos with delayed development displayed significantly higher $\Delta \Psi_m$ than embryos developing at normal rates. This high $\Delta \Psi_m$ may be a result of a compensatory mechanism due to insufficient energy.
- Slow growing zygotes also showed a significant increase in the presence of ROS, which is likely due to the high activity of the mitochondria.
- There was no difference in the expression of GLYCOX and OXPHOS genes between slow and fast growing embryos at the 4-cell stage, but at the 8-cell stage there was an increase in the expression of ATP5b in SG embryos suggesting that SG 8-cell embryos are lacking in ATP and are up-regulating the OXPHOS pathway.
- SG morula and blastocysts had a significant increase in the expression of GAPDH. This can be explained by a potential mitochondrial impairment preventing the production of ATP and up-regulating the GLYCOX pathway in response.
- Treatment of embryos with CoQ10 resulted in a significant increase in cleavage rates.
- When treated with CoQ10, SG 4-cell embryos had significantly lower expression of COX5a. The treatment of CoQ10 may be relieving the SG embryos of the need to up-regulate OXPHOS genes.
• FG 8-cell embryos displayed lower expression of GAPDH when treated with CoQ10, which can indicate that FG embryos are receiving sufficient energy from the OXPHOS pathway with the help of CoQ10. SG 8-cell embryos treated with CoQ10 had significantly higher expression of ATP5b.

• The addition of CoQ10 may be assisting SG embryos in utilizing the OXPHOS pathway and thus up-regulating ATP5b.

Overall our results indicate that embryos developing at delayed rates have impaired mitochondria and that the embryo responds to the lack of energy production by altering the expression of GLYCOX and OXPHOS genes. This gives a better understanding of the molecular mechanisms governing delayed embryonic development. The use of CoQ10 as a potential treatment and supplementation in the practice of IVF is a promising method to improve mitochondrial function and the overall efficiency of IVF.
FUTURE DIRECTIONS

In the future, it would be beneficial to evaluate the translation of OXPHOS and GLYCOX proteins, which can be accomplished with western blotting. This will supplement our qPCR data and provide a more comprehensive view of the embryos response to mitochondrial dysfunction. Although we demonstrated valuable information regarding the expression of metabolic genes, the analysis of a higher number of genes for both the GLYCOX and OXPHOS pathway will provide a better understanding of the complicated and dynamic metabolic responses of the early embryo.

It would be useful to carry out live-stain imaging for the embryos undergoing treatment with CoQ10 to observe the effects on ΔΨm and presence of ROS. Additionally, future experiments with the supplementation of CoQ10 to media would likely benefit from the techniques described by Stojovic et al. (1999) to avoid exposing the embryos to harmful solvents.

Our study was able to demonstrate the embryo’s ability to develop and adapt to culture without exogenous glucose. However, it would be interesting and valuable to carry out a similar set of experiments with the presence of glucose in the culture media. This may also more accurately represent the embryos environment in vivo.

Finally, although the GLYCOX and OXPHOS pathways are very significant in providing most of the energy for the developing embryo, it is still important to consider
the roles of lipids, fatty acids and amino acids in producing ATP. Future studies will hopefully cover these processes in more detail to expose the dynamic metabolic interactions of the early embryo.
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