DISSERTATION

METABOLIC CHARACTERISTICS AND GENE EXPRESSION OF IN VITRO AND IN VIVO MATURED MAMMALIAN OOCYTES

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

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Fall 2006

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Oh, it's delightful to have ambitions....And there never seems to be any end to them, that is the best of it. Just as soon as you attain to one ambition you see another one glittering higher up still. It does make life so interesting.

- ANNE SHIRLEY (L.M. MONTGOMERY)

Many thanks to my family, friends, advisers, and colleagues who have helped me achieve a portion of my never-ending ambitions.

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ABSTRACT OF DISSERTATION

METABOLIC CHARACTERISTICS AND GENE EXPRESSION OF IN VITRO AND IN VIVO MATURED MAMMILIAN OOCYTES

Current in vitro maturation (IVM) systems for oocytes impair cellular function, leading to compromised developmental potential. Identification of components in IVM systems that affect cell function requires development of a defined maturation medium capable of supporting both nuclear and cytoplasmic maturation. The extent to which factors such as medium supplementation, oxygen tension, and priming oocyte donors with exogenous gonadotropins affect oocyte viability is unclear. Characterization of these aspects will facilitate optimization of a defined maturation system capable of producing oocytes of equivalent developmental competence to in vivo matured counterparts. The main objective of this thesis was to examine these factors in IVM systems and subsequent developmental rates after fertilization. Metabolic and gene expression patterns of in vitro matured oocytes were studied, and characteristics of in vivo matured oocytes were used as a baseline. A defined maturation system was developed, yielding equivalent blastocyst development to systems including blood serum. In the defined system, metabolism of individual mouse oocytes was positively correlated with subsequent developmental potential. Inclusion of EGF during maturation of mouse oocytes was correlated with increased developmental competence, while maturation under 20% O_2 instead of 5% O_2 resulted in reduced cell numbers of resulting embryos, as well as decreased oxygen metabolism and mitochondrial membrane potential. Variations in medium

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supplementation during bovine in vitro maturation altered the quantity and quality of blastocysts produced. Gene expression of bovine oocytes matured in vitro at 20% O_2 differed from that of in vivo matured oocytes. Reduced O_2 (5%) during bovine oocyte maturation resulted in similar carbohydrate metabolism and gene expression; however, oxygen metabolism was lower for oocytes matured at 5% rather than 20% O_2. These studies indicate that maturation conditions profoundly affect the quality of oocytes, as well as the quantity and quality of resulting embryos. Metabolic and gene markers will aid in development of optimal IVM systems and will serve as viability markers of oocyte quality and subsequent developmental potential.

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CHAPTER I

INTRODUCTION

In vitro maturation (IVM) of immature oocytes has been conducted in animal research and breeding programs for many years. Although results of IVM vary among species, generally the majority of in vitro matured oocytes fail to develop following fertilization. Interest regarding maturation of human oocytes has increased significantly over the last decade; however, progress in this area remains limited. According to the World Health Organization, infertility affects more than 80 million people world wide. The majority of assisted reproductive technologies involve ovarian stimulation through administration of exogenous gonadotropins. Disadvantages to stimulation with exogenous gonadotropins include high treatment cost, extensive physician monitoring, medication side effects, and risk of ovarian hyperstimulation. The development of a successful in vitro maturation system (IVM) would allow collection of immature oocytes from patients not undergoing ovarian stimulation. Although in vitro maturation is currently being offered by some human fertility clinics, success rates are low, suggesting that current systems of in vitro maturation do not adequately support nuclear and/ or cytoplasmic maturation of the immature oocyte. In addition to benefits to human clinical laboratories, research

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laboratories and animal breeding programs would greatly benefit from an optimized maturation system.

Current in vitro maturation systems impair cellular function, leading to compromised developmental potential of the oocyte. The development of a successful IVM system requires a thorough understanding of the physiology of the oocyte during maturation. Removal from the follicle dramatically alters the hormonal environment of the oocyte, resulting in varying degrees of abnormality. The objective of an IVM system is to limit the degree of insult to the oocyte, while supporting nuclear, cytoplasmic, and molecular maturation.

Many factors affect the success of an IVM system. Prior to oocyte retrieval, oocyte quality may be impacted by administration of exogenous gonadotropins. Once oocytes are recovered, factors such as medium composition, source of macromolecule, pH, temperature, and oxygen tension can dramatically influence oocyte quality and subsequent development. Several groups have studied oocyte maturation in mice (Downs et al., 1998), cattle (Lonergan et al., 1994), pigs (Abeydeera et al., 2000) and humans (Mikkelsen et al., 1999). Unfortunately, a significant amount of research on oocyte maturation has been conducted under ill defined conditions, due to the inclusion of serum in the maturation medium, leading to inconsistencies in results between laboratories and an incomplete understanding of oocyte physiology. The development of a defined maturation system will facilitate analysis of specific effects of growth factors,

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gonadotropins, and alternative additives to be assessed in terms of oocyte maturation and subsequent embryo development.

The development of an optimized IVM system will require a rapid, sensitive and reliable method to evaluate oocyte quality following maturation. Currently IVM systems are assessed through the evaluation of oocyte morphology, fertilization rates and subsequent embryo development. Quantitative analysis of metabolism, mitochondrial membrane potential and gene expression could potentially provide markers of oocyte viability and provide a more comprehensive understanding of the effects of IVM conditions on oocyte physiology. Establishment of baseline characteristics of oocyte physiology with in vivo matured oocytes will provide valuable information regarding any aberrant characteristics displayed by oocytes matured under different IVM conditions. Identification of components of IVM systems that affect cell function will aid in the optimization of the IVM conditions that ultimately will produce oocytes of equivalent developmental competence to their in vivo matured counterparts.

The use of quantitative markers will not only increase our understanding of oocyte physiology in order to develop a more optimized IVM system, but they could also serve as a tool for selecting the healthiest oocytes. For clinical laboratories, non-invasive techniques, such as carbohydrate and oxygen metabolism, will provide the opportunity to assess oocyte viability without compromising fertilization and development. Non-invasive techniques for evaluating oocytes, followed by established techniques to evaluate embryo viability, will greatly enhance the selection of the most viable embryos

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for transfer. Transfer of viable embryos will increase the number of successful single embryo transfers and ultimately eliminate the risk of multiple pregnancies.

The main objective of this research was to characterize the metabolism and gene expression of in vivo and in vitro matured oocytes. To achieve this goal, a defined maturation system was modified to support the meiotic and developmental competence of the immature murine and bovine oocyte. In addition, immature oocytes from donors primed with exogenous gonadotropins and unprimed donors were collected, matured and evaluated, thus yielding information regarding all types of oocytes currently being recovered in both human and animal laboratories.

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CHAPTER II

LITERATURE REVIEW

Oocyte Maturation in Mammals

Maturation of oocytes involves several complex changes in the nucleus, as well as several less studied but not less important changes in the cytoplasm. The resumption of meiosis is blocked at the germinal vesicle (GV) stage at the end of the first meiotic division (Sorensen, 1973). The first visible sign of resumption of meiosis is germinal vesicle breakdown (GVBD; Donahue, 1968; Szollosi et al., 1972). In vivo, oocytes are arrested at the GV stage until a signal is received that will overcome the inhibiting factors maintaining arrest (Downs, 1998). In vitro, however, the oocyte resumes meiosis following removal from the follicle (Pincus and Enzmann, 1935), suggesting that there are inhibitors of meiotic resumption in the follicular environment. After resumption of meiosis, the oocyte develops to metaphase II (MII) when half of the chromosome complement is extruded in the first polar body. Furthermore, the complex process of maturation is accelerated in vitro compared to in vivo. For example, in bovine oocytes GVBD occurs between 3-6 h following removal from the follicle; however, in vivo this process takes between 7-10h following the LH surge (Sirard et al., 1989; Hyttel et al., 1986). As many cellular events must occur within a short time interval, in vitro

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maturation systems must be optimized to support both nuclear and cytoplasmic maturation to achieve the equivalent viability of in vivo matured oocytes. The development of an optimal maturation system will require a thorough understanding of the cellular events, both nuclear and cytoplasmic, that take place during maturation.

Regulation of meiotic arrest

Within the follicle, the oocyte is held in the GV stage by inhibitory signals that originate from the theca and granulosa cells (Whitaker, 1996). These inhibitory signals are positively influenced by the hormonal environment and are transported to the oocyte via gap junctions and follicular fluid (Thibault et al., 1987). Inhibitory molecules in follicular fluid were evidenced when bovine oocytes were matured in follicular fluid obtained either prior to or 20 h post LH surge. Increased development was observed following maturation in follicular fluid obtained post LH surge (Romero-Arredondo and Seidel, 1996). In addition to follicular fluid, a meiosis arresting molecule, oocyte meiotic inhibitor (OMI), is present in the granulosa cells (Thibault et al., 1987). High levels of cyclic adenosine monophosphate (cAMP) within the oocyte maintain arrest (Conti et al., 1998). Gonadotropins stimulate cAMP production in the granulosa cells, and this cAMP is transported to the oocyte via gap junctions (Sunahara et al., 1996). In the oocyte, cAMP activates protein kinase A (PKA), which is inhibitory to oocyte maturation (Corbin et al., 1975). A decrease in cAMP is required for resumption of meiosis. Phosphodiesterases (PDE) inactivate cyclic nucleotides, in this case cAMP, by hydrolytically cleaving the 3'phosphodiesterase bond to form an inactive product (Tsafriri et al., 1996). Inactivation of PKA and the subsequent reinitiation of meiosis are

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thought to be due to a decrease in cAMP resulting from hydrolytic cleavage by PDE (Downs and Hunzicker-Dunn, 1995; Shitsukawa et al., 2001).

Resumption of meiosis

Upon removal from the follicle, the immature oocyte is arrested at the GV stage. The GV stage is characterized by diffuse chromatin surrounded by a nuclear membrane (Calarco et al., 1972). The first sign of resumption of meiosis is breakdown of the germinal vesicle (Donahue, 1968; Sorensen, 1973). The lamins of the nuclear envelope are broken down until the nuclear envelope disappears. Chromosome condensation occurs at the same time, which implies cessation of transcription (Szollosi et al., 1972).

Resumption of meiosis is dependent on maturation promotion factor (MPF; Nurse, 1990). Maturation of oocytes and mitosis of somatic cells is regulated by MPF. Activity of MPF changes throughout the cell cycle (Wasserman and Smith, 1978). There is an increase in MPF during metaphase I (MI) and MII, while there is a decrease in MPF levels when oocytes enter interphase between MI and MII (Nurse, 1990). Understanding of the role of MPF has been gained through the study of the cell cycle in the yeast, *S. pombe*. Components of MPF in *S. pombe* have extensive sequence homology with mammalian components, and they are thought to have similar structural and regulatory mechanisms (Lodish et al., 2000). The accumulation of MPF occurs in G-2 of the cell cycle, simultaneously with the increase in the 2 subunits of MPF: a homolog of $P34^{cdc2}$ and cyclin B (Figure 1; Jessus and Beach, 1992).

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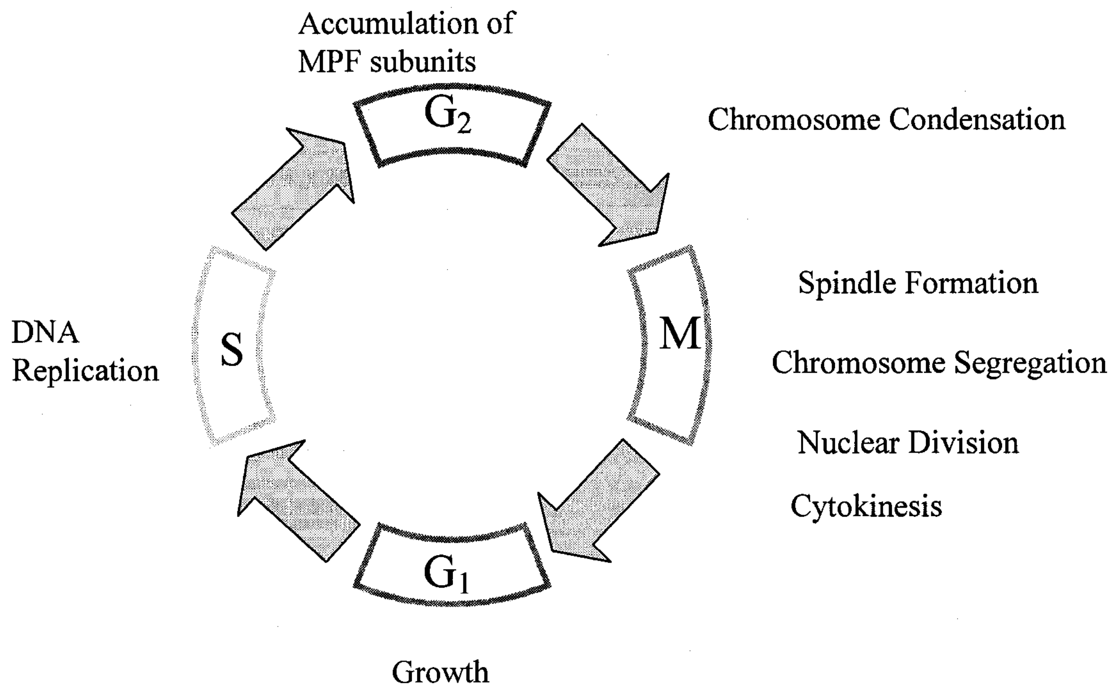
Figure 1. Main events of the mitotic cell cycle.



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Cyclins are proteins that occur at markedly different concentrations at different stages of the cell cycle. Cyclin dependent kinases form heterodimers with cyclins and are activated by specific phosphorylations or dephosphorylations (Lodish et al., 2000). Once active, cyclin dependent kinases phosphorylate specific target proteins that control the cell cycle.

The activation of MPF is regulated by a cascade of phosphorylations. The first event is the fusion of cyclin B and $P34^{cdc2}$ subunit (Figure 2; Jessus and Beach, 1992). Once bound to cyclin B, the catalytic $P34^{cdc2}$ subunit can be phosphorylated at 2 regulatory sites: Tyrosine-15 (Y15) and Thereonine-161 (T161; Gautier et al., 1988). The Wee1 protein kinase first phosphorylates Y15, followed by cdc2 activating kinase which phosphorylates T161 (Lodish et al., 2000). Only when $P34^{cdc2}$ is monophosphorylated at T161 is MPF active. Therefore, the final step required for MPF activation is the dephosphorylation of Y15 by cdc25 (Galaktinov and Beach, 1991).

Active MPF phosphorylates target proteins such as nuclear lamins, histones, cohesin, and the myosin light chain (Gautier et al., 1988). Phosphorylation of nuclear lamins by MPF leads to nuclear envelope breakdown (Parrish et al., 1992). Cohesin, when phosphorylated, holds the sister chromatids together and phosphorylation of the myosin light chain holds the chromatin at the metaphase plate (Lodish et al., 2000). The diversity of phosphorylation targets of MPF can explain the coordination between events during meiosis. The affinity of MPF for the proteins such as laminae B, histone H1 and certain proteins associated with microfilaments are directly responsible for GVBD, chromosome condensation, and remodeling of the cytoskeleton (Lodish et al., 2000). MPF is equally

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responsible for phosphorylation of RNA polymerase II and elongation factors; therefore, inducing the modifications of protein synthesis observed in meiosis resumption (Parrish et al., 1992).

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Figure 2. Interaction of pathways necessary for the normal progression of meiosis. MPF is activated by a series of phosphorylations and dephosphorylations (right), sending a positive signal for GVBD. MAP kinase pathway (lower left) interacts with MPF and through a series of phosphorylations stimulates nuclear maturation (MII). In addition, MAPK activates c-mos/CSF (lower/ middle left) which maintains MII arrest through the inhibition of Anaphase Promotion Complex (APC; top center). Calcium (top left) generated from oocyte activation following fertilization activates calpain, which degrades CSF and releases the oocyte from MII arrest. Upon degradation of CSF, APC is activated, leading to cyclin B degradation and MPF inactivation.

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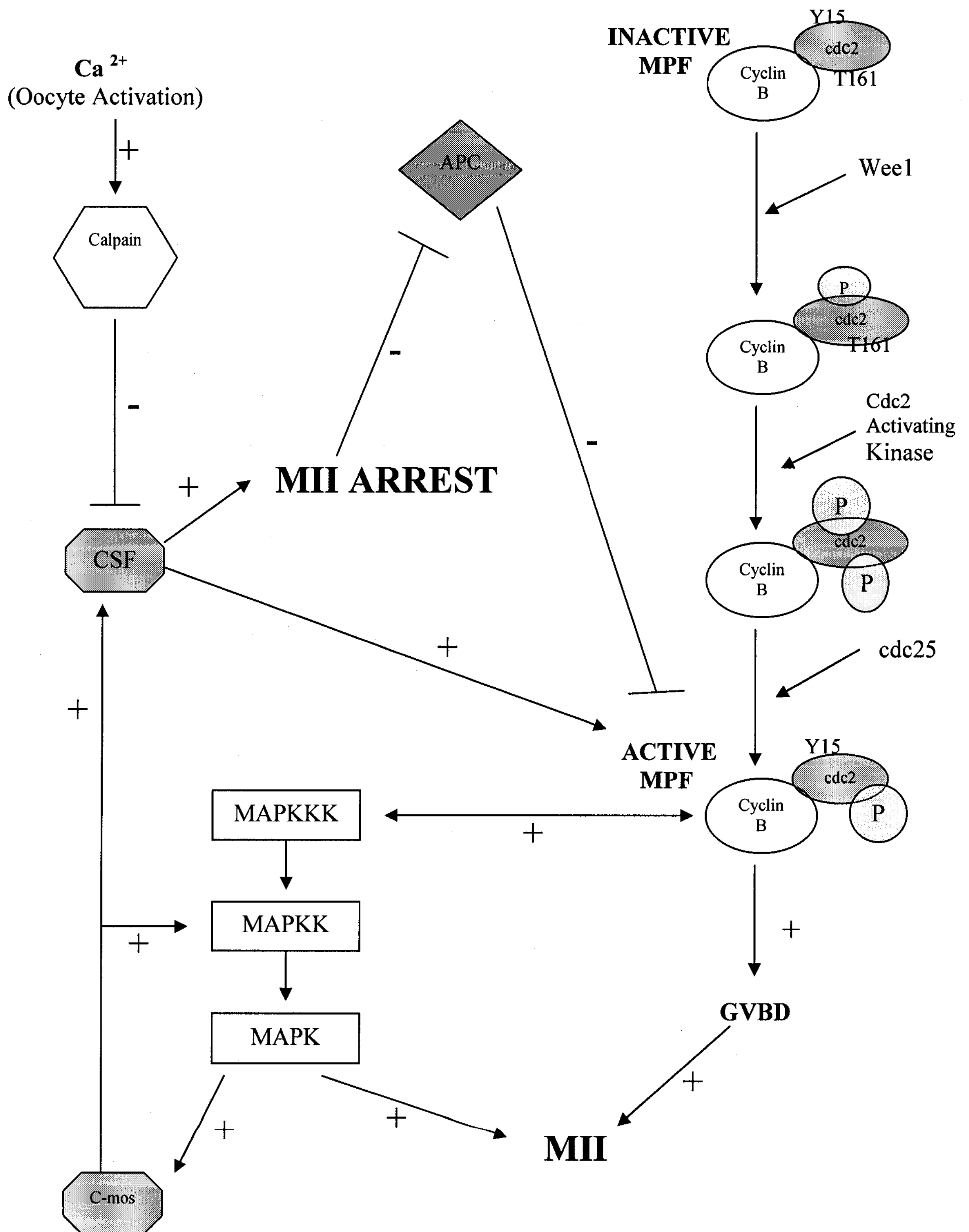
Figure 2. MPF and MAPK regulation of meiosis.



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MAPK and MPF interaction

One pathway that is integral in the maturation process is that of mitogen activated protein kinase (MAPK; Figure 2). A complex cascade of kinases results in the activation of MAPK, starting with MAP kinase kinase (MAPKK), which is phosphorylated by MAP kinase kinase kinase (MAPKKK; Gotch et al., 1995). The targets of MAPK are numerous cytosolic and nuclear proteins (Posada et al., 1993; Colledge et al., 1994). Nuclear targets act via transcriptional regulation (Segar and Krebs, 1995), while cytosolic targets include cystoskeletal proteins (Verlhac et al., 1993) and lamins (Peter et al., 1992). There is an increase in kinase activity simultaneous with the occurrence of GVBD, chromatin condensation, spindle assembly and progression to MII (Crosby et al., 1994; Bornslaeger et al., 1986). MPF is responsible for the phosphorylation of many proteins involved in those functions; however, there is a decrease in MPF activity between MI and MII, suggesting that other kinases are responsible for chromatin activity (Figure 3; Kubiak et al., 1992). MAPK activity is elevated throughout maturation and targets proteins involved in chromosomal condensation, as well as microtubule organization and spindle formation (Smitz et al., 2004).

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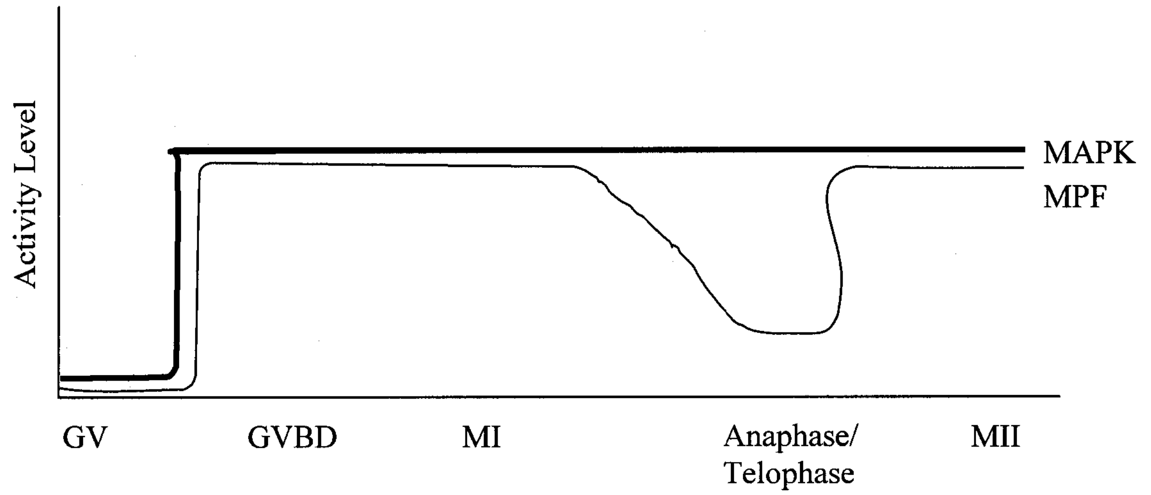
Figure 3. Pattern of MPF and MAPK activity within oocytes through the stages of maturation.



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Maintenance of MII arrest

Following the extrusion of the first polar body, mature oocytes arrest at MII prior to fertilization. Failure to arrest at MII results in parthenogenic activation of the oocyte. MII arrest requires high levels of active MPF, accumulated during maturation. Degradation of cyclin B results in deactivation of MPF and release from MII arrest (Figure 2; Taieb et al., 1997). The factor responsible for degradation of cyclin B is anaphase promoting complex (APC, Turnquist and Maller, 2003). Therefore, to hold oocytes in MII arrest, a factor must be responsible for the inhibition of APC; this factor is cytostatic factor (CSF, Masui and Markert, 1971). Cytostatic factor, a heavily phosphorylated protein kinase, stabilizes MPF (Taieb et al., 1997). Cytostatic factor is destroyed by calpain, a calcium dependent kinase. Calpain is activated by an increase in intracellular free calcium triggered by oocyte activation following fertilization (Lodish et al., 2000).

Through gain and loss of function experiments, a potential candidate for CSF activity is c mos (Sagata et al., 1989). Mos is a germ-cell specific MAPK kinase kinase synthesized during oocyte maturation in response to progesterone administration (Turnquist and Maller, 2003). Unfertilized oocytes from $mos^{-/-}$ mice undergo parthenogenic activation due to failure to arrest at MII (Colledge et al., 1994; Hashimoto et al., 1994). The mos/MEK/MAPK pathway has been implicated in the activation of MPF (Figure 2; Tong et al., 2003). This same pathway has been implicated by several researchers as essential for the activation of CSF (Jones, 1994; Chen and Cooper, 1995; Maller et al., 2002). Each component of the mos/MEK/MAPK pathway is a necessary requirement to

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establish MII arrest in oocyte extracts (Sagata et al., 1989). Activation of the MEK/MAPK cascade maintains MII arrest in mouse oocytes, as the presence of the MEK inhibitor U0126 resulted in the release from MII arrest, as well as MAPK dephosphorylation (Tong et al., 2003).

Cytoplasmic maturation

Although nuclear maturation may be successful, numerous events occurring in the cytoplasm are required for developmental competence. Developmental competence is the ability of an oocyte to be fertilized and develop into a viable embryo. During the growth phase, arrested oocytes accumulate large stores of mRNA and proteins that function after fertilization to support and regulate preimplantation embryonic development (De Sousa et al., 1998). Immature oocytes recovered from small follicles have not yet completed cytoplasmic maturation. Although such oocytes can complete meiosis, because of cytoplasmic deficiencies they fail to develop after fertilization (Hendriksen et al., 2000).

Characterized events of cytoplasmic maturation include cortical granule migration to the periphery of the plasma membrane after resumption of meiosis (Lodish et al., 2000), enlargement of the perivitelline space and rearrangement of membrane-bound vesicles and mitochondria, which give the mature oocyte a polarized appearance (Grondahl et al., 1995). Additional cytoplasmic events include mRNA transcription (Hunter and Moor, 1987), protein translation (Sirard et al., 1989), and post-translational modification of proteins (Levesque and Sirard, 1995). Some genes transcribed during growth of the

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oocyte are involved in the progression to MII (Wickramasinghe and Albertini, 1993) while other gene transcripts and proteins are involved in cellular processes for development post fertilization (Barron et al., 1989; Watson et al., 1999).

Oocyte maturation is a lengthy process, requiring coordination of nuclear and cytoplasmic events, culminating in a mature oocyte capable of fertilization and subsequent embryo development. As the process of in vitro maturation alters the physiological environment of the oocyte, careful consideration must be taken when developing an in vitro maturation system to ensure minimal negative effects on the oocyte. Many factors affect oocyte quality, such as medium components and oxygen tension. However, aberrant effects are not only induced by conditions to which the oocytes are exposed following removal from the follicle, but also from the hormonal conditions within the follicle prior to oocyte retrieval. Therefore, in order to optimize a maturation system one must begin with understanding the impact of hormonal manipulation of oocyte donors on subsequent oocyte quality.

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Superovulation

Superovulation is routinely used in both research and clinical laboratories. The use of exogenous gonadotropins is attractive as the number of oocytes and embryos recovered from a donor are dramatically increased, thus allowing greater opportunity for success after a single cycle. Oocytes recovered from superovulated mice have been reported to have higher embryo development rates than oocytes recovered from non-superovulated mice (De la Fuente et al., 1999). Calves born following transfer of embryos recovered from superovulated cows were similar to non-embryo transfer calves (King et al., 1985). Similarly in humans, use of superovulation protocols has resulted in the birth of thousands of healthy babies over the last 28 years. Although the use of exogenous gonadotropins increases the quantity of oocytes and embryos per cycle, it is important not to overlook evidence suggesting that the quality of these oocytes and embryos are compromised. Understanding the benefits and costs of superovulation will aid in development of improved stimulation protocols.

Numerous reports have cited benefits of exogenous stimulation in both animals (Schramm and Bavister, 1994) and humans (Gomez et al., 1993; Wynn et al., 1998; Junk et al., 2003), with regard to the increase in number of oocytes collected and subsequent improvement in maturation rates, when compared to oocytes recovered from non-stimulated donors. Further reports, however, suggested that although maturation rates may be improved, subsequent developmental competence was not enhanced by priming with exogenous gonadotropins in mice (Junk et al., 2003), bovine (Moor et al., 1984; Blondin et al., 1996), and human (Mikkelsen et al., 1999).

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Exogenous gonadotropins have been shown to increase follicular size and decrease follicular atresia (Blondin et al., 1996) supporting the idea that superovulation alters the normal maturation process of the oocyte (Hyttel et al., 1986; Dieleman and Bevers, 1993). In vivo, oocytes undergo the final process of nuclear and cytoplasmic maturation only hours prior to ovulation (Dieleman and Bevers, 1993). Follicular recruitment through exogenous FSH, therefore, not only changes the hormonal environment of the maturing oocyte, but causes variation in timing of ovulation (Allen and McLaren, 1971); therefore, oocytes may lack sufficient time to acquire developmental competence.

Embryonic development has been examined extensively with respect to superovulation. Increased pre-implantation mortality has been reported following superovulation in mice (Beaumont and Smith, 1975; Ertzeid and Storeng, 1992), rats (Miller and Armstrong, 1981), and hamsters (Mckiernan and Bavister, 1998). Embryos that implant frequently do so in a delayed or impaired manner (Ertzeid et al., 1993), and fetuses surviving to birth often have low birth weight and fetal growth retardation (Ertzeid and Storeng, 1992). Decreased developmental competence of embryos recovered from superovulated donors was associated with chromosome abnormalities in murine embryos (Elbling and Colot, 1985; Luckett and Mukherjiee, 1986) and rat embryos (Tain et al., 2000). Additionally, gonadotropins are involved in meiosis control (Picton et al., 1998), as well as in regulating gene expression (Warner et al., 1998). Therefore, exposure of oocytes to supra-physiological levels of gonadotropins results in altered oocyte physiology, subsequently altering embryo quality.

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Exogenous gonadotropins not only affect the follicle and oocyte, but also alter the uterine environment. In animal models this physiological response is often not of concern, as oocytes and embryos collected from superovulated donors are replaced into non-superovulated recipients; however, for humans, the donor and recipient are usually synonymous. Successful embryonic implantation depends not only on embryo quality, but also uterine receptivity and the synchronization of embryo and endometrium (Ertzeid and Storeng, 2001). Higher implantation rates were reported when embryos from superovulated mice were transferred into non-superovulated recipients (Fossum et al., 1989). Also, post implantation fetal mortality was higher and average fetal birth weights were lower when embryos were transferred into superovulated recipients (Ertzeid and Storeng, 2001; Kelley et al., 2006). Rats superovulated with PMSG had increased pregnancy failure due to changes in the uterine environment (Walton et al., 1982). Similarly in women, high estradiol levels following superovulation were correlated with decreased implantation and pregnancy rates (Pellicer et al., 1996; Ng et al., 2001).

It is evident from these studies that exogenous gonadotropic stimulation affects oocyte and embryo quality, oviduct and uterine environment, as well as the synchrony between the embryo and endometrium at implantation. Additionally, oncology patients with steroid dependent tumors wishing to preserve their fertility prior to undergoing chemotherapy are unable to receive exogenous FSH, as the gonadotropins will exacerbate the cancer. Furthermore, the use of exogenous gonadotropins substantially increases the cost of an IVF cycle, requires extensive physician monitoring, and places particular patients at risk for ovarian hyperstimulation syndrome (OHSS). OHSS is a complication

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of ART that can range from mild to severe, with the overall occurrence between 0.6 to 14% of IVF cycles (Brinsden et al., 1995; Al-Ramahi, 1999; Fluker et al., 2000). The most severe cases of OHSS occur at a rate of 0.2 to 5% of IVF cycles (Navot et al., 1992; Al-Ramahi, 1999; Forman, 1999), with a few cases (approximately 1 in 450,000 to 500,000) resulting in death (Brinsden et al., 1995). Therefore, as a result of the negative physiological, emotional, and financial aspects of superovulation, the field of human ART requires either a modification of stimulation protocols, or a shift to the development and optimization of protocols which do not involve superovulation of the donor.

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Origin of Oocyte

Follicular development in a monovular species is characterized by recruitment of primordial follicles for an extended period of follicular growth, selection of a dominant follicle for ovulation and atresia of the remaining cohort. In non-rodent species, the ability to resume meiosis is acquired when follicle diameter is 9-13% of ovulation diameter (Gilchrist et al., 1995). The ability to complete meiotic maturation and developmental competence is acquired progressively as follicle diameter increases (Eppig et al., 1992). Cow oocytes from follicles > 6mm have increased developmental potential than oocytes from 2-6 mm follicles (Lonergan et al., 1994). Cow oocytes recovered from follicles <3mm have poor maturation and fertilization rates and fail to cleave beyond 8-16-cell stage (Pavlok et al., 1992; Blondin and Sirard, 1995).

Not only does follicular size impact developmental competence, but the source of oocytes also impacts developmental rates. Abattoir ovaries are recovered from animals of various ages, health, and stage of cycle. Typically, developmental rates of bovine COCs recovered from abattoir ovaries are between 30 and 40% (Lechniak, 2002). However, when oocytes were recovered by transvaginal aspiration following administration of FSH and a 48h coasting period, there was no difference in embryo development between in vitro matured and in vivo matured oocytes (Bousquet et al., 1999). It is thought that the intrinsic quality of the oocyte determines the proportion of oocytes that develop to the blastocyst stage (Rizos et al., 2002). In addition to initial quality of oocytes, subsequent maturation conditions also greatly affect the developmental competence of in vitro matured oocytes.

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Conditions of Oocyte Maturation

Many factors, such as pH, temperature, osmolality, and oxygen tension, affect success of an in vitro maturation system (Eppig and Wigglesworth, 1995; Sutton et al., 2003). The components of maturation media are also key factors in determining the quality of oocytes following IVM (Rose-Hellekant et al., 1998). Most maturation media are composed of varying concentrations of salts, amino acids, vitamins, and carbohydrates. Supplements to maturation media vary greatly between species and between laboratories (Merriman et al., 1998; Rieger et al., 1998; Roberts et al., 2002); however, no consensus has been reached regarding optimal supplementation. In addition to media, oxygen tension during culture can also impact the quality of the oocyte. Reduced oxygen tension during culture has been shown to improve embryo development (Quinn and Harlow, 1978; Thompson et al., 1990; Batt et al., 1991; Noda et al., 1994; Gardner and Lane, 1996); however, optimal oxygen tension for maturation remains to be determined. Much research has been performed on maturation conditions; however, the low blastocyst development of IVP embryos indicates that the system is less than optimal.

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Components of Maturation Medium

Serum

Components of maturation media and conditions of maturation affect meiotic regulation of mammalian oocytes (Kito and Bavister, 1997; Downs and Mastropolo, 1997). Commonly, maturation media are supplemented with blood serum or albumin (Younis et al., 1989; Bavister, 1992; Thompson et al., 2000). Generally, it is perceived that serum can compensate for whatever essential elements are missing from the medium. Serum is comprised of a complex mixture including metabolites, hormones, growth factors and macromolecules (Perloff et al., 1955; Caravaglios and Cilotti, 1957). However, serum can be derived from different sources, resulting in significant variation in composition of amino acids, hormones, growth factors, cytokines, and vitamins. For example, quantity and quality of components are altered by species, age, health, and nutrition of the donor animals. Serum can vary greatly from batch to batch, even when acquired from the same supplier (Sirard and Lambert, 1985); therefore, the effectiveness on in vitro maturation and in vitro fertilization will be inconsistent. This variability leads to poor reproducibility of results between and within laboratories.

Postulated roles of serum are as a fixed nitrogen source, a chelator of toxic metal ions and an antioxidant (Gardner, 1994). The inclusion of serum in oocyte maturation medium is also postulated to provide a source of albumin that acts as a free radical scavenger (Downs et al., 1986; Dunglison et al., 1995; Keskintepe et al., 1995). Additionally, serum protein present in the medium prevents oocytes and embryos from sticking and floating (Chanson et al. 2001). However, unknown substances and contaminants may be

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present in serum (Barnes and Sato, 1980), thus making it difficult to accurately define and control the contents of maturation medium. A quantitative understanding of cellular physiology can not be obtained with ill defined and variable conditions. Since serum contains components that provide protection to oocytes and embryos during culture, a better understanding of the beneficial components is required, therefore allowing for the inclusion of these specific components in a defined medium while excluding potential contaminants. To increase our understanding of specific nutrient and hormonal requirements involved in both nuclear and cytoplasmic maturation, a shift to a defined maturation system is required.

In addition to batch variability and limitations regarding the understanding of oocyte physiology, the inclusion of serum in embryo culture medium has been correlated with pathological development (Thompson et al., 1995; Young et al., 1998; McEvoy et al., 2000; Khosla et al., 2001). In sheep, in vivo derived zygotes cultured in the presence of 20% serum resulted in fetuses that were significantly larger than their in vivo counterparts or embryos cultured without serum (Sinclair et al., 1999). These size differences were proportionally greater at d125 of gestation than at d61, therefore indicating altered growth patterns resulting from the treatment differences during early embryonic life. In the bovine, no obvious ultrastructural changes were observed in embryos up to the 8-cell stage, but an increase in large lipid droplets was observed in morula that were cultured in the presence of serum compared with morula cultured without serum (Abe et al., 1999). The same study found that blastocysts had increased lipid accumulation in the trophectoderm cells when compared to in vivo blastocysts, a

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finding confirmed by De la Torre Sanchez et al. (2006). Serum has been shown to decrease blastocyst cell number and increase apoptotic activity in bovine embryos (Byrne et al., 1999). In addition to morphology, serum alters metabolic activity of embryos, resulting in the increase in lactate production when compared to in vivo or embryos cultured in a defined medium (Gardner et al., 1994).

It is clear that serum negatively impacts embryonic development; however, the exact nature through which serum exerts an effect on the embryo is not yet known. There is some evidence that serum has a detrimental effect on mitochondria. Compact morula produced in the presence of serum had fewer mitochondria, but more lipid than in vivo embryos (Crosier et al., 2000). Mitochondrial degeneration has also been reported at the blastocyst stage when embryos are cultured in serum (Dorland et al., 1994; Thompson et al., 1995). Damage to mitochondria may lead to altered oxidative phosphorylation, as well as potentially impair utilization of lipid reserves (Dorland et al. 1994; Leese et al., 1998). Increased glycolytic activity observed in embryos cultured in serum may reflect this mitochondrial damage (Gardner et al., 1994; Leese et al., 1998). An increase in triglyceride formation has also been reported in bovine embryos cultured with serum (Ferguson and Leese, 1999; McEvoy et al., 2000), which has been associated with increased risk of oxidative damage and impaired mitochondrial function (McEvoy et al., 2001). In addition, the inclusion of serum in mouse embryo culture medium was correlated with altered expression of imprinted genes (Khosla et al., 2001). Perhaps these events, along with other less characterized events, contribute to the developmental issues observed in later embryonic development following embryo culture with serum.

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With all the negative evidence associated with the use of serum in culture medium, a shift to an effective alternative is necessary. In serum-free medium we can begin to understand how other conditions of maturation impact oocyte physiology, gene expression and development.

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Gonadotropins: Follicle Stimulating Hormone and Luteinizing Hormone

Nuclear maturation is frequently achieved by in vitro matured oocytes; however, poor embryonic development suggests that cytoplasmic maturation is inadequate. Removal of the oocyte from the follicle stimulates GVBD (Pincus and Enzmann, 1935); however, a positive signal may be required to initiate cytoplasmic maturation. Gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), have been shown to regulate oocyte maturation in mice (Eppig, 1979), cattle (Younis et al., 1989; Zuelke and Brackett, 1990; Saeki et al., 1991), sheep (Moor and Trounson, 1977), pigs (Okazaki et al., 2003; Ye et al., 2005), and humans (Anderiesz et al., 2000; Hreinsson et al., 2003).

In vivo, FSH plays a critical role in selection and development of the dominant follicle. FSH controls selection of the dominant follicle by stimulating FSH receptors (FSHR) which are localized on cumulus cells (Zeleznik et al., 1974; Amsterdam et al., 1975; Richards and Midgley, 1976). The FSHR is a transmembrane receptor that regulates the activity of heterotrimeric G proteins (Abou-Issa and Reichert, 1979). The binding of FSH to FSHR results in the conformational change of FSHR, which subsequently triggers a cascade of signal transductions (Ford and LaBarbera, 1988; Figure 4). First, GDP is exchanged for GTP, leading to the activation of the Gά subunit of the G protein. The Gά-GTP subunit interacts with adenylate cyclase (AC) to generate cAMP. The cAMP binds to PKA, releasing the catalytic subunits of PKA, which subsequently phosphorylates cAMP response element binding protein (CREB). This protein binds to upstream DNA regulatory elements (cAMP response element; CRE) where it regulates gene expression.

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The FSH control of gene activity in the granulosa cells is the basis of dominant follicle growth and development (Zeleznik et al., 1974).

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Figure 4. Illustration of the signal transduction cascade following gonadotropin stimulation in vivo. Prior to follicle selection, LH receptors (LHRs) are located predominantly on the theca cells. LH stimulation of these receptors leads to the production of androstendione, which subsequently enters the granulosa cell, where it is converted to estradiol 17 beta. FSH receptors are located on granulosa cells. Stimulation of these receptors results in the activation of PKA. Through a series of steps, PKA enhances mitosis of the granulosa cells and stimulates the expression of LHR on the granulosa cells. In addition, gene regulation of P450 aromotase is enhanced by PKA, which is involved in the conversion of androstendione to estradiol 17 beta.

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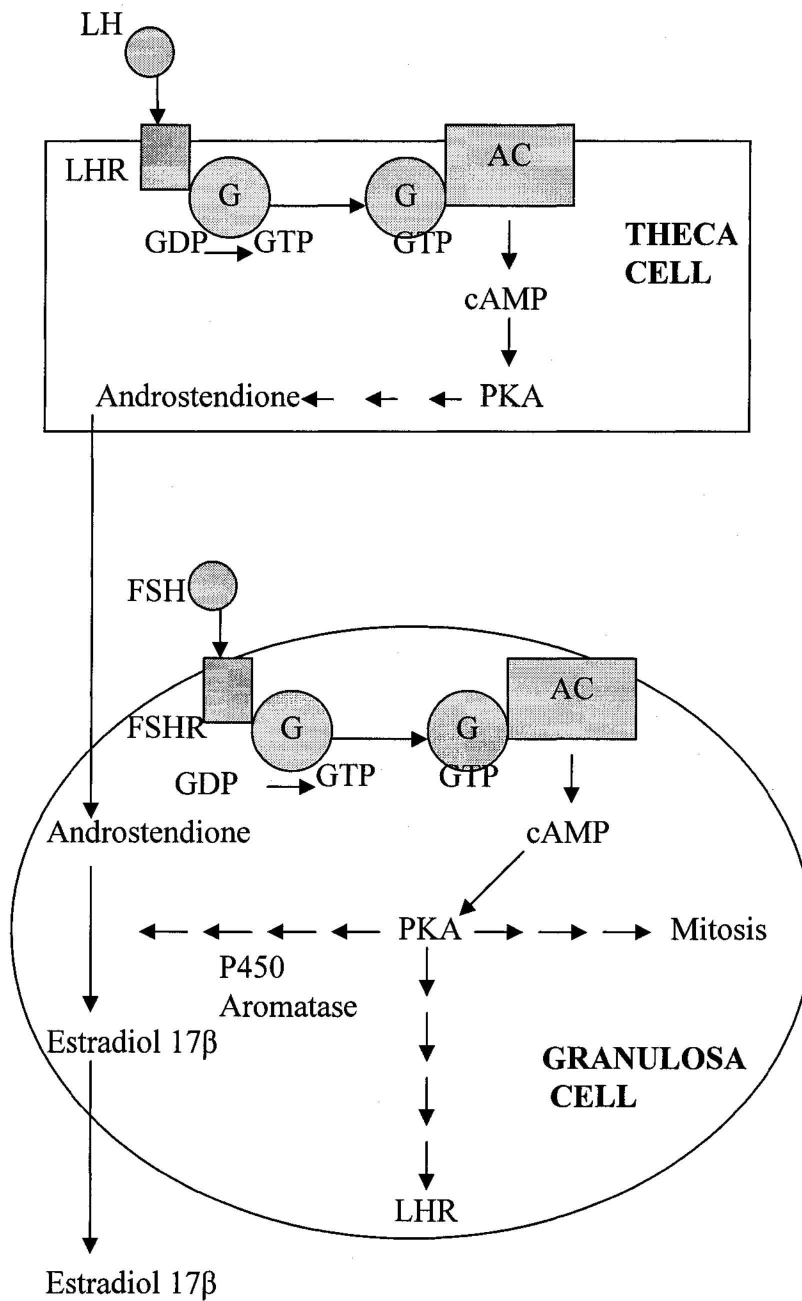
Figure 4. Signal transduction following gonadotropin stimulation of theca and granulosa cells.



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In vitro, it is thought that FSH acts by stimulating cAMP in the isolated COC, in the same manner as it does in vivo (Eppig, 1979). As FSH is an important stimulator of extra cellular matrix (Salustri et al., 1989), the addition of FSH to maturation medium of COCs results in mucification and cumulus cell expansion (Buccione et al., 1990). FSH has been shown to not only improve maturation, but also in vitro fertilization (IVF) and subsequent embryo development in mammals (Merriman et al., 1998); therefore, FSH appears to enhance not only nuclear maturation, but also cytoplasmic maturation of in vitro maturated oocytes.

In vivo, oocytes are exposed to both FSH and LH. The ability of the pre-ovulatory follicle to respond to the LH surge by undergoing ovulation involves the expression of LH receptors (LHR) on the theca and granulosa cells. Like FSH, the LHR is a G protein coupled receptor. When LH binds the LHR, a similar signaling pathway as FSH is activated, leading to phosphorylation of CREB, and modulation of gene activity. However, LH signaling pathways in the theca cells are involved in the activation of genes in the biochemical pathway leading to androstendione biosynthesis (Figure 3). In addition, LH stimulation of theca and mural granulosa cells is proposed to stimulate the production of EGF-like growth factors (Park et al., 2004).

The inclusion of hypophysial LH in maturation medium without serum has been shown in some cases to enhance maturation, fertilization and blastocyst development (Younis, 1989; Zuelke and Brackett, 1990; Saeki et al., 1991). However, other groups have found that recombinant LH (Anderiesz et al., 2000) or hypophysial LH (Choi et al., 2001; Ali

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and Sirard 2002) do not increase blastocyst development. Differences in findings may be due to the source of LH, as unpurified forms of LH may be contaminated with FSH or other growth factors that have been linked to enhanced blastocyst development (Harper and Brackett, 1993; Rieger et al., 1998).

In many cases, FSH and LH are combined in maturation medium. The combination of FSH and LH enhance maturation and early development in bovine (Saeki et al., 1991) and rat oocytes (Vanderhyden and Armstrong, 1990). However, other reports have found no benefit of gonadotropins on subsequent blastocyst development in cattle (Choi et al., 2001). Differences in results could be due to variations in base culture medium or concentration of gonadotropins. For example, in the literature FSH supplementation ranged from 1 ng/ml (Calder et al., 2003) to 10 μg/ml (Bing et al., 2001), and LH supplementation ranged from 0.1 IU/ml (Accardo et al., 2004) to 10 IU/ml (Anderiesz et al., 2000). Other sources of variation between studies may be due to the source and purity of the gonadotropins. The use of recombinant gonadotropins will eliminate some of the variation between studies, but not all variation due to different degrees of gylcosylation. Maturation rates of sheep oocytes were similar with hypophysial or recombinant gonadotropins (LH and FSH); however, blastocyst development was higher with hypophysial gonadotropins (Accardo et al., 2004). In the mouse, recombinant gonadotropins, either singly or in combination, did not improve development rates; however, a combination of rFSH and rLH at ratio of 1:10 did improve the developmental competence of human oocytes (Anderiesz et al., 2000). As hypophysial gonadotropins are unpurified, they may be contaminated with other growth factors that have been linked

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to enhanced blastocyst development (Harper and Brackett, 1993; Rieger et al., 1998); therefore, additional supplementation of medium with growth factors may result in enhanced developmental rates across species.

Benefits of gonadotropins in maturation medium may not only be concentration dependent, but also time dependent. Interestingly, LH alone stimulated oocyte maturation and increased blastocyst development in the mouse; however, when LH was added 2 h after culture with FSH, blastocyst development significantly increased to equal in vivo matured oocytes (Jinno et al., 1990). In the bovine, blastocyst development was significantly improved when COCs were matured in the presence of rFSH for the first 6 h of maturation only, compared to COCs matured for 24 h in the presence of rFSH (Ali et al., 2005). The results of these studies suggest the ideal maturation conditions may require not only modification of gonadotropin concentrations, but perhaps timing as well.

Although the inclusion of gonadotropins in maturation medium is routine in many laboratories and maturation and developmental benefits have been reported, a recent study found that the supplementation of FSH in maturation medium induced aneuploidy in IVM mouse oocytes (Roberts et al., 2005). Therefore, alternative supplements, such as growth factors, should be examined for their impact on oocyte physiology. Inclusion of alternative supplements may negate the requirement of gonadotropins in the medium or work in concert with gonadotropins, thus resulting in fewer abnormalities in the in vitro matured oocyte.

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Epidermal Growth Factor

Growth factors may be a key factor in mediating oocyte nuclear and cytoplasmic maturation. In particular, epidermal growth factor (EGF) and EGF-like growth factors have been proposed to have a positive impact on oocyte maturation in vivo and in vitro. EGF-like growth factors are a large family of closely related proteins that include transforming growth factor ά (TGFά), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AREG), epiregulin (EREG), and beta-cellulin (BTC; Yarden and Sliwkowski, 2001).

EGF and EGF-like growth factors have been found in follicular fluid of several species (Fang et al., 1987; Westergaard et al., 1990). De novo synthesis of an EGF-like peptide has been shown in the culture of rat theca and interstial cells (Skinner et al., 1987). The origin of intra-follicular EGF is not clear, although it is speculated to be via local production in the granulosa or theca cells (Westergaard et al., 1990). Inclusion of EGF in maturation medium (concentration range 1 to 100 ng/ml) resulted in an increase of cumulus expansion and maturation in the mouse (Downs, 1989), rat (Dekel and Sherizly, 1985), bovine (Lorenzo et al., 1994; Lonergan et al., 1996), sheep (Grazul-Bilska et al., 2003), and human (Das et al., 1991; Gomez et al., 1993; Li et al., 2004). Developmental competence was also improved when cumulus-enclosed oocytes were matured in the presence of EGF (concentration range 1 to 100 ng/ml; Harper and Brackett, 1993; Lonergan et al., 1996; Abeydeera et al., 2000; Guler et al., 2000).

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EGF modifies cell proliferation and operates in both a paracrine and autocrine fashion (Carpenter and Cohen, 1990). EGF was shown to be a potent mitogenic agent in rabbit, porcine, bovine and human granulosa cells (Gospodarowicz and Bialecki, 1979). LH stimulation of granulosa cells in vitro leads to production of EGF-like growth factors (AREG, EREG, BTC), suggesting that these growth factors are paracrine mediators of LH signaling in vivo (Park et al., 2004). In vitro, AREG, EREG, and BTC induced oocyte maturation in follicle culture as effectively as LH (Park et al., 2004); however, the kinetics of maturation following LH stimulation of COCs was slower than COCs exposed to EGF-like growth factors, suggesting that these growth factors act downstream of LH (Downs et al., 1988).

EGF receptors (EGFR) have been located on both the oocyte (Goff et al., 2001) and cumulus (Fang et al., 1987). Maturation with EGF resulted in decreased interval to GVBD by denuded mouse oocytes (Das et al., 1991) and had a positive effect on the maturation of denuded bovine oocytes (Lonergan et al., 1996). However, in the majority of reports, EGF had no effect on denuded oocytes (Parks et al., 2004; Lorenzo et al., 1994; and Sakaguchi et al., 2000), therefore suggesting that the site of action of EGF resides in the cumulus cells. In support of this theory, EGF-like growth factors have been reported to bind the EGFR on cumulus cells, promoting cumulus expansion and GVBD (Johnson et al., 1993). As the effect of EGF is mediated by the cumulus, it becomes clear the importance of evaluating the oocyte and cumulus as an intact functional unit. Removal of cumulus cells in vitro likely induces physiological changes within the oocyte,

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making it difficult to understand events of maturation and potentially limits the success of in vitro maturation.

Unlike the receptors for FSH and LH, the EGFR is a tyrosine kinase receptor. When EGFR is activated, signal transduction can occur via activation of substrates involved in different pathways. The most prominent pathway activated is the MAPK signal cascade. Treatment of COCs with EGF produces an increase in MAPK phosphorylation, resulting in the activation of the signaling cascade (Conti et al., 2005). A similar increase in MAPK phosphorylation was observed following LH exposure. The MAPK pathway is not only important in regulating the activity of transcription factors, but also in stimulating the activation of MPF, which is required for the completion of nuclear maturation (refer to nuclear maturation section).

A second pathway activated by EGFR is phospholipase C (PLC). Tyrosine residues on the EGFR on the cumulus cells phosphorylate and activate PLC. Activated PLC cleaves phosphatidylinositol bisphosphate (PIP_2), resulting in inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG; Lodish et al., 2000). IP_3 is a second messenger, which functions to liberate calcium from the smooth endoplasmic reticulum; while membrane bound DAG is involved in the activation of protein kinase C (PKC; Carpenter and Cohen, 1990). PKC and cAMP act synergistically to activate phosphodiesterase 4D (PDE 4D) promoter (Vicini and Conti, 1997).

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Insulin-Like Growth Factor I

Insulin-like growth factor I (IGF-I) plays a multifunctional role in follicle development and oocyte maturation. IGF-I is a differentiating and mitogenic agent (Hill et al., 1989), whose role in follicular development has been examined in several species. Pre-antral porcine follicles cultured with IGF-I resulted in increased granulosa cell proliferation, decreased apoptosis and increased follicular integrity, compared to follicle culture without IGF-I (Mao, et al., 2004). In the mouse, blastocyst development and cell number were increased following the culture of pre-antral follicles with IGF-I (Domeestere et al., 2004). Intra ovarian injection of IGF-I in prepubertal cattle resulted in blastocyst rates comparable to adult cows (Oropeza et al., 2004). In hypophysectomized sheep treated with gonadotropins, growth of follicles beyond 2 mm was arrested and follicles greater than 2 mm underwent atresia unless IGF-I was administered (Eckery et al., 1997). In addition, IGF-I enhanced rabbit follicular development and estrogen production (Yoshimura et al., 1996), as well as enhancing androgen biosynthesis in rat ovarian follicles (Adashi et al., 1985; Hernandez et al., 1988). These studies demonstrate the importance of IGF-I in follicular and subsequent embryonic development through its action on the oocyte.

IGF-I has also been examined with respect to medium supplementation used for the maturation of oocytes recovered from antral follicles. Maturation rates were increased when COCs were matured in the presence of IGF-I in the human (Gomez et al., 1993), rabbit (Lorenzo et al., 1996; Yoshimura et al., 1996), and cow (Lorenzo et al., 1994). Subsequent embryonic development was also improved when IGF-I was added to

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maturation medium (Herrler et al., 1992) and embryo culture medium (Lima et al., 2005; Moreira et al., 2002).

IGF-I receptors are located on granulosa cells (Kumamoto et al., 2005) and oocytes (Balbono et al., 1987). In the mouse, IGF-I significantly increased maturation of denuded oocytes (Kiapekou et al., 2005). However, the majority of work has examined IGF-I activity in follicle culture and maturation of COCs; therefore, more work may be necessary to determine the level of effect on the oocyte.

The pathway of IGF-I in the cell is mediated through receptor tyrosine kinase activity. Similar to EGF, IGF-I also works via two main pathways: PKC and MAPK. These pathways provide a potent proliferative signaling system that stimulates growth and modifies transcription factor activity.

The research on gonadotropins and growth factors clearly indicates their importance in both in vivo and in vitro maturation of the cumulus oocyte complex. Though their roles in vivo are well established, their requirements in vitro are less clear. In order to enhance oocyte quality, continued research using defined maturation systems is required to determine the necessity, as well as the concentration and timing of these factors in vitro. Further research must focus on the functional unit of the COC in order to understand the physiological requirements of the oocyte. These cell types (cumulus and oocyte) work in concert; therefore, it is important that the COC be studied intact. This type of research will not only provide a better understanding of medium requirements during oocyte

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maturation, but also increase knowledge regarding other conditions that potentially impact oocyte quality, such as oxygen tension.

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Oxygen Tension

In vitro maturation and culture are generally conducted under atmospheric oxygen tension (~20%), which is significantly higher than the in vivo environment (Bavister, 1995). Reduced oxygen concentration has been shown to have beneficial effects on pre-implantation embryos (Quinn and Harlow, 1978; Batt et al., 1991; Noda et al., 1994; Olsen and Seidel, 2000). Both the number of cells and the proportion of embryos developing to the blastocyst stage increased when the oxygen tension was between 5-7% (Thompson et al., 1990; Li and Foote, 1993). Further restricting the oxygen from 7% to 2% was beneficial for bovine embryos following compaction (Thompson et al., 2000). In addition, metabolic activity of mouse embryos cultured under reduced oxygen correlates more closely with in vivo oocytes (Hooper et al., 2001), than embryos cultured under 20% oxygen.

Although reduced oxygen concentration is beneficial for embryo culture, optimal oxygen concentration during oocyte maturation is undetermined. The maturation of denuded hamster oocytes was higher under 5% O_2, compared to either 0 or 10% O_2 (Gwatkin and Haidri, 1974). Contradictory findings have been observed in the mouse for cumulus oocyte complexes (COCs) recovered from both antral follicles or from pre-antral follicle culture. For example, fertilization and blastocyst development by COCs recovered from antral follicles were unaffected by maturation under various O_2 atmospheres (5, 10, 15, 20%; Eppig and Wigglesworth 1995). In a contradictory report, mouse oocytes collected from antral follicles showed improved blastocyst development when in vitro maturation and embryo culture were conducted under 5% O_2 (Adam et al., 2004). A similar

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contradiction has been reported with follicle culture, where low O_2 was beneficial to early stages of follicle culture (Eppig and Wigglesworth, 1995) or deleterious to both maturation rates and chromosomal alignment of MII oocytes recovered from pre-antral follicle culture (Betzendahl et al., 2001; Hu et al., 2001). Following 12 days of follicle culture under 5% O_2, only a few mouse oocytes matured to MII (Smitz et al., 1996). At 20% O_2, diffusion in pre-antral follicles appears sufficient to provide essential oxygen supply without inducing evident damage to the oocyte (Cortvrindt et al., 1996). The majority of reports on in vitro production of bovine blastocysts matured COCs under 20% O_2. Increasing glucose concentration during maturation improved blastocyst development when COCs were matured under low oxygen tension (Hashimoto et al., 2000; Oyamada and Fukui, 2004); however, when comparing blastocyst rates achieved in these studies to other studies which utilized 20% O_2, blastocyst development was higher when maturation occurred under ambient oxygen tension (Harper and Brackett, 1993; Lonergan et al., 1996; Ali et al., 2005). Currently most reports regarding the maturation of human oocytes, either for research or clinical procedures, utilize high oxygen tension (Das et al., 1991; Gomez et al., 1993; Roberts et al., 2002).

In vitro maturation and culture under 20% O_2 is speculated to increase production of reactive oxygen species (ROS) within the mouse oocyte and embryo, resulting in developmental arrest (Nasr-Esfahani and Johnson, 1991; Kwon et al., 1999). An increase in H_2O_2 production in the bovine oocyte was reported following maturation under 20% O_2 (Hashimoto et al., 2000). ROS are able to diffuse and pass through cell membranes, altering lipids, proteins, and nucleic acids, consequently resulting in mitochondrial

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alterations, developmental arrest, ATP depletion, and apoptosis (Guerin et al., 2001). To counteract ROS, oocytes express antioxidant genes and proteins (El Mouatassim et al., 1999), and additional antioxidants and proteins are found in follicular fluid and granulosa cells (Briggs et al., 1999; Jozwik et al., 1999). Under certain conditions, these antioxidants may provide protection to the oocyte during follicle culture and in vitro maturation, contributing to inconsistent results in oocyte viability between studies examining oxygen tension.

It is evident from the research that many factors impact oocyte quality both prior to and during oocyte maturation. Evaluation of oocytes both during and following IVM conditions is critical for determining which factors have positive or negative effects on the oocyte. The gold standard evaluation of an IVM system would entail fertilization of oocytes, transfer of the resulting embryos, and evaluation of the offspring. However, this method of evaluation is not only financially impractical, but would require an enormous amount of time, especially with respect to species with longer gestational periods. Therefore, it is critical to develop reliable viability markers to quickly and accurately assess oocyte quality. These markers could not only be used to assess the effectiveness of a variety of IVM conditions, but also be used to aid in the selection of healthy oocytes and embryos, ultimately reducing the number of embryos transferred in clinical settings.

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Viability Markers of Developmental Potential

The development of a successful in vitro maturation system will be expedited when reliable methods to assess oocyte quality are established. Aside from fertilization and blastocyst development, morphological characteristics are the most widely used criteria to assess oocyte quality. Attempts have been made to correlate cytoplasmic appearance (De Sutter et al., 1996; Balaban et al., 1998), perivitelline space (De Sutter et al., 1996), or thickness of zona pellucida (Talevi et al., 1997; Gabrielsen et al., 2001) with subsequent developmental potential; however, no clear correlation has been found. In addition, the composition of follicular fluid has failed to correlate with subsequent developmental potential (Artini et al., 1994; Friedman et al., 1998). Morphology of bovine abattoir ovaries has been correlated with oocyte developmental competence. Oocytes recovered from ovaries containing at least 1 follicle larger than 10 mm in diameter or 10 follicles between 2-5 mm in diameter have greater developmental potential than oocytes recovered from other types of ovaries (Gandolfi et al., 1998). Also an oocyte diameter of 120 μm in the cow has been correlated with subsequent developmental potential (Otoi et al., 1997). In addition, many laboratories use the morphology of cumulus cells surrounding the oocyte as a selection criterion prior to in vitro maturation (Lonergan et al., 1994; Goud et al., 1998) and the degree of expansion as a morphological indicator of oocyte quality after maturation. However, it is contentious as to whether cumulus expansion directly relates to developmental capacity of the oocyte (Ali and Sirard, 2002). Although much research has focused on the correlation of morphological characteristics, few relationships have been established that would accurately indicate oocyte developmental potential. Therefore, a shift from

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morphological to quantitative markers of oocyte development may hold greater potential for assessment of IVM systems and oocyte selection.

Oocyte Metabolism

Understanding energy substrate metabolism of the cumulus oocyte complex throughout in vitro maturation may aid in optimizing maturation conditions and serve as a potential viability marker of developmental competence. A critical aspect of oocyte maturation involves the development and activation of appropriate metabolic pathways. A transition occurs during maturation from predominately COC metabolism to oocyte centered metabolism. This transition ensures the metabolic independence of the oocyte following ovulation and the loss of the gap junction communication between the cumulus cells and the oocyte, allowing the oocyte to support early embryo development. Although much research has been done to correlate embryo quality and metabolism (Renard 1980; Gardner and Leese, 1987; Lane and Gardner, 1996; Gardner et al., 2001), few groups have examined the relationship between oocyte metabolism and developmental competence.

Although there has been an increased interest in in-vitro maturation over the past decade, little is known about the metabolism of the oocyte, and even less about the metabolism of the cumulus-oocyte complex. Energy requirements of the COC are unique, as the cumulus cells and oocyte have different metabolic needs. Pyruvate is the major energy source for denuded oocytes in several species including the mouse (Biggers et al., 1967), cow (Rieger and Loskutoff, 1994), pig (Sturmey and Leese, 2003), and cat (Spindler et

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al., 2000). While denuded mouse oocytes require pyruvate or oxaloacetate in the medium in order to mature, COCs develop in medium containing lactate, phosphoenolpyruvate, or glucose (Biggers et al., 1967). Cumulus cells are able to convert these alternative energy substrates into pyruvate, which is then utilized by the oocyte (Leese and Barton, 1985; Gardner and Leese, 1990; Gardner et al., 1996).

Metabolism of the Denuded Oocyte

Metabolism of the denuded oocyte has been examined by several groups. In general, relative energy substrate utilization is dictated by meiotic stage of the oocyte. In humans, maturing oocytes take up 20-30 pmol/oocyte/h of pyruvate and produce lactate at a rate of 2-10 pmol/oocyte/h, pending on the stage of maturation, with pyruvate and lactate metabolism greater during GVBD than during polar body extrusion (Roberts et al., 2002). The amount of pyruvate consumed during the maturation process is similar to human embryos in their fist cleavage division (Leese et al., 1986), but higher than human zygotes following ICSI (Devreker et al., 2000). Similar findings have been reported in the mouse, where denuded oocytes arrested in prophase I or metaphase II consumed less pyruvate than actively maturing oocytes (Downs et al., 2002). Pyruvate metabolism peaked at 12 h of maturation in the bovine oocyte (Steeves and Gardner, 1999). In oocytes recovered from ovaries of non-superovulated cattle, GVBD occurs between 3 and 9 h maturation and metaphase II takes place at approximately 22 h maturation (Sirard et al., 1989); therefore, a peak in pyruvate metabolism at 12 h is consistent with findings of increased metabolism in the actively maturing oocyte, as seen in both the human and the mouse.

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Carbohydrate metabolism is altered by the composition of maturation medium. In human denuded MII oocytes, pyruvate uptake was 30% lower when oocytes were matured in a medium (modified Eagle medium with Earle's modified salts; MEME) containing an increased glucose concentration and fewer non essential amino acids and vitamins than a medium (TCM199) containing less glucose and both essential and non essential amino acids and vitamins (Roberts et al., 2002). The composition of MEME has a 4 fold greater concentration of glutamine compared to TCM199. Glutamine has been shown to be metabolized by both bovine and feline oocytes (Steeves and Gardner, 1999; Spindler et al., 2000). Glutamine and pyruvate are both precursors for the TCA cycle. Therefore, the increased glutamine levels in MEME may have competed with pyruvate, subsequently decreasing pyruvate uptake. The activation of the TCA cycle is critical during oocyte maturation; therefore, medium must be able to support TCA cycle activity, whether via pyruvate or an alternative precursor of the TCA cycle. As maturation medium affects the absolute uptake of carbohydrates, caution should be taken when comparing metabolic results between laboratories.

In addition to increasing pyruvate metabolism during maturation, glutamine and glycine metabolism also increase over the course of bovine maturation (Rieger and Loskutoff, 1994; Gandolfi et al., 1998). All three of these substrates are involved in oxidative metabolism, thus suggesting the importance of this pathway in the oocyte. Malate dehydrogenase, a metabolic enzyme involved in oxidative metabolism, also increased over the course of maturation in the rat (Tsutsumi et al., 1992). Furthermore, when inhibitors of oxidative phosphorylation were present, denuded rat oocytes were arrested

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at the GV stage (Zeilmaker and Verhamme, 1974). The cytoplasm of the oocyte must provide enough cellular energy to support maturation, fertilization, and early embryo development. The oocyte is able to achieve these high levels of energy production via oxidative phosphorylation.

Although pyruvate is the primary energy source, the oocyte is able to metabolize small quantities of glucose. Denuded oocytes do not effectively utilize glucose. A proposed theory for the inefficient use of glucose is limited hexokinase activity in the oocyte (Brinster, 1968). A second theory suggests the oocyte has a deficiency in glycolytic enzymes, which has been shown in early preimplantation embryos (Barbehenn et al., 1974). The limited quantity of glucose consumed by the oocyte can be used in three pathways: oxidative metabolism, glycolysis, or the pentose phosphate pathway (PPP). Glycolysis leads to the production of ATP, as well as substrates such as pyruvate, which then proceed in oxidative metabolism for further energy production. Metabolism through the PPP supplies substrates for ooplasmic integrity and is required for the formation of ribose moieties for DNA and RNA synthesis (Sutten et al., 2003). Bovine and porcine oocytes matured in vivo have increased glucose utilization through glycolysis and the PPP, relative to their in vitro counterparts (Gandolfi et al., 1998; Durkin et al., 2001). Activity of glucose 6 phosphate dehydrogenase (G6PDH), a key regulatory enzyme of the PPP, was high in bovine oocytes compared to cumulus cells, suggesting a preference by the oocyte for this pathway (Cetica et al., 2002). Interestingly, blocking glycolytic activity within the mouse oocyte did not influence meiotic maturation (Downs et al., 1996); however, stimulators of the PPP increased

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GVBD and glucose uptake, thus suggesting that the role of glucose in meiotic induction is via the PPP (Downs et al., 1998). Total glucose metabolism by denuded bovine oocytes through glycolysis and the PPP has been reported to be low and not change over the course of maturation (Rieger and Loskutoff, 1994; Gandolfi et al., 1998). However, in a different report, glucose utilization by denuded bovine oocytes was shown to increase over the course of maturation (Steeves and Gardner, 1999). Differences in maturation protocols between laboratories make comparisons difficult, as metabolism will be altered depending on maturation conditions.

Although glucose is not a major energy source for the denunded oocyte, the pathway of glucose utilization has been correlated with subsequent development. Throughout maturation, glucose oxidation in denuded bovine oocytes was low (Zuelke and Brackett, 1992; Krisher and Bavister, 1999) or absent (Khurana and Niemamn, 1992). Higher glycolytic rates by in vitro matured bovine oocytes were associated with increased developmental competence (Krisher and Bavister, 1999; Sutton et al., 2003). Blastocyst development in cats was also associated with higher rates of glycolysis by denuded MII oocytes (Spindler et al., 2000). Therefore, while glycolysis is not necessary for meiotic induction, it may be important for cytoplasmic maturation, thus imparting increased developmental capacity on the subsequent embryo.

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The Metabolism of the Cumulus Oocyte Complex (COC)

Much work has been done to evaluate the metabolism of the denuded oocyte; however, the metabolic profiles of COCs have been examined to a lesser extent. It is well known that the cumulus cells are critical to the maturation process, by supplying the oocyte with essential metabolites, amino acids, as well as other factors (Donahue and Stern, 1968). Removal of the cumulus may inherently change the oocyte's physiological responses; therefore, an accurate understanding of oocyte physiology requires examining the oocytes and cumulus as an intact complex. Although pyruvate is the primary energy source of the oocyte, cumulus cells metabolize alternative substrates such as glucose, which ultimately produce substrates that the oocyte requires for maturation. Metabolic studies have confirmed that pyruvate is produced by cumulus cells (Leese and Barton, 1984; Gardner and Leese, 1990; Gardner et al., 1996).

Over the course of maturation, pyruvate uptake increased, but glucose consumption remained constant in mouse COCs (Downs et al., 2002). In cattle, glucose and pyruvate consumption by the COC increased over the course of maturation; however, lactate production remained constant (Sutten et al., 2003). It is proposed that the primary fate of glucose in the COC is the production of lactate and pyruvate, which will then be utilized by the oocyte (Biggers et al., 1967; Downs and Utecht, 1999). Upon resumption of meiosis, both pyruvate and glucose enhance maturation. Although both substrates are not required for meiotic resumption, the inclusion of glucose and pyruvate enhance both nuclear and cytoplasmic maturation in bovine and murine COCs (Rose-hellekant et al., 1998; Downs and Hudson, 2000).

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Similar to the denuded oocyte, the type of medium used for maturation greatly affects the metabolism of the cumulus enclosed oocyte. Inclusion of LH increased glycolysis and glucose oxidation within the bovine COC (Zuelke and Bracket, 1992). When FSH was added to the medium, glycolysis increased in the mouse COC (Roberts et al., 2004). FSH increased hexokinase activity, therefore increasing glucose uptake through the glycolytic pathway in the mouse COC (Downs et al., 1996).

More investigation is required with respect to understanding how medium components affect metabolism of the COC and oocyte, in order to understand the physiology as well as to develop better culture conditions. As maturation and developmental competence are enhanced for COCs compared to denuded oocytes, more studies are required to determine metabolism of the COC in response to different maturation conditions.

Metabolic Enzymes

The activity and allosteric regulation of metabolic enzymes impact the metabolism of the oocyte. Knowledge of activity of key enzyme pathways in the maturing oocyte will increase understanding of oocyte physiology and assist in the development of maturation systems that enhance the functioning of required pathways. In cattle, phosphofructokinase (PFK) and G6PDH were found in both the oocyte and cumulus cells (Cetica et al., 2002). PKF is a rate limiting enzyme regulating the glycolytic pathway (Schirmer and Evans, 1990), while G6PDH regulates activity of the PPP (Wood, 1986). PKF was the most abundant enzyme in the bovine cumulus, suggesting glycolysis is the primary pathway in the cumulus; however activity of PKF in the bovine oocyte was very

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low (Cetica et al., 2002), which corroborates previous findings in human oocytes (El Mouatassim et al., 1999). In the oocyte, G6PDH was four fold greater than PKF, suggesting that the PPP has an important role in the synthesis of ribose moieties (Cetica et al., 2002). Similarly, rat oocytes have higher G6PDH activity than PKF (Tsutsumi et al., 1992). The activity of the enzymes changed over the course of maturation, becoming greater in the oocyte and lesser in the cumulus cells (Kruip et al., 1983). This is consistent with the idea that the oocyte is preparing stores of metabolic substrates to sustain it through early embryonic development.

An enzyme present in excess in cumulus cells and oocytes is lactate dehydrogenase (LDH). There was no significant difference in LDH activity in mouse and rat oocytes over the course of maturation (Mangia et al., 1975; Tsutsumi et al., 1992). Gonadotropins enhanced the production of lactate in rat, mouse and human follicles by increasing the glycolytic pathway in cumulus (Billig et al., 1983; Boland et al., 1993; Harlow et al., 1987). Also, a high concentration of lactate was found in human follicular fluid (Leese and Leton, 1990). When denuded bovine oocytes were matured in medium supplemented with pyruvate, lactate, or lactate plus NAD^+, the only group failing to realize an increase in maturation was the lactate only group. Therefore, the denuded bovine oocyte can utilize lactate as long as there are adequate supplies of NAD^+ present either de novo in the oocyte or supplemented in the medium (Cetica et al., 1999; see Figure 5)

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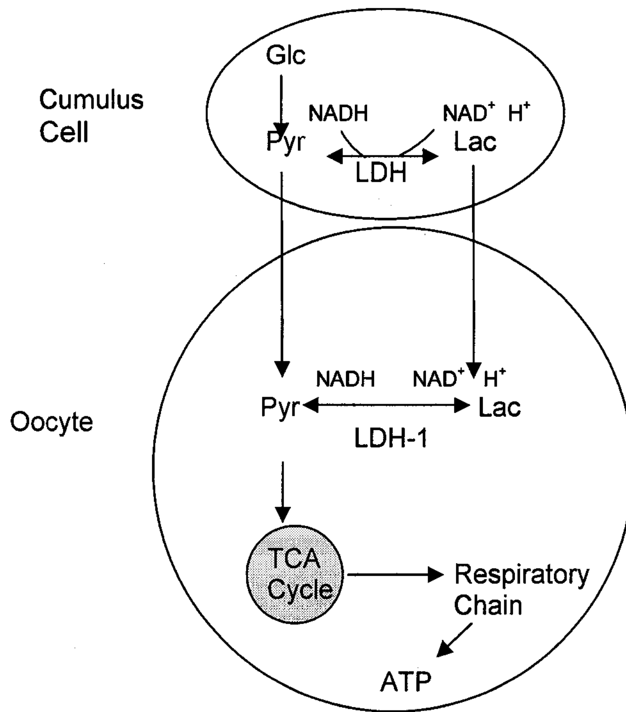
Figure 5. Mechanism of glucose metabolism by the cumulus cells, resulting in substrates (pyruvate and lactate) that can be readily metabolized by the oocyte to generate ATP via oxidative respiration.



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A clear understanding of metabolic requirements of the COC will not only aid in the optimization of maturation medium, but potentially serve as an indicator of oocyte viability. The use of metabolism as a viability marker will require further research correlating developmental competence with COC metabolism at specific time points during maturation. Previous work examining COC metabolism during maturation was performed in groups, thus making it difficult to correlate with subsequent developmental potential (Downs et al., 2002). Researchers that have examined individual COC metabolism only assessed maturation rates, not developmental potential (Roberts et al., 2004). Therefore, future research needs to focus on the metabolism of the individual COC and the subsequent developmental potential of those COCs. The correlation of metabolic markers to oocyte developmental competence would allow for the selection of the highest quality oocytes, which would be beneficial for procedures such as gamete intra-fallopian transfer (GIFT) or oocyte transfer (OT). For oocytes undergoing fertilization in the laboratory, oocytes of higher viability could be cultured together, as the oocytes would benefit from paracrine and autocrine factors. Metabolic markers of developmental competence offer potential to increase success rates of both animal breeding programs and clinical IVF programs.

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Embryo Metabolism

The quality of in vitro produced embryos is variable and is influenced by numerous factors including superovulation of the donor, donor age and health, as well as in vitro conditions of maturation and embryo culture. Currently, selection of embryos for transfer is largely based on embryo morphology, frequently resulting in suboptimal pregnancy rates. Quantitative viability markers would greatly increase the selection of healthy embryos. The ideal viability marker must meet four requirements. First, the technique must be completely non-invasive. The measurement of the marker must have no detrimental effect on the subsequent health of the embryo. Second, the method must be sensitive enough to evaluate a single embryo. Methods requiring multiple embryos are helpful for increasing our understanding of embryo physiology; however, these methods do not allow for individual embryo selection. Third, the technique, including analysis, must be completed relatively quickly to allow for selection and transfer of the embryo at the appropriate time. Finally, the technique must be highly predictable and reproducible, allowing the viable embryos to be accurately separated from the non-viable embryos.

Metabolism of the embryo has been studied to a greater extent than that of the oocyte. Pre-implantation mouse embryos provided with a physiological mixture of nutrients normally switch from pyruvate to glucose as the preferred substrate during the late morula stage (Thomson and Brinster, 1966). Glucose solely supported mouse embryo development after the 4-8 cell stage (Brinster, 1965). Leese and Barton (1984) developed a non-invasive ultramicrofluormetric technique to study the uptake of nutrients by single mouse embryos. With this technique, it was confirmed that pyruvate is the preferred

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nutrient consumed by early cleavage stage mouse embryos prior to switching to glucose consumption at the blastocyst stage (Gardner and Leese, 1986). In the rat, pyruvate was the dominant substrate until the morula stage; however, glucose became the primary metabolite during the formation of the blastocoel cavity (Brinson and Leese, 1991). Ruminants show a similar metabolic pattern, with pyruvate being the primary energy source until the 8-16 cell stage, followed by a dramatic increase in glucose uptake until the blastocyst stage in both cattle (Rieger et al., 1992; Tiffin et al., 1991) and sheep (Thompson et al., 1991; Gardner et al., 1993).

Due to the paucity of material, human embryo metabolism has also been examined to a lesser extent using this non-invasive technique. Although human embryos consume larger quantities of substrates than mouse embryos, proportionately substrate consumption is the same. Pyruvate is essential to support early cleavage stage human embryos, as medium devoid of pyruvate resulted in 84% developmental arrest (Conaghan et al., 1993). Early stage human embryos primarily consume pyruvate prior to switching to glucose consumption at the blastocyst stage (Hardy et al., 1989). Pyruvate uptake in healthy human oocytes was higher than that of zygotes; however, pyruvate consumption of the early embryo subsequently increased and peaked at the morula stage (Leese et al., 1986). In normal human embryos that develop into blastocysts, pyruvate uptake exceeded glucose in early development (d 2-5 post insemination), after which glucose was the dominant substrate (d 6; Gott et al., 1990; Gardner et al., 2001).

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Nutrient uptake has been correlated with developmental capacity after transfer of embryos in cattle, mice and humans (Renard et al., 1980; Gardner and Leese, 1987; Conaghan et al., 1993; Turner et al., 1994). In the mouse, blastocysts with higher glucose uptake in vitro implanted and developed at higher rates than embryos with lower glucose uptake (Gardner and Leese, 1987). Increased glucose uptake in d 10 cattle embryos (Renard et al., 1980) was positively correlated with viability post transfer. When glycolytic activity, estimated through glucose uptake and lactate production, was used to select individual d 4 blastocysts, those with lower glycolytic rates resulted in a 4 fold increase in pregnancy rates following transfer (Lane and Gardner, 1996). At each stage of development, pyruvate uptake by healthy human embryos was greater than embryos that subsequently arrested (Leese et al., 1986; Gott et al., 1990). Human embryos that arrest in culture consume 20% less pyruvate on d 2.5 compared to those that go onto develop to the blastocyst stage; additionally polyspermic embryos, parthenogenic embryos and unfertilized oocytes all had lower pyruvate uptake than their viable counterparts (Hardy et al., 1989). On d 2, pyruvate uptake was higher for untransferred embryos of pregnant women compared to non-pregnant women (Devreker et al., 2000). Furthermore, arrested embryos failed to switch from pyruvate to primarily glucose based metabolism (Gott et al., 1990). Although there was a correlation between pyruvate uptake and embryo health, the use of pyruvate uptake as the sole criterion for embryo selection is not advisable, as great variability exists between embryos (Conaghan et al., 1993). However, the selection of embryos based on morphology and metabolic characteristics proved to be a more reliable predictor of embryo viability following transfer (Turner et

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al., 1994). Metabolism, in conjunction with other established markers, is a valuable tool for embryo selection.

Pyruvate consumed by the embryo is primarily oxidized; however, glucose consumed by the embryo can be utilized in more than one pathway. A large percentage of glucose is utilized in the glycolytic pathway, although the oxidation of glucose has been reported in the blastocyst (Rieger et al., 1992; Donnay and Leese, 1999). The first increase in oxidation of glucose by cattle embryos was between the 12-16 cell stage, with a significant increase at compaction and blastocyst expansion (Khurana and Niemamn, 2000). In addition to oxidation and glycolysis, glucose can enter the PPP. Metabolism of glucose through the PPP increased 15 times between the 2-cell embryo and expanded blastocyst (Rieger et al., 1992). The activity of these metabolic pathways is indicative of embryo health; therefore evaluation of these pathways can aid in embryo selection.

In addition to glucose and pyruvate metabolism, lactate production has also been examined in the pre-implantation embryo. Lactate production by the embryo increased as glucose consumption increased (Brinsin and Leese, 1991). In the mouse, approximately half of the glucose consumed was converted to lactate at the blastocyst stage (Gardner and Leese, 1988). A single mouse blastocyst grown in vitro produced almost twice as much lactate as blastocysts that were freshly collected (Gardner and Leese, 1990). In the bovine blastocyst, close to 100% of glucose is converted to lactate (Thompson et al., 2000). Radio-labeled metabolic measurements of single human embryos showed that 90% of the glucose consumed at the blastocyst stage was converted

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to lactate (Wales et al., 1987). Higher levels of lactate production in later stages of development may prepare the embryo for the reduced oxygen environment of the uterus, relative to the oviduct (Brown and Mattner, 1984) and the anoxic environment at implantation (Rogers et al., 1983).

The combination of oocyte and embryo metabolism may provide valuable markers that will assist in the selection of the healthiest embryos for transfer. As most techniques have limitations, it is valuable to develop additional quantatative markers that can be used in conjunction with metabolism to enhance in vitro maturation and culture systems. Potential candidates for oocyte viability markers include the assessment of mitochondrial arrangement and mitochondrial membrane potential, oxygen consumption, and gene expression. The development of each marker will continue to increase our understanding of oocyte physiology, and combining the information from these potential markers will allow development of better maturation conditions, and ultimately selection of the most viable oocytes and embryos.

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Mitochondrial Redistribution

Energy within mammalian cells is provided through the production of adenosine triphosphate (ATP). ATP can be produced via mitochondrial independent or mitochondrial dependent pathways. Embryos, depending on species and stage of development, have different requirements for ATP production. Mitochondrial respiration strongly influences human pre-implantation embryo development (Van Blerkom et al., 2000; Wilding et al., 2001); however, mouse embryos are capable of developing without mitochondrial generated ATP (Li et al., 2000). Though mouse embryos can develop with out mitochondrial respiration, embryos that rely predominantly on glycolysis are less viable following transfer (Gardner and Lane, 1998).

Developmental stage of the embryo often dictates the pathway of ATP production. The addition of mitochondrial respiration inhibitors improves the development of porcine embryos (Machaty et al., 2001) and bovine embryos (Rieger et al., 2002; Thompson et al., 2000) at compaction. However, inclusion of uncouplers of oxidative phosphorylation in early cleavage stage embryos is detrimental, suggesting a larger reliance on mitochondrial ATP (De La Torre et al., 2006). Ultimately, gametes and embryos of all species are dependent upon oxidative phosphorylation at some point in their development; therefore, assessment of mitochondrial organization and activity may provide insight into the quality of oocytes and embryos.

Organization and metabolic activity of mitochondria are necessary features of nuclear and cytoplasmic maturation (Van Blerkom and Runner, 1984; Hyttel et al., 1986),

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affecting subsequent development after fertilization (Van Blerkom et al., 1995). Altered structural changes in mitochondrial redistribution in oocytes after fertilization have been correlated with abnormal embryo development (Barnett et al., 1996). Stage specific changes in mitochondrial distribution in maturing oocytes and newly fertilized oocytes may be indicative of the need in those areas for increased ATP (Van Blerkom and Runner, 1984). The mitochondrial arrangement in immature oocytes has been reported to be on the periphery in the bovine (Stojkovic et al., 2001) and human (Wilding et al., 2001). In the mouse, mitochondria have been reported to be perinuclear in MI oocytes and homogeneous in MII oocytes (Van Blerkom, 1991; Tokura et al., 1993). Mitochondrial arrangement has been correlated with developmental competence in cattle (Stojkovic et al., 2001), mouse and human oocytes (Van Blerkom et al., 2002). In addition to oocyte quality, in vitro maturation conditions may affect the redistribution of the mitochondria.

Mitochondrial activity can be indirectly assessed by determining mitochondrial inner membrane potential. Mitochondrial respiration involves pumping protons across the inner mitochondrial membrane, creating an inner membrane potential. The energy created from this process is utilized to drive the conversion of ADP to ATP for cellular functions. Mitochondrial respiration is highly correlated with membrane potential of the inner mitochondrial membrane (Mitchell and Moyle, 1967). Both nuclear and cytoplasmic maturation are dependent on the organization and metabolic activity of mitochondria (Van Blerkom and Runner, 1984; Hyttel et al., 1986). Oocyte mitochondrial activity subsequently affects development after fertilization (Van Blerkom et al., 1995). Higher

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mitochondrial membrane potential has been correlated with increased developmental competence in oocytes (Wilding et al., 2001) and embryos (Ahn et al., 2002; Jones et al., 2004; Wilding et al., 2003). As membrane potential is indicative of the health of the mitochondria, and healthy mitochondria are required for oxidative respiration, it could be a potential marker of oocyte quality, and therefore serve to evaluate the effectiveness of an IVM system.

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Oxygen Consumption and ATP production

Oxygen consumption provides a method of determining total ATP production by the oocyte. ATP is produced by two pathways in the cell: oxidative phosphorylation and glycolysis. The main substrates for oxidative phosphorylation are pyruvate, glutamine, oxaeloacetate, and other pre-cursors of the TCA cycle. Glycolysis requires glucose, which is converted to lactate. In the absence of oxygen, glycolysis is able to solely produce ATP, yielding 2 ATP per glucose molecule. Oxidative phosphorylation is a more efficient mechanism of producing ATP, generating 38 ATP molecules for every glucose molecule that enters glycolysis and is subsequently oxidized. Another key component of ATP production, often overlooked, is oxygen. Oxygen is essential for the conversion of ADP to ATP in oxidative phosphorylation through its role as an electron acceptor in the electron transport chain. However, the benefits of oxygen are coupled with a negative effect. The use of oxygen for energy production results in production of reactive oxygen species (ROS), particularly superoxide anion (O_2-) and hydroxyl radical (OH-; Guerin et al., 2001). The generation of intracellular ROS during culture has been proposed to be detrimental to embryo culture (Johnson and Nasr-Esfahani, 1994).

As oxygen consumption is critical for oocyte and embryo metabolism, quantification of oxygen could serve as a potential viability marker. Oxygen consumption, combined with glucose uptake and lactate production, provides a measurement of ATP production (Thompson et al., 1996). The measurement of oxygen uptake is non-invasive and the short period of incubation required for the technique has no detrimental effect on subsequent development (Thompson et al. 1996). Several groups have measured oxygen

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consumption of the preimplantation embryo. During early stages of embryonic development, low levels of oxygen are consumed; however, there is significant increase in oxygen consumption by the blastocyst stage of murine (Nilsson et al., 1982; Houghton et al., 1996) porcine (Sturmey and Leese 2003) and bovine (Sutton et al., 2003) embryos. Although glycolysis is critical during post compaction stage embryo development, the increase in oxygen uptake suggests that oxidative metabolism is still being utilized at the blastocyst stage. In the bovine, d 7 in vivo blastocysts were reported to consume between 0.66+0.08 nl/embryo/hr O_2 (Thompson et al., 1990) and 1.47+0.44 nl/embryo/hr (Overstrom et al., 1992). Oxygen uptake by in vitro produced blastocysts was reported at 0.9+0.13 (Thompson et al., 1996), suggesting that under the culture conditions of the experiment, there was little difference between in vivo and in vitro produced bovine blastocysts.

Although oxygen uptake of the embryo is well established, less information is available regarding the oxygen uptake of the oocyte. No difference was seen in the oxygen uptake of denuded pig oocytes before or after maturation (Sturmey and Leese, 2003). In the bovine, oxygen uptake by the COC or denuded bovine oocyte was not affected by follicle size or stage of maturation (Lequarre et al., 2005). However, when number of cumulus cells per COC was taken into account, COCs from smaller follicles had higher oxygen uptake. As COCs from smaller follicles consistently have lower developmental rates, the increased oxygen uptake by these COCs may result in excessive ROS production, resulting in irreversible damage to DNA of the oocyte.

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The measurement of oxygen uptake may serve as a potential marker of oocyte viability. More research needs to focus on the oxygen uptake of in vivo and in vitro matured oocytes. As with carbohydrate metabolism, in order to fully understand the physiological requirements, research should focus on the intact unit of the COC. Additionally, this technique could be used to assess various conditions of maturation, for example there is currently no information regarding oxygen uptake by oocytes matured under different oxygen tensions. Comparisons between in vivo matured COCs and COCs matured under various maturation conditions will not only aid in optimization of IVM systems, but this non-invasive measurement may aid in selection of healthy oocytes.

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Gene Expression

Gaining insight into the control and regulation of developmental processes of oocyte maturation requires knowledge of molecular events during this dynamic period of time. The acquisition of molecular information has been limited by the small quantity of RNA contained within oocytes. A physiological time table of gene expression requires accurate measurement of mRNA over various time points of maturation, which in the past would require thousands of oocytes. Previous analytical methods to analyze gene expression in oocytes included measurement of the product of nucleic acid concentration and time (Cot; Davidson and Hough, 1969), northern blotting (Thomas, 1980), and dot or slot blots (White and Bancroft, 1982); however, all of these approaches lacked the sensitivity to detect mRNA levels in single cells. Reverse transcription PCR (RT-PCR) has the ability to detect low levels of mRNA in individual cells (Rappolee et al., 1988); however, the end point analysis used by this approach may yield altered product information, not accurately reflecting the concentration of mRNA in the starting material. Real time PCR overcomes the dilemma of end point analysis of RT-PCR, while retaining the sensitivity to analyze single cells. In real time, fluorescence is monitored as the reaction progresses and analysis is conducted at the threshold cycle, or the cycle at which the fluorescence is increased above the background (Steuerwald et al., 1999). The threshold cycle indicates the start of exponential growth and is the most reliable point at which to take measurements (Wittwer et al., 1997). Unlike previous approaches, real time PCR allows for the study of gene expression in single oocytes; however, it requires picking discrete candidate genes. Therefore, progress in understanding the variety of genes involved in oocyte maturation is limited by the number of genes and the specific

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genes chosen for an experiment. The advent of DNA micro array technology circumvents this problem by allowing assessment of thousands of genes simultaneously. Gene arrays are solid supports upon which a collection of gene specific nucleic acids have been placed in defined locations. In array analysis, an RNA sample is labeled with a fluorescent probe and then allowed to hybridize with the gene targets on the array. Gene arrays are valuable when examining differential gene expression between two or more treatment groups. Gene arrays are commercially available for several species, including human, mouse and more recently the cow. In addition, cross species hybridization can be done to examine species for which arrays are not available or to examine genes that are conserved between species.

In the past, analysis of oocyte gene expression has been performed using many methods. It is thought that the intrinsic quality of the oocyte determines the proportion of oocytes that develop to the blastocyst stage (Rizos et al., 2002). For example, oocytes recovered from larger follicles are more competent than oocytes recovered from small follicles (Pavlok et al., 1992; Lonergan et al., 1994). Also, in vivo matured oocytes are more developmentally competent than in vitro matured oocytes (Van de Leemput et al., 1999; Blondin et al., 2002). Therefore, examining gene expression between populations of oocytes of varying developmental potential may reveal specific genes required for developmental competence.

During oocyte growth and maturation, mRNA and protein molecules are synthesized for immediate use or stored for use during early embryo development (Eichenlaub-Ritter and

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Peschke, 2002). Storage of mRNA takes place during oocyte growth, and the extent of the poly (A) tail at the 3' end of the each transcript has emerged as important regulatory element for determining the stability of the mRNA (Gray and Wickens, 1998). The poly (A) tail is responsible for the initiation of translation and the regulation of mRNA degradation (Jackson and Standart, 1980). Activation of mRNA is associated with polyadenylation of pre-existing short poly (A) tail, while deadenylation is correlated with cessation of transcription (Paynton et al., 1988). The mRNA transcripts of particular proteins that are important during oocyte maturation, such as cyclin B1, are reported to undergo polyadenylation during maturation in mouse oocytes (Tay et al., 2000). In the bovine, high cyclin B1 levels are reported in immature oocytes (Levesque and Sirard, 1995); however, cyclin B1 mRNA decreases with increasing follicular size (Robert et al., 2000). This suggests active translation of cyclin B1 by larger follicles, which would indicate polyadenylation of this transcript as observed in mice.

In growing mouse oocytes RNA with short poly (A) tails (<90 residues) were more stable than those with >150 residues, which were for immediate use (Bachvarova, 1992). In one study, bovine oocytes of reduced developmental competence (based on ovary morphology) had RNA with shorter poly (A) tails at both the GV stage and MII stage when compared to oocytes of higher developmental competence (Brevini-Gandolfi et al., 1999). The same study also found that the length of the poly (A) tail in MII oocytes was shorter for 6 out of 8 genes of interest following in vitro maturation, compared to GV oocytes. Perhaps oocytes of reduced developmental competence have impaired maturation dependent polyadenylation, therefore resulting in the inability to translate the

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mRNA, as their counterparts of higher developmental potential were able to do. Analysis of bovine oocytes revealed a difference in relative abundance in mRNA transcripts of Na+ K+- ATPase between unselected and selected oocytes, with the later having significantly less transcripts (DeSousa et al., 1998). Although this difference in abundance could be due to mRNA degradation, it may be the result of adenylation patterns that are conducive to protein translation. Interestingly, another study found that the total poly (A) content was similar between calf and cow oocytes, with the later group of known superior developmental competence (Lequarre et al., 2004). However, total content does not reflect the length of the poly (A) tail, which would indicate its translational ability. As the control of polyadenylation appears to be a key regulatory step in gene expression and is known to be important in early embryo development, more research is required to determine how maturation conditions impact polyadenylation patterns.

During the growth phase, oocytes accumulate large quantities of mRNA (Marello et al., 1992; Verrotti and Strickland, 1997). In the mouse, stored mRNA had a half life of 28 days (Wassarman et al., 1996). Transcription of new mRNA occurred up to the GVBD, while polyadenylation of transcripts occurred up to the MI (Tomek et al., 2002). The quantity of mRNA within the mouse oocyte was decreased by half between the GV and MII stages (Bachvarova et a., 1985). Changes in the pattern of gene expression during meiosis have been associated with increased utilization of mRNAs which were previously accumulated during the oocyte growth phase (Heke and Richter, 1994) or an increase in mRNA degradation (Bachvarova and De Leon, 1980). The total RNA content

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of bovine oocytes did not change over the course of maturation (2 ng); however, similar to the mouse, poly (A) RNA was reduced by half (53 pg to 25 pg/oocyte; Lequarre et al., 2004). Interestingly, in the mouse, there was a concurrent 20% decrease in total RNA content over the course of maturation (Paynton et al., 1988). However, discrepancies between species and between studies may be due to methods of RNA assessment, rather than true differences.

Much of the research to date on gene expression has been performed with the selection of specific candidate genes. Growth differentiation factor 9 (GDF-9), which is essential for normal follicular development (Dong et al., 1996), has been examined by several groups. GDF-9 is present in bovine cumulus and granulosa (Fontouris and Campbell, 2002), as well as in sheep, cattle, and mouse oocytes (Bodensteiner et al., 1999; McGrath et al., 1995). The abundance of GDF-9 transcripts is lower in in vivo matured bovine oocytes, compared to in vitro matured oocytes (Lonergan et al., 2003), suggesting that this gene transcript could serve as a potential marker of developmental competence.

Other genes that are frequently studied during oocyte maturation are genes related to oxidative stress. RNA from five genes related to oxidative stress (mnSOD, Cu/ZnSOD, GPX, GCS, and SOX) were all present in bovine oocytes (Lonergan et al., 2003). Similar studies have found GCS, GPX, Cu/Zn SOD, and MnSOD RNA in MII human oocytes and mouse oocytes (El Mouatassim et al., 1999; Goto et al., 2002). This cross species conservation of gene transcription suggests the importance of these genes in oocyte physiology. Bovine oocytes that were matured in vitro had an increase in expression of

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some of these genes, suggesting either an increase in the need to protect these oocytes from oxidative stress, or a down regulation of degradation pathway of these enzymes (Lequarre et al., 2001). Additionally, three gene transcripts that function to protect cells from oxidative stress were down regulated in bovine oocytes following in vitro maturation (Dalbies-Tran and Mermillod, 2003), possibly suggesting an increased translation of these transcripts to protect IVM oocytes.

Although there is a large amount of data regarding gene expression of oocytes during different stages of oocyte growth and maturation (Bachvarova et al., 1985; Marello et al. 1992; Robert et al., 2000), there is a paucity of information regarding the level of gene expression in oocytes matured under varying maturation conditions. Maturation medium was shown to affect two out of six gene transcripts (Watson et al., 2000), while ten out of twelve gene transcripts were shown to differ in abundance between in vivo matured and in vitro matured oocytes (Lonergan et al., 2003). As determined by heterologous hybridization onto a human cDNA array, 70 transcripts underwent significant differential regulation during in vitro maturation of the bovine oocyte (Dalbies-Tran and Mermillod, 2003).

Lack of developmental competence in the majority of in vitro matured oocytes may reflect the composition and abundance of specific mRNA transcripts in the oocyte (De Sousa et al., 1998). The use of DNA microarray technology allows identification of candidate genes that may play a role in oocyte maturation and subsequent developmental competence. Comparisons between in vivo matured oocytes and oocytes matured under

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varying conditions will aid in the understanding of the impact of these factors on the regulatory elements controlling both meiotic and developmental competence.

It is evident from the literature that current in vitro maturation systems impair cellular function, leading to compromised developmental potential of the oocyte. The main objective of this thesis was to characterize the metabolism and gene expression of in vivo and in vitro matured oocytes. Identification of components in IVM systems that affect cell function will aid in the optimization the IVM system, with the goal of producing oocytes of equivalent developmental competence to their in vivo matured counterparts.

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CHAPTER III

RESEARCH DESIGN AND METHODS

Chemicals and Hormones

Unless otherwise specified, all chemicals and hormones used in the research described within this thesis for murine and bovine experiments were purchased from Sigma (St. Louis, MO, USA) and prescreened for embryo toxicity using a sequential mouse pronucleate oocyte assay (Gardner et al., 2005).

Plasticware

The plasticware used in the experiments is listed in Appendix A. All plasticware was prescreened for embryo toxicity using a sequential mouse pronucleate oocyte assay (Gardner et al., 2005).

Mice

All mice used in this research were F1 hybrid mice (C57BL/6 x CBA). Mice were housed in an environment of 14 h of daylight and 10 h of dark. Room temperature of the mouse facility was maintained at 21 ± 2 °C. Mice were provided with feed and water ad libitum. All research was approved by the Institutional Animal Care and Use Committee (IACUC).

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Collection of immature and mature mouse oocytes

Cumulus-enclosed oocytes were collected from 28 d old F1 (C57BL/6 x CBA) females. Mice were sacrificed by cervical dislocation, and the ovaries were removed and placed into G-MOPS (20 mM MOPS-buffered G medium) with 5% fetal calf serum (FCS). FCS was included in the collection medium to prevent hardening of the zona pellucida. Antral follicles were punctured with sterile needles, releasing COCs into the collection medium. After all follicles were punctured, COCs were isolated and washed three times through G-MOPS prior to placement in maturation medium. COCs were selected on the basis of cumulus morphology; only COCs having several layers of cumulus completely surrounding the oocyte were selected for experiments. Depending on the experiment, COCs were recovered from mice primed with equine chorionic gonadotropin (eCG) or mice that were not primed with eCG. Collection of immature oocytes from primed mice, occurred 48 h post eCG (5 IU) injection. For collection of in vivo matured oocytes, 5 IU eCG was administered i.p. followed by the administration of 5 IU human chorionic gonadotropin (hCG; i.p.) 48 h later and by oocyte collection 10 h later.

In vitro maturation of mouse oocytes

Maturation medium (Appendix B) for all experiments was prepared on the day of oocyte collection and equilibrated a minimum of 4 h. For individual maturation, oocytes were matured in 2 µl drops under paraffin oil (Ovoil, Vitrolife, Gothenburg, Sweden). For group maturation, 5 oocytes were matured in 30 µl drops of maturation medium under paraffin oil. Oocytes were matured at 37 °C, in 6% CO_2. Oxygen tension was either 5%

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or 20% O_2, depending on the experiment. Oocytes were matured for 17 h, at which time maturation rates were assessed.

In vitro fertilization of mouse oocytes

Fertilization medium was prepared on the evening prior to, or the day of fertilization and equilibrated a minimum of 4 h. Sperm were collected from 3-6 month old F1 males (C57BL/6 x CBA) on the morning of in vitro fertilization. Males were sacrificed by cervical dislocation, and the epididymis and vas deferens were dissected and placed into a dish containing 3 ml G-MOPS supplemented with 5mg/ml human serum albumin solution (HSA; Vitrolife, Gothenburg, Sweden). Following dissection, the epididymis and vas deferens were placed into 800 μl of equilibrated fertilization medium (G-Fert; Lane and Gardner, 2004) under paraffin oil. Sperm were removed by sliding forceps along the vas deferens, as well as by puncturing the epididymis several times with forceps to release the sperm. Recovered sperm were capacitated for a minimum of 1 h in G Fert medium at 37°C, under 6 % CO_2 and 5% O_2. Mature COCs (at 17 h maturation) were placed into fresh drops of G-Fert medium under paraffin oil. For individual fertilization, one COC was placed into a 20 μl drop. For group fertilization, up to 30 COCs were placed in a 90 μl drop of G-Fert medium. To count sperm for insemination, 95 μl of water were combined with 5 μl sperm suspension. A hemocytometer was used to count the number of sperm in the sample. All counts were between 30-40 sperm in a total of 5 squares on the hemocytometer chamber, for a final concentration between 1.2 to 1.6×10^6 sperm/ml. Sperm were then added to fertilization medium containing the COCs. For individual fertilization, 1 μl of sperm suspension was added to the

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fertilization medium, while 10 μl was added to fertilization medium containing a group of oocytes. Gametes were co-incubated for 6 to 8 h depending on the experiment.

Mouse embryo culture

Following in vitro fertilization, oocytes were placed into G-MOPS. A fine-bore pipette was used to clean cumulus and sperm from the presumptive zygotes. Zygotes were then placed into G1.3 culture medium (Vitrolife, Gothenburg, Sweden). For individual culture, one zygote was cultured in 5 μl of G1.3. For group culture, 10 zygotes were cultured in a 20 μl drop of G1.3. Embryos remained in G1.3 for 48 h, at which time embryo development was assessed, and embryos were placed into G2.3 (Vitrolife, Gothenburg, Sweden) for an additional 48 h. At 96 h of culture, blastocyst development was assessed.

Mouse Embryo Transfer

Vasectomy procedure

The male was anesthetized with Avertin (0.02ml/g body weight) and laid on his back on a dissecting microscope stage. A 2-3 mm incision was made ventrally along the scrotal sac below the penis and through the body wall and peritoneum. A small incision was made in the bursa sac, and the epididymis was pulled through the incision. Using a pair of forceps, the vas deferens was dissected away from the testes and the blood vessel and a 1 cm section of the vas deferens was cut and removed. The procedure was then repeated on the other testicle. Absorbable suture was used to stitch the body wall and skin. Males were given 2-3 weeks to recover from the procedure and then they were placed with two

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superovulated females. Then next day females were checked for vaginal plug. On d 2, the oviducts of the mated females were flushed. The presence of unfertilized eggs in the females indicated that the males were sterile and could be used to obtain pseudopregnancies.

Preparation of pseudopregnant recipients

Two mature females (8-12 weeks old) were placed with vasectomized males the evening before the desired d 1 of pseudopregnancy. The following morning, mating was confirmed by the presence of a vaginal plug. This was designated as d 1 of pseudopregnancy.

Embryo Transfer

In vitro produced embryos were cultured for 96 h. Blastocysts were transferred to d 4 pseudopregnant F1 (C57BL/6 x CBA) female mice (-1 d asynchronous). Recipients for embryo transfer were anesthetized with Avertin (0.02ml/g of body weight) and laid face down on the microscope stage. The back of the recipient was sprayed with 70% ethanol. An incision was made ventrally down the midline of the back, exposing the body cavity. The incision was wiped with 70% ethanol to remove any loose hairs. With watchmaker's forceps, the body cavity was bluntly dissected from the skin on the left side of the mouse. The opening of the skin was moved over the area of the ovary, and an incision was made into the peritoneum. Watchmaker's forceps were used to grab the fat pad and expose the ovary, oviduct and beginning of the uterus. The fat pad was clipped to the back of the mouse, while the embryos were loaded in embryo glue (Vitrolife, Gothenburg, Sweden)

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into a fine-bore glass transfer pipette. A large air bubble was placed in the transfer pipette and the embryos were loaded in a small volume of embryo glue immediately following the air bubble. A second air bubble was created after the embryos were loaded into the pipette. Embryos (≤ 6) from each treatment were allocated to separate uterine horns. After embryos were loaded into the transfer pipette, a 27-gauge needle was used to make a hole in the uterus approximately 1/3 of the way down from the oviduct. Upon removal of the needle, the tip of the transfer pipette was inserted into the hole in the uterus, and the embryos were expelled. Following transfer, the pipette was checked to ensure that all embryos were expelled. The reproductive tract was then placed back into the body cavity. The same procedure was followed on the right side of the mouse. At the end of the procedure, the incision was closed with wound clips. Recipients were ear tagged for subsequent identification.

Recipient Dissection

On day 15, recipients were sacrificed by cervical dislocation. An incision was made in the abdomen and the uterine horns were dissected. The number of fetuses and implantation sites were recorded. Each fetus was removed from the uterus using forceps and scissors. Weights of resultant fetuses were determined.

Collection of mature and immature bovine oocytes

Cows

Cows were housed at Colorado State University in an approved facility. All research was approved by the Animal Care and Use Committee (ACUC) at Colorado State University.

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Abattoir Ovaries

Unless otherwise noted in the experiment, abattoir ovaries were collected from cows that did not receive exogenous hormones such as melengesterol acetate (MGA). Ovary transport time ranged from 2-8 h; exact times are specified in each experiment.

Transvaginal Aspiration (TVA)

Cumulus-enclosed oocytes were collected from cows undergoing the following regimen: 6 injections of 50 mg FSH (Folltropin-V; Bioniche, Onterio, CA), were administered twice daily for 3 days. For collection of immature oocytes, TVA was performed 48 h post final FSH injection. For the collection of in vivo matured oocytes, cows were administered 25 mg prostaglandin (PG; Lutalyse, Pfizer, NY, USA) simultaneously with the last FSH injection. GnRH (50ug; Cystorelin, Merial Ld, Duluth, GA, USA) was administered 37 h post PG. TVA was performed 24 h post GnRH administration.

<u>Bovine in vitro maturation, fertilization of abattoir oocytes, and embryo culture</u>

COCs were aspirated from 3-6 mm follicles with a vacuum pump set at 50 psi. COCs were recovered and washed in G-MOPS plus 5 mg/ml human serum albumin solution (HSA; Vitrolife, Gothenburg, Sweden) a minimum of 3 times prior to being placed into G maturation medium (Lane and Gardner, 2004). Only COCs with cumulus completely surrounding the oocyte were selected. Cumulus enclosed oocytes were matured in four-well dishes (33 COCs per 500 μl) at 38.5°C under 6% CO_2 and air for 24 h, at which time they were inseminated with frozen thawed semen. Frozen sperm was prepared using a Puresperm gradient (45/90%; Nidacon, Sweden). After centrifugation through

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Puresperm for 20 min, the pellet was washed (centrifuged for 5 min) twice in sperm TALP. Following the second sperm wash, the sperm concentration was determined using a hemocytometer. Sperm concentration was adjusted to 5×10^6 sperm/ml by adding the pertinent amount of G-Fert. Sperm suspension (50µl) was then added to the fertilization dish already containing the COCs (50 COCs/ 450 µl), for a final concentration of 5×10^5 sperm/ml. Gametes were co-incubated for 18 h at 38.5°C, 6% CO_2 in air (50 COCs/500 µl). Presumptive 1-cell embryos were then vortexed for 2 min in G-MOPS and placed into G1.3 culture medium supplemented with 8 mg/ml BSA (Bovine Albumin Crystallized; Serologicals Proteins Inc, Kankakee, IL) for 72 h (50 embryos/500 µl) at 38.5°C, 6% CO_2, 5% O_2, 89% N_2. After 72 h, embryos were washed and placed into G2.3 medium supplemented with 8 mg/ml BSA with minimal medium carry over. At 120 h post IVF, blastocyst development was evaluated.

In vitro maturation of bovine COCs recovered by transvaginal aspiration

COCs (x10) were placed in 100 µl drops of maturation medium for 23 h at 38.5°C in 6% CO_2 in air.

Determination of total blastocyst cell number

The staining procedure for cell number analysis was performed at room temperature. Expanded blastocysts or hatching blastocysts were placed into Triton X 100 solution (20 µl in 100 ml G-MOPS) for 45 sec. Embryos were then moved into a wash dish containing G-MOPS and transferred into 20 µl propidium iodide (PI; 0.2 mg/ml PI in G-MOPS) drops under paraffin oil, where they remained for a minimum of 10 min. PI specifically

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binds the DNA and thus stains the cell's nucleus. Stained embryos were mounted in glycerol on a siliconized glass slide. Cell number was determined by counting the cell nuclei using a fluorescence microscope with a UV filter.

Oocyte and Embryo Metabolism

Oocytes and embryos were washed a minimum of three times in G2 metabolism medium (modified G2 with 0.5 mM glucose, 0.23 mM pyruvate, and 0 mM lactate), 2.5 mg/ml recombinant human serum albumin, 0.5 mM sodium citrate (Appendix B). Oocytes and embryos were thoroughly washed in 800 μl of metabolic medium and then each was individually placed in a fresh 20 μl drop of metabolic medium prior to placement in incubation volumes. Thorough washing of oocytes and embryos was required, as any media transfer would alter the starting concentrations of carbohydrates in the metabolic medium, resulting in inaccurate data. Oocytes and embryos were then individually placed in 0.5 or 1 μl drops of medium for 1-4 h, depending on the experiment.

Quantification of oocyte and embryo metabolism

Fluorometric assays were used to determine carbohydrate levels in the metabolism medium. Fluorometric assays are based on the generation or consumption of the reduced pyridine nucleotides NADH or NADPH, which fluoresce when excited by light at 340 nm (Leese and Barton, 1984; Gardner and Leese, 1990). The oxidized counterparts NAD^+ and $NADP^+$ do not fluoresce. The concentration of several carbohydrates can be individually analyzed using this method. Each carbohydrate requires a specific assay.

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The following assays were prepared: glucose, pyruvate, and lactate. The complete composition of these reagents, buffers, and standards are in Appendix C.

For the assay, a sample of metabolic medium, in which the oocyte or embryo was incubated for a specific period of time, was added to a reagent drop. Volumes for the assays were in the nanoliter range, and typically 5 nl of sample were added to 30 nl of reagent. Prior to adding the sample to the reagent, the fluorescence of the reagent was measured upon exposure to light at 340 nm. Sample was then added to each reagent drop (one reagent drop per embryo or oocyte sample). The reaction of sample and reagent proceeded for 3 min, and a second measurement was taken by exposing the drop to light at 340 nm. The difference of the two measurements was recorded and compared to a standard curve performed on the same day as the analysis.

The following reactions provide the basis of measurement for lactate, pyruvate and glucose.

<u>Lactate and Pyruvate</u>:

Lactate Dehydrogenase

Lactate + NAD^+ $\leftrightarrow$ Pyruvate + NADH + H^+

Both lactate and pyruvate assays utilize the same reversible reaction. The direction of the reaction is dictated by the components of the cocktail assays: NAD^+ (lactate assay) or NADH (pyruvate assay). When measuring the amount of lactate within sample medium, the sample is added to lactate reagent. The lactate in the sample will then interact with

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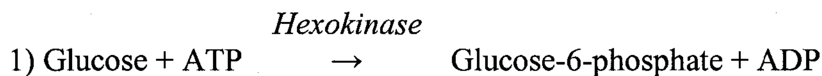
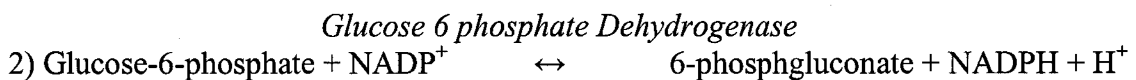
the NAD^+ in the reagent, driving the reaction to the right and producing NADH+ H^+. There is a linear relationship between lactate concentration and NADH + H^+; therefore, increased lactate in the sample will result in a higher level of fluorescence. Consequently, with less lactate in the sample, less NADH will be formed, and a lower level of fluorescence will be observed.

When measuring the quantity of pyruvate in a sample, the sample is added to pyruvate reagent. As the pyruvate reagent contains NADH, the reaction is driven to the right upon addition of a sample containing pyruvate, resulting in the production of NAD^+. An inverse linear relationship exists between pyruvate and NADH; therefore, as the concentration of pyruvate in the sample increases, the resulting fluorescence will decrease. Lower pyruvate present in a sample will preserve the original concentration of NADH in the pyruvate reagent, and thus will result in a high level of fluorescence.

<u>Glucose:</u>





When a sample containing glucose is added to the glucose reagent, two successive reactions will proceed. Since ATP is present in the glucose reagent, the addition of glucose from the sample will drive the first reaction to the right. The product of the first reaction (glucose-6-phosphate) will then interact with the $NADP^+$ in the glucose reagent

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and drive the second reaction to the right. A product of the second reaction is NADPH, which fluoresces upon exposure to light at 340 nm. There is a linear relationship between glucose concentration and NADPH; therefore, as the amount of glucose increases, a higher level of fluorescence will be recorded.

Quantitation of fluorescence with standard curves

Fluorescence measurements are rarely reported in absolute units. Fluorescence is affected by numerous factors, such as quantity of light, temperature, as well as the type of fluorescence detector (CCD camera or photomultiplier tube). Therefore, most fluorescence measurements are calibrated with standard curves.

A separate standard curve for each carbohydrate was performed on the day of sample analysis. Each standard curve contained 6 points of measurement: 0 mM, 0.1 mM, 0.2 mM, 0.3 mM, 0.4 mM, and 0.5 mM. The fluorescence of each known concentration was plotted, with an acceptable curve having an $r^2 \geq 0.99$ (Figure 1a, b, and c). The concentration of carbohydrates in samples was determined through linear regression of sample values and values obtained from the standard curve for the carbohydrate of interest.

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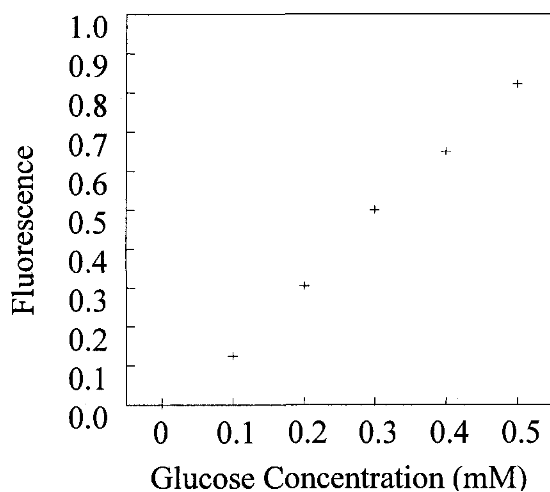

Figure 1a.
Glucose standard curve. $r^2 = 0.999$

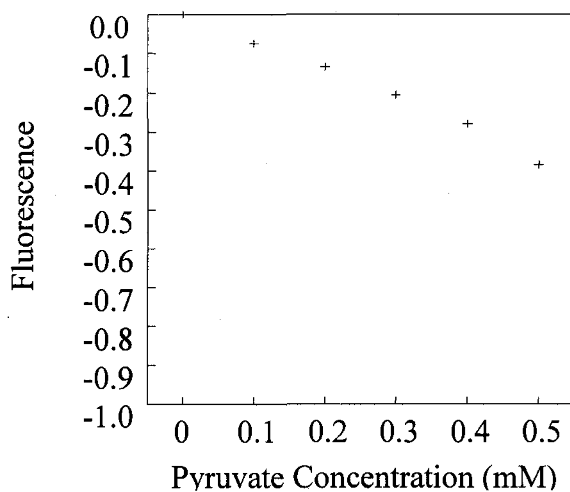

Figure 1b.
Pyruvate standard curve. $r^2 = 0.991$

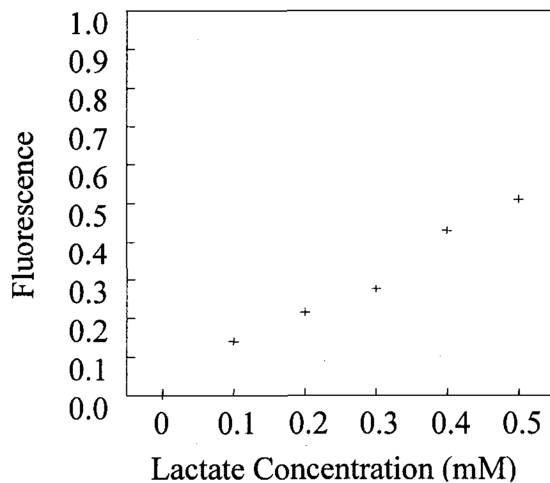

Figure 1c.
Lactate standard curve. $r^2 = 0.992$

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Micropipettes for Metabolic Analysis

Constriction pipettes of submicroliter volume were constructed for metabolic analysis. Pipettes were made from borosilicate glass capillaries (o.d. 1.0 mm, i.d. 0.8mm) pulled over a flame to produce an inner diameter of 50-100 μm. The tubing was snapped in half and a small hook was made on each end. Using a microforge, a constriction was made in the glass by placing the heated filament close to the glass capillary. A paperclip was placed onto the end of the hook of the capillary and the tip was made by again heating the glass with the microforge filament. As the glass heated up, the weight of the paperclip resulted in the glass pulling to a tip. The tip was broken with a pair of watchmaker's forceps. Glass pipettes were mounted in 16 gauge stainless steel tubing and sealed using hot wax. Filling and expelling fluids from the pipettes was achieved by using an air filled syringe attached to the pipette via thin tubing. Micropipettes were siliconized and then calibrated with tritiated water.

Oxygen Uptake

Pyrene fluorescence was used to determine the level of oxygen consumption (Houghton et al., 1996). Fluorescence of pyrene diminishes in the presence of oxygen; therefore, as oocytes consume oxygen, the amount of fluorescence increases. A pyrene solution was made by dissolving 1 mg/ml pyrene in paraffin oil (Vitrolife, Gothenburg, Sweden). To measure oxygen uptake a metal plunger was inserted into a glass polymerase chain reaction (PCR) micropipette (Drummond, PA, USA). One microliter of pyrene solution was loaded into the micropipette, followed by 2 μl G-MOPS containing COCs or denuded oocytes (Figure 2). Rubber tubing was placed over one end of the capillary and

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sealed with a tube clamp. The other end of the capillary was sealed with hot wax. Sealed capillaries were incubated at 37˚C.

Positive and negative controls were set up for each experiment. The positive control consisted of 1 mg/ml baker's yeast in modified G-MOPS containing 60 mM glucose. The negative control consisted of only G-MOPS. For both the positive and negative controls, 1 µl of pyrene solution was loaded into the micropipette followed by 2 µl of yeast solution/or G-MOPS. Control pipettes were sealed in the same manner as the sample pipettes. The positive control was set up the night prior to the experiment which allowed the yeast to consume 100% of the oxygen. The negative control was set up at the same time the samples were assayed, and served as a base line for no oxygen uptake.

Two readings of pyrene fluorescence were measured for each sample and both controls every 40 min for 160 min. One reading was taken at the pyrene/ plunger interface, and the second reading was taken at the pyrene/ medium (G-MOPS) interface. The level of fluorescence for samples and controls was entered into a finite difference computer model which calculated the amount of oxygen uptake in nanoliters per oocyte per hour.

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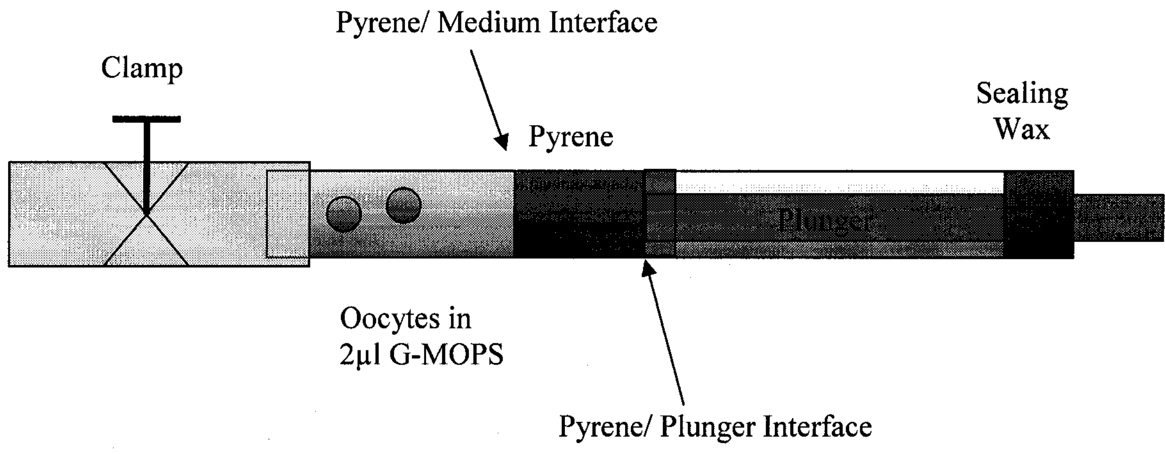
Figure 2. Illustration of a PCR capillary pipette for measurement of oxygen consumption. The pipette is sealed on both ends, thus allowing for the quantification of oxygen uptake by the oocytes contained within the pipette.



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Mitochondrial Membrane Potential

Cumulus cells were removed from metaphase II oocytes by pipetting COCs in G-MOPS containing 1mg/ml hyaluronidase. Denuded oocytes were washed (x3) in G-MOPS and then incubated in maturation medium supplemented with 0.5 µmol 5,5'6,6'-tetrachloro-1,1,3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1; Molecular Probes, Invitrogen, Carlsbad, CA, USA) for 30 min.

JC-1 exists in two forms. When mitochondrial membrane potential is high, indicating healthy mitochondria, JC-1 enters the mitochondrial matrix and accumulates in an aggregated form which fluoresces red. When the mitochondrial membrane potential is low, JC-1 remains in the cytoplasm in a monomeric form which fluoresces green (Figure 3).

Following 30 min incubation in JC-1, oocytes were washed in G-MOPS and placed into fresh 5 µl drops of G-MOPS under paraffin oil on a glass slide. JC-1 is a ratiometric dye that is internalized as a monomer dye (green fluorescence, emission wavelength 530 nm) and is concentrated by respiring mitochondria with negative inner membrane potential into J-aggregate dye (orange fluorescence, emission wavelength 590 nm). The ratio of JC-1 fluorescence was measured on an Eclipse TE2000 (Nikon, Melville, NY, USA) microscope with a 20x Plan Fluor objective. Fluorescent images were captured with a CCD camera: Cascade 512B (Roper Scientific, Duluth, GA, USA) and analyzed using MetaFluor (Universal Imaging, Downingtown, PA, USA) software.

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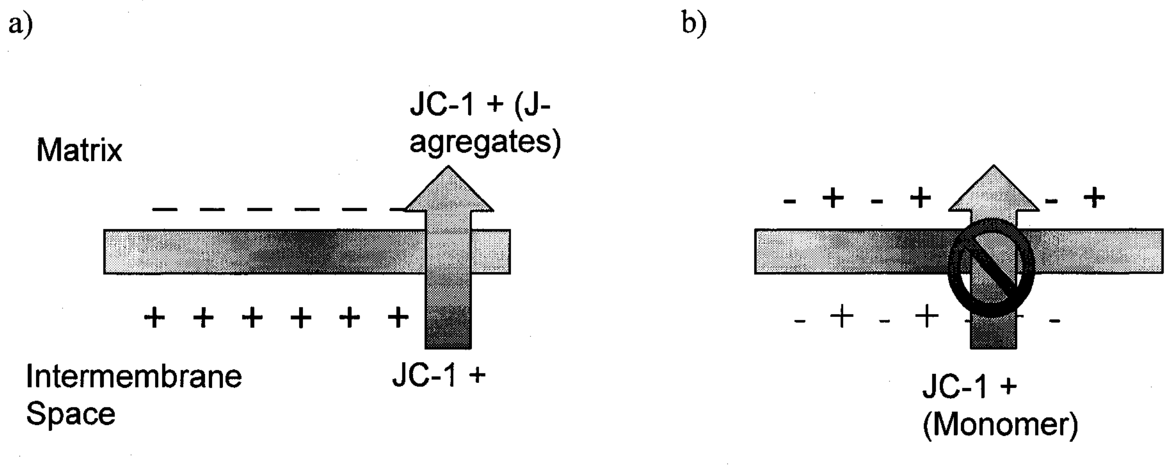
Figure 3. Illustration of JC-1 activity within mitochondria. In healthy mitochondria (a), protons are pumped across the inner mitochondrial membrane, creating an inner membrane potential. In mitochondria of high membrane potential, JC-1 enters the mitochondrial matrix and forms J-aggregates. The inner membrane potential of degenerate mitochondria (b) is disrupted, depleting available energy for the conversion of ADP to ATP that is required for cellular functions. Disruption of mitochondrial membrane potential prevents JC-1 from crossing the inner membrane of the mitochondria, resulting in the build up of J monomers in the intermembrane space.



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RNA Isolation and Extraction

Pronase (0.5% in G-MOPS) supplemented with 5% HSA and phosphate-buffered solution (PBS) supplemented with 10% BSA were warmed for 60 min at 37°C. BSA used in RNA procedures was molecular biology grade, free of RNases and DNases. Denuded oocytes were repeatedly pipetted in 500 μl pronase until the zona dissolved. Upon removal of the zona, oocytes were moved to 500 μl PBS. With a clean pipette, oocytes were washed in PBS (x3) to remove any residual pronase. Oocytes (single or groups of ≤9) were transferred with minimal PBS into a 0.65 ml tube containing 10 μl of Arcturus extraction buffer (Arcturus, CA, USA). A clean pipette was used to transfer each group of oocytes or single oocytes to the tube containing extraction buffer. Contents of the tube were mixed by pipetting with a clean pipette tip and then snap frozen and stored at -80°C. RNA extraction was performed using the Arcturus PicoPure RNA isolation kit (Arcturus, CA, USA) with modifications (Appendix D). Each purification column extracted RNA from a combined 10 oocytes of the same treatment group or single oocytes in each treatment group. Extracted RNA was stored at -80°C until further processing.

Microarray Analysis

RNA quantification, amplification and microarray analysis were performed by the Health Sciences Center, at the University of Colorado. RNA samples were quantified using a nanodrop spectrophotometer. RNA integrity was measured on the Agilent 2100 Bioanalyzer, which requires only nanogram quantities of RNA. The bioanalyzer fractionates RNA molecules according to size, and calculates the amount of 18S and 28S

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rRNA. Theoretically, intact RNA will have 28S:18S ratio of 1.7-2.0. The quality of the RNA was evaluated before and after amplification using the Agilent 2100 Bioanalyzer.

Bovine oocyte samples underwent two rounds of RNA amplification, incorporating a T7 polymerase promoter. T7 RNA polymerase is a linear amplification method which results in minimal bias due to the size of the template. Following amplification, samples were fluorescently labeled and allowed to hybridize with gene-specific targets on bovine-specific gene arrays (GeneChip Bovine Genome Array; Affymetrix, Santa Clara, CA). The bovine genome array contained approximately 24,128 bovine transcripts.

Quantitative real-time PCR

Samples for real-time PCR included the amplified RNA generated from the microarray experiment and total RNA extracted from single oocytes from each treatment group. A cDNA template was generated from all samples using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics; Appendix E), according to manufactor's instructions. Relative quantification of specific genes was performed on the cDNA generated from RT-PCR using Roche Faststart DNA Master kit (Roche Diagnostics, Appendix F), generating Ct values and normalized ratios using light cycler software (Roche Diagnostics).

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CHAPTER IV

METABOLIC MARKERS OF DEVELOPMENTAL COMPETENCE FOR IN VITRO MATURED MOUSE OOCYTES

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Published: October, 2005 in Reproduction

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ABSTRACT

In vitro maturation (IVM) of oocytes has enormous potential in assisted reproductive technology, but its use has been limited due to insufficient knowledge of oocyte physiology during this dynamic period and lack of an adequate maturation system. The aim of this study was to characterize metabolic profiles of three groups of oocytes throughout maturation: 1) cumulus oocyte complexes (COCs), 2) denuded oocytes, and 3) denuded oocytes co-cultured with cumulus cells. Mouse oocytes were collected from 28 day old unstimulated females and matured in a defined medium. Oocytes were matured individually and transferred into fresh 0.5μl drops of medium at 4 h intervals until 16 h. Ultramicrofluorimetry was used to quantitate carbohydrate consumption from and metabolite release into the medium. Glucose consumption and lactate production of COCs increased ($P<0.001$) over the maturation interval (0-16 h). Glucose consumption by COCs that subsequently fertilized was higher between 8-12 h of maturation than by COCs that did not fertilize (38 versus 29 pmol/COC/h, respectively; $P<0.01$). Lactate production by COCs that subsequently fertilized was higher between 8-16 h of maturation, than by oocytes that did not fertilize (8-12 h, 66 versus 46 pmol/COC/h, $P<0.01$; 12-16 h, 56 versus 40 pmol/COC/h, respectively; $P<0.05$). These data indicate that the final hours of maturation may hold a unique marker of oocyte competence, as during this time fertilizable COCs take up more glucose and produce more lactate than those not subsequently fertilized.

Key Words: Oocyte, glucose, pyruvate, lactate metabolism, viability, maturation

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INTRODUCTION

Meiotically competent oocytes will mature spontaneously once released from the follicle if matured under appropriate conditions (Pincus and Enzmann, 1935). Maturation rates vary between, as well as within, species and depend on medium composition and whether immature oocytes were collected from females primed with FSH prior to oocyte collection or unprimed females, i.e. those not receiving FSH. Reported ranges in the mouse are 67-73% MII, unprimed (De la Fuente et al., 1999) and 89% MII, primed (Fagbohun and Downs, 1992). Maturation rates are routinely determined by the expulsion of a polar body, representing nuclear maturation. However, developmental competence of oocytes is acquired through completion of both nuclear and cytoplasmic maturation. Only a small portion of IVM oocytes go on to develop into blastocysts, where as oocytes matured in vivo have much higher embryo development rates (Van de Leemput et al., 1999; Blondin et al., 2002). Low blastocyst formation by IVM oocytes indicates that current oocyte maturation systems do not adequately support either nuclear and/or cytoplasmic maturation.

Understanding energy substrate metabolism of the oocyte throughout IVM may aid in optimizing maturation conditions. Energy requirements of the cumulus oocyte complex (COC) are unique, as the cumulus cells and oocyte have different metabolic needs. Denuded mouse oocytes require pyruvate or oxaloacetate in the medium in order to mature, whereas COCs develop in medium containing lactate, phosphoenolpyruvate, or glucose (Biggers et al., 1967). Cumulus cells metabolize alternative substrates, such as glucose, which ultimately produce substrates that the oocyte requires for maturation.

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Metabolism has been assessed in the mouse oocyte by several groups; however, experimental designs allowed for only a single time point measurement of individual oocytes (Roberts et al., 2004) or multiple measurements throughout maturation using groups of oocytes, thus making correlation with meiotic status and subsequent embryo development difficult (Downs et al., 2002). Additionally, oocytes used in most studies were collected from mice primed with exogenous gonadotropins, which may alter the metabolic profile of the recovered oocytes (Downs et al., 1996; Fagbohun and Downs, 1992). Oocytes recovered from mice primed with eCG have higher maturation, 2-cell and blastocyst rates (De la Fuente et al., 1999). However, ovarian stimulation in mice has been associated with delayed embryonic development, fetal growth retardation, and increased fetal loss (Edwards et al., 2004; Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). Supplementation of maturation medium with FSH has varying effects on oocyte developmental competence based on age of donor mice and in vivo priming with gonadotropins. After in vitro maturation in the presence of FSH, oocytes collected from primed 22-24 day old mice displayed increased developmental competence (Downs et al., 1986). However, in older mice (26 day) FSH supplementation in the maturation medium produced improved developmental competence only in oocytes collected from unprimed mice, with no effect seen in oocytes collected from primed mice (Eppig et al., 1992). Additionally, when gonadotropins are supplemented in the maturation medium of oocytes recovered from both primed and unprimed mice, the metabolic profiles differ between groups. When immature oocytes were collected from unprimed mice and subsequently matured with or without FSH, the FSH groups had higher glucose uptake and higher production of both lactate and pyruvate (Roberts et al.,

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2004). In addition to altering carbohydrate uptake and production, maturation in the presence of FSH changes substrate metabolism. COCs principally metabolize glucose via glycolysis, and the addition of FSH in the medium increases glycolysis 2.7 fold (Downs et al., 1999). Furthermore, supplementation of FSH into maturation medium induced aneuploidy in IVM mouse oocytes (Roberts et al., 2005).

In addition to assisting medium optimization, metabolic profiles may also serve as a potential marker of oocyte viability. Oocyte morphology is often used as a predictor of development; however, this is not a very accurate method of selection. Nuclear and cytoplasmic maturation are often asynchronous. Cytoplasmic maturation is required for activation of the oocyte at fertilization and subsequent embryo development. The oocyte's cytoplasm provides the appropriate metabolic machinery for production of energy for cellular functions during maturation, fertilization, and embryo development. Metabolic profiles of mouse embryos have been established as valuable indicators of embryo viability (Gardner and Leese, 1987; Lane and Gardner, 1996). The use of noninvasive metabolic assays would quantify oocyte characteristics and potentially result in a method of predicting developmentally competent oocytes.

The metabolic profiles of individual mouse oocytes throughout maturation have not been studied in the unstimulated mouse. The first objective of this study was to characterize the metabolic profile of the individual oocyte throughout maturation. Three categories of oocytes were included: 1) cumulus oocyte complexes (COCs), 2) denuded oocytes, and 3) denuded oocytes matured in the presence of cumulus cells. The second objective was

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to compare metabolic profiles of COCs that matured and fertilized versus COCs that exhibited nuclear maturation, but failed to fertilize.

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MATERIALS AND METHODS

Experiment 1: Characterization of oocyte metabolism throughout in vitro maturation

Oocyte Collection, InVitro Maturation, Fertilization, and Culture

Oocytes were collected from unstimulated 28 day old (C57BL/6 x CBA) F1 female mice. Antral follicles were punctured from ovaries with sterile 30 gauge needles and COCs were released into the collection medium (G-MOPS plus 5% bovine calf serum; Lane and Gardner, 2004). Oocytes were rinsed 3 or 4 times in a defined maturation medium (Modified G2 with 0 mM lactate, 0.23 mM pyruvate, 0.5 mM glucose 2.5 mg/ml human recombinant serum albumin (Delta Biotechnology Limited, Nottingham, UK), 0.5 mM citrate, 0.5 mM cysteamine, 100 ng/ml EGF, ITS, 2 mg/ml fetuin; Gardner et al., 2001), prior to being placed into final culture drops. Unless otherwise noted, all chemicals and hormones were purchased from Sigma (St. Louis, MO USA) and prescreened for embryo toxicity using a sequential mouse pronucleate embryo assay (Gardner et al., 2005). Oocyte maturation, fertilization, and embryo culture were performed in 60 mm (3652) Falcon dishes (Becton Dickenson, Franklin Lakes, NJ, USA). Oocytes were matured individually at 37°C in 6% CO_2, 5% O_2, 89% N_2 in 0.5 µl drops of medium under paraffin oil (Ovoil, Vitrolife, Gothenburg, Sweden). Oocytes were transferred into a fresh 0.5µl drop of medium every 4 h, until 16 h. After oocytes were removed, the medium was frozen and stored at -80°C for subsequent analysis. Oocytes were transferred into individual drops of fertilization medium at 17 h post retrieval. For fertilization, all oocytes were denuded, and no cumulus cells were replaced into the medium. Sperm was

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collected from the epididymis and vas deferens of 3-6 month old F1 (C57BL/6xCBA) male mice and allowed to capacitate in fertilization medium for 1 h prior to insemination. Gametes were co-incubated for 8 h, after which presumptive zygotes were transferred individually into 5 μl drops of G1 culture medium (Gardner and Lane, 2004). Embryos were transferred into fresh G1 medium after 24 h and into G2 medium 48 h post fertilization. Embryos were cultured individually until 96 h at which time blastocyst rates were recorded. Oocyte maturation rates were determined by the presence of a polar body at the end of the 17 h maturation period and again at the end of the fertilization period. All experiments were approved by the Institutional Animal Care and Use Committee.

Assessment of Oocyte Metabolism

Oocytes (n=604) were matured in one of nine treatment groups (Fig. 1). A minimum of 50 oocytes were matured in each group. Group 1 was matured with cumulus cells intact throughout maturation (0-17 h). Cumulus cells were removed from remaining groups at various intervals, and stripped oocytes were matured with or without the presence of cumulus cells. Groups 2, 3, 4, and 5 were denuded at 12, 8, 4, and 0 h, respectively, and subsequently matured with no cumulus cells. Groups 6, 7, 8, and 9 were denuded at 12, 8, 4, and 0 h, respectively and their cumulus cells were replaced into the medium for the duration of maturation. Oocytes were denuded in G-MOPS with 1 mg/ml hyaluronidase. Oocytes and cumulus cells were rinsed 3 or 4 times in the defined maturation medium prior to incubation. Control oocytes (COC, denuded, and denuded plus cumulus) were individually matured in 5 μl drops and remained undisturbed in the

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incubator until 17 h of maturation. Fertilization and embryo culture of control oocytes were identical to treatment oocytes.

Metabolism was assessed from a minimum of 14 oocytes per group. Ultramicrofluorimetry was used to quantify the level of carbohydrate production and consumption from maturation medium. Fluorometric assays are based on the generation or consumption of the reduced pyridine nucleotides NADH or NADPH, which fluoresce when excited by 340 nm light (Leese and Barton, 1984; Gardner and Leese, 1990). Assay cocktails included all necessary enzymes and cofactors needed to carry out the reaction, depending on the carbohydrate being analyzed. All cocktail to sample ratios were 10:1. For the assay, a 30 nl cocktail drop was placed on a siliconized slide under mineral oil and exposed to 340 nm light to obtain a base reading. A 3 nl sample was then added to the cocktail drop. The reaction was allowed to proceed for 3 min, and a second reading was taken. The difference was determined between the initial reading of the cocktail and subsequent reading of the cocktail plus sample. Concentrations of substrate were calculated though comparison of the change with known quantities calculated from a standard curve ($r > 0.99$) obtained for each carbohydrate on each day of analysis.

Cumulus Cell Counts

Cumulus-oocyte complexes (n=56) were denuded upon removal from the follicle and divided into four groups. Denuded oocytes were matured with their cumulus cells in 0.5µl drops of medium, with a medium change over every 4 h as described above. In group 1, cumulus cells were stained and counted following a 4 h maturation interval.

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Cumulus cells in groups 2, 3, and 4 were stained following 8, 12, and 16 h of maturation, respectively. For staining, cumulus cells from individual oocytes were placed into Triton X for 45 seconds and then placed into a 20 µl drop of propidium iodide for a minimum of 5 min. Cells for individual oocytes were counted, and cumulus cell numbers from the same group were averaged.

Experiment 2: Assessment of glucose metabolism at 8-12 h maturation for COCs that fertilized versus COCs that matured and failed to fertilize

Cumulus oocyte complexes (n=59) were matured in 5 µl drops of medium until 8 h. At 8 h, oocytes were transferred into 0.5 µl drops for 4 h. Following the 4 h incubation, oocytes were transferred back into the original 5 µl drops of maturation medium until 17 h. Oocytes were then fertilized, and presumptive zygotes and embryos were cultured for 96 h. Medium drops from the 4 h incubation were frozen and stored for glucose determination.

Experiment 3: Assessment of lactate production at 8-16 h maturation for COCs that fertilized versus COCs that matured and failed to fertilize

Cumulus oocyte complexes were matured in 5 µl drops of medium until 8 or 12 h. At 8 h (n=30) or 12 h (n=44), oocytes were transferred into 0.5 µl drops for 4 h. Following the 4 h incubation, oocytes were transferred back into the original 5 µl drops of maturation medium until 17 h. Oocytes were then fertilized, and presumptive zygotes and embryos were cultured for 96 h. Medium drops from the 4 h incubation were frozen and stored for lactate analysis.

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Statistical Analysis

Preplanned comparisons of maturation and fertilization rates between specific treatment groups were compared using Fisher's exact test. Metabolic profiles were analyzed by one way analysis of variance or by Kruskal-Wallis test, in cases where variances were heterogeneous. Cumulus cell numbers were analyzed by one way analysis of variance. Comparisons between metabolic profiles of fertilized and unfertilized oocytes (experiments 2 and 3) were done by Student's t-test.

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RESULTS

Experiment 1

Oocytes in treatment 5 had lower maturation rates than oocytes in group 1 (P<0.01) and lower fertilization rates than oocytes in groups 1, 9, and control denuded oocytes (P<0.01; Table 1). Oocytes in group 1 did not differ from the COC control group in maturation; however, fertilization rates were lower in group 1 than its respective control (P<0.05). Group 9 had a higher maturation rate than the respective control (P<0.05); however, the fertilization rates were similar. Oocytes denuded after 4 h of maturation and matured with cumulus (group 8), had higher maturation rates than oocytes denuded at the same time point, but matured alone (group 4; P<0.01). Fertilization rates between groups 4 and 8 did not differ. Oocytes denuded at 8 h (groups 3 and 7) did not differ in maturation or fertilization rates. Blastocysts developed in all groups, except group 5. Of groups that formed blastocysts, rates of development were similar.

Glucose and lactate profiles of denuded oocytes matured in the presence of cumulus cells followed a similar, though suppressed, metabolic patterns compared to cumulus oocyte complexes. The two groups of oocytes with cumulus cells had higher glucose uptake throughout maturation, compared to denuded oocytes without cumulus (Fig. 2a). COCs and denuded oocytes with cumulus had similar glucose uptakes until 8 h of maturation, at which point COCs had higher glucose uptake for the duration of maturation (P<0.01). A similar pattern was seen in lactate production (Fig. 2b). COCs and denuded oocytes with cumulus had higher lactate production than the denuded group. For the first 8 h of maturation, COCs and denuded oocytes with cumulus had similar lactate production.

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However, after 8 h, COCs had higher lactate production (P< 0.01). Pyruvate uptake was similar for all three groups over the course of maturation (Fig 2c). Numbers of cumulus cells replaced back into the maturation medium decreased with each medium change (P<0.01) except the final change at 12 h (0-4 h, 1200±57; 4-8 h, 745±59; 8-12 h, 487±57; 12-16 h, 273±44).

Glucose uptake of COCs increased throughout maturation (27 pmol/COC/h at 0-4 h versus 49 pmol/COC/h at 12-16 h; P<0.01; Fig. 3). Glucose consumption between 0-4 h and 4-8 h was not different (27 versus 30 pmol/COC/h); however, by 8-12 h of maturation, glucose uptake increased (39 pmol/COC/h; P<0.05). Lactate production of COCs also increased over the maturation interval (38 pmol/COC/h at 0-4 h versus 62 pmol/COC/h at 12-16 h; P<0.01; Figure 3). Like glucose, lactate production began to increase between 8-12 h (44 pmol/COC/h at 4-8 h and 60 pmol/COC/h at 8-12 h; P<0.01). For the cumulus oocyte complexes, pyruvate consumption did not change throughout maturation (13 pmol/COC/h at 0-4 h versus 16 pmol/COC/h at 12-16 h).

The metabolic profiles of oocytes that fertilized and oocytes that matured but failed to fertilize differed over the course of maturation. Fertilized oocytes had significantly higher glucose uptake from 8-16 h versus the uptake at 0-8 h (P<0.05). For oocytes that failed to fertilize, glucose uptake was similar throughout maturation (Figure 4). For lactate, oocytes that failed to fertilize also had no significant changes throughout maturation; however, oocytes that fertilized had higher lactate production from 8-16 h vs. 0-4 h of maturation (P<0.01; Figure 5). Glucose uptake by COCs that subsequently

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fertilized was higher between hours 8-12, than by oocytes that did not fertilize (38 versus 29 pmol/COC/h; P<0.01). Although lactate profiles had a tendency to differ between 12-16h; pyruvate profiles were not different between oocytes that subsequently fertilized or did not.

Experiment 2

Experiments 2 and 3 were carried out to confirm results of experiment 1, but only one 4 h metabolic incubation was performed during the maturation process. COCs undergoing metabolic analysis had 82% (89/109) maturation and a 69% (61/89) fertilization rate compared to 70% (21/30) and 100% maturation and fertilization rates of control oocytes. Eight blastocysts developed in the treatment group (13%) versus 11 in the control (37%). At 8-12 h of maturation, fertilized oocytes consumed an average of 37 pmol/COC/h, and unfertilized oocytes, 29 pmol/COC/h (P<0.01; Table 2), confirming the finding of experiment 1.

Experiment 3

COCs undergoing metabolic analysis had an 86% (49/57) maturation rate, identical to controls. Fertilization and blastocyst rates were not different between treatment and control (68% and 24% vs. 47% and 27%, respectively). Lactate production during 8-16h was significantly higher for oocytes that fertilized compared to those that did not fertilize. At 8-12 h maturation, oocytes that fertilized produced on average 66 pmol/COC/h vs. 46

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pmol/COC/h by oocytes that failed to fertilize, and at 12-16 h oocytes that fertilized produced 56 pmol/COC/h vs. 40 pmol/COC/h by oocytes that did not fertilize ($P<0.05$; Table 2).

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DISCUSSION

In the present study, there were several differences in maturation and fertilization rates among treatments. When oocytes were denuded upon removal from the follicle, maturation and fertilization were enhanced in the presence of cumulus cells and were not different from intact COCs. The results confirm previous work where the frequency of fertilization was lower when mouse oocytes (Schroeder and Epigg, 1984) and bovine oocytes (Zhang et al., 1995; Fatehi et al., 2002) were matured in the absence of cumulus cells. Denuding oocytes later in the maturation process and maturing without cumulus cells may have deleterious effects on maturation rates; however, developmental potential was not impacted. Oocytes in all groups were fertilized without cumulus, indicating that cumulus cells are not required for fertilization.

Blastocyst development was lower in this study compared to other studies for several reasons. First, oocytes and embryos were matured and cultured individually as opposed to groups. Oocytes and embryos in groups may benefit from the sharing of paracrine factors among one another, potentially enhancing developmental competence. Second, the high level of oocyte manipulation throughout the course of in vitro maturation could have resulted in reduced developmental rates. Finally, the literature has reported a wide range of blastocyst development from in vitro matured mouse oocytes, with higher percentages of blastocysts being produced from protocols including in vivo gonadotropin priming and/or inclusion of FSH in the maturation medium (Eppig et al., 1992; Merriman et al., 1998; Schroeder et al., 1988).

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Throughout maturation change-overs took place every 4 h. Numbers of cumulus cells in group 9 decreased over time. Previous estimates of the number of cumulus cells surrounding an individual mouse oocyte averaged 2,060 cells (Leese and Barton, 1985). The average number of cells initially placed with each oocyte was 1200. During the maturation process some cells plated to the dish, preventing transfer to the subsequent medium drop. Loss in cumulus cells affected metabolic analysis, perhaps leading to the differences between the COC and the denuded group matured with cumulus during the last 8 h of maturation. Additionally, cumulus cells decrease glucose oxidation once removed from the oocyte (Zuelke and Brackett, 1992). Both of these factors would influence the metabolic profile of this group of oocytes and may account for the differences between the COC group.

This study shows that the metabolic profile of developmentally competent oocytes changes throughout maturation. Metabolic activity of COCs increased toward the end of maturation. It is important to acknowledge that the maturation conditions and medium, as well as the source of the oocytes (primed versus unprimed mice) will affect metabolism of the oocyte. Under the present conditions of maturation in a defined system, the beginning hours of maturation are relatively quiescent, and towards 11 h of maturation, the COC increases in metabolic activity. The increased uptake and production of nutrients by the COC during this time may reflect the need to support various processes associated with completion of both nuclear and cytoplasmic maturation. As stated above, a change in the maturation conditions may result in changes in the metabolic profile. For example, FSH primed mice or oocytes matured in the

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presence of FSH may show an increase in metabolism at an earlier time point in the maturation process, as oocytes matured in the presence of FSH reach maturation sooner than non FSH treated oocytes (Roberts et al., 2004). In future studies, the use of this defined maturation system will allow for changes in medium composition, such as the addition of FSH, to be directly correlated to changes in oocyte metabolism and developmental potential.

In the present study, metabolic activity of oocytes during later stages of maturation correlated with developmental competence of in vitro matured COCs. Few groups have examined the relationship between metabolism, oocyte maturation, and developmental competence. In the mouse, COCs progressing through meiosis and denuded oocytes have a greater requirement for pyruvate than prophase I or MII arrested oocytes (Downs et al., 2002). Higher rates of glycolysis by bovine oocytes denuded at the end of a 24 h maturation period were associated with increased developmental potential (Krisher and Bavister, 1999). Blastocyst development in cats was also associated with higher rates of glycolysis by denuded oocytes; however, no correlation was determined between glycolytic activity or glucose oxidation by cumulus cells and subsequent oocyte development (Spindler et al., 2000). In the present study, COCs that were able to fertilize consumed more glucose and produced more lactate than the oocytes that failed to fertilize. This increase in metabolism may indicate healthier oocytes.

Lactate production increased in fertilized COCs throughout the maturation process, indicating an increase in glycolysis in the later half of maturation. During the first 12 h of

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maturation of COCs, approximately thee quarters of glucose is being converted to lactate, whereas during the final 4 h of maturation, less than two thirds of the glucose is being converted to lactate. However, actual glycolytic rates can not be calculated from this study, as pyruvate was included in the maturation medium. Pyruvate in the medium could have been converted to lactate, thus artificially increasing the lactate production. Synthesis of extracellular matrix by cumulus cells in the latter stages of maturation leads to mucification and expansion of cumulus cells. It has been shown that the addition of glucosamine to medium reduces the total glucose uptake by bovine COCs during the final stages of maturation (Sutton-McDowall et al., 2004). When glucosamine is not included in the medium, as in the present study, a portion of glucose must be utilized in this pathway to produce the extracellular matrix component hyaluronic acid. The same phenomenon has been demonstrated in the mouse under FSH induction of meiosis (Salustri et al., 1989); however, in the present study no FSH was added to the medium, although EGF was included, which has also been shown to act on cumulus cells to trigger GVBD (Downs et al., 1988; De La Fuente et al., 1999). Another route of glucose consumption that has been shown to play a large role in oocyte maturation is the pentose phosphate pathway (PPP) (Downs and Utecht, 1999; Downs et al., 1998). Towards the end of maturation, the increase flux of glucose though the PPP allows increased production of substrates involved in nuclear maturation (Sutton et al., 2003). COCs that fertilized consumed a larger amount of glucose in the later half of maturation than those that did not fertilize, thus allowing these COCs to generate increased energy not only though glycolysis, but also through alternative biosynthetic pathways that yield products necessary for complete nuclear and cytoplasmic maturation.

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In conclusion, developmental and metabolic data generated from this study have implications for both research and clinical settings. First, when COCs are denuded for procedures such as ICSI and are found to be immature, they are sometimes placed back into maturation until they reach metaphase II. The data from this study suggests that developmental competence will be enhanced when cumulus cells are placed in the medium with GV or MI denuded oocytes. Second, metabolic data differed between COCs that subsequently fertilized versus those COCs that matured but failed to fertilize. Not only did the glucose and lactate profiles of developmentally competent COCs change throughout maturation, but glucose uptake and lactate production were higher for oocytes that subsequently fertilized compared to those that failed to fertilize. Therefore, glucose and lactate profiles may provide a non-invasive measurement of oocyte viability. At this point, the overlap between COCs that fertilize and those that fail to fertilize does not allow this assessment to prospectively predict which oocytes will fertilize. However, the use of metabolic assessments would allow for COCs with higher glucose uptake and lactate production to be subsequently grouped together, allowing the healthier oocytes to benefit from shared paracrine factors. The use of metabolic markers, in conjunction with other established markers of oocyte viability, should facilitate improvements in the in vitro maturation system, allowing evaluations of IVM systems to expand from solely morphological criteria to non-invasive quantitative assessment.

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Acknowledgements

The authors would like to thank Dr. Mark Larman for providing valuable advice and comments on the manuscript and the Colorado Center for Reproductive Medicine for support of this study.

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Table 1. Maturation, fertilization, and development rates per oocyte

Treatment	total # oocytes	# MII	% MII	# Fertilized	%Fert/Oocyte	# Blast	%Blast/Oocyte
1	50	48	96[a]	28	56[d,g]	6	12[h]
2	50	38	76	32	64	12	24
3	50	47	94	41	82	12	24
4	50	31	62[b]	30	60	7	14
5	53	39	74[a]	12	23[d,e,f]	0	0[h,i]
6	50	48	96	38	76	18	36
7	50	46	92	34	68	12	24
8	50	47	94[b]	30	60	7	14
9	50	42	84[c]	32	64[e]	7	14[i]
control COC	51	42	82	40	78[g]	12	24
control DO	50	33	66	24	48[f]	2	4
control DO+	50	31	62[c]	26	52	2	4

Like pairs within a column are significantly different; [c,g] $P<0.05$; [a,b,d,e,f,h,i] $P<0.01$.
Denuded oocytes (DO), Denuded oocytes matured with cumulus cells (DO+).

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Table 2. Glucose uptake and lactate production by COCs that fertilized vs COCs that failed to fertilize at 8-12 h and 12-16 h

	8-12h, Fertilized	8-12h, Unfertilized	12-16h, Fertilized	12-16h, Unfertilized
Glucose Uptake pmol/COC/h±SEM	37±1.5 [a]	29±2.1 [a]	48±2.9	44±2.9
Lactate Production pmol/COC/h±SEM	66±6.9 [b]	46±6.3 [b]	56±5.3 [c]	40±3.3 [c]

Like pairs within rows are significantly different; [c] $P<0.05$; [a,b] $P<0.01$

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FIGURE LEGENDS

Figure 1. Experimental design for oocytes throughout maturation. COCs (black square), denuded oocytes (white square), and denuded oocytes matured in the presence of cumulus cells (hatched square).

Figure 2a-c. Carbohydrate profiles (±SEM) throughout maturation of COCs (■), denuded oocytes (●), and denuded oocytes matured in the presence of cumulus (▲). Different letters represent differences between groups at individual time points. a) For glucose uptake, denuded oocytes differed from COCs ($P<0.001$) at all time points of maturation. Denuded oocytes matured in the presence of cumulus cells differed from COCs at 8-12 h and 12-16 h maturation ($P<0.001$). b) Denuded oocytes produced significantly less lactate throughout maturation ($P<0.001$) than COCs. Denuded oocytes matured in the presence of cumulus cells produced less lactate at 8-12 h and 12-16 h maturation than COCs ($P<0.05$). c) Pyruvate profiles did not differ between groups at any time point.

Figure 3. Carbohydrate metabolism of COCs throughout maturation. Glucose uptake (■) ($P<0.05$) and lactate production (▲) were higher at 8-12 h ($P<0.01$) and 12-16 h ($P<0.01$) than 0-8 h. Pyruvate concentration (●) in medium remained similar through maturation. Significant differences in relation to individual carbohydrate (CHO) metabolism over time are indicated by * ($P<0.05$) and ** ($P<0.01$).

Figure 4. Glucose uptake (±SEM) by COCs that fertilized (▲) versus COCs that failed to fertilize (●). Glucose uptake increased over the maturation period for COCs that fertilized (0-8 h v 8-12 h, *$P<0.05$; 0-8 h v 12-16 h, **$P<0.01$). COCs that failed to fertilize had similar glucose uptake throughout maturation.

Figure 5. Lactate production (±SEM) by COCs that fertilized (▲) versus COCs that failed to fertilize (●). COCs that fertilized had increased lactate production over the final half of maturation (0-4 h v 8-16 h, **$P<0.01$). COCs that failed to fertilize did not have an increase in lactate production throughout maturation.

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Figure 1.

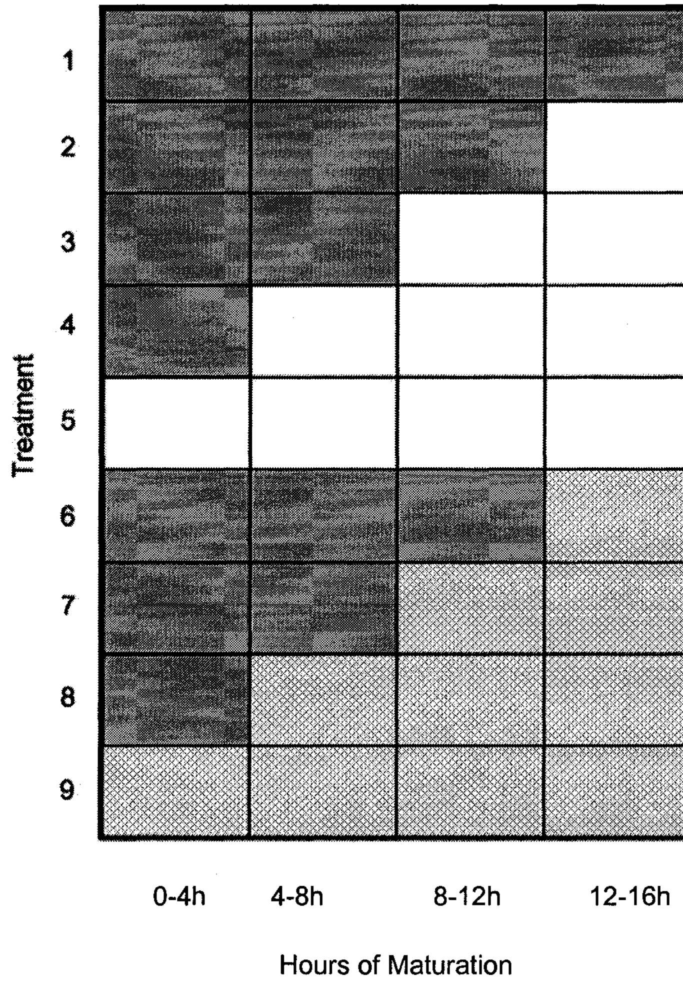

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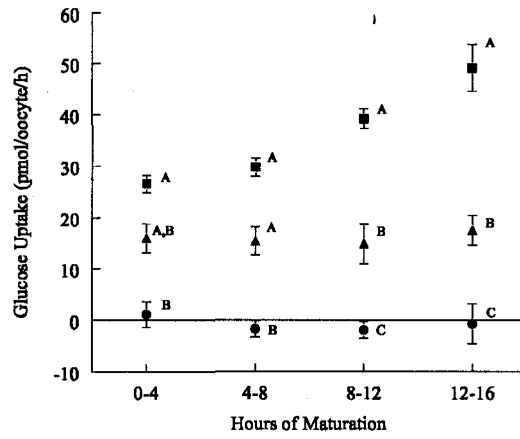
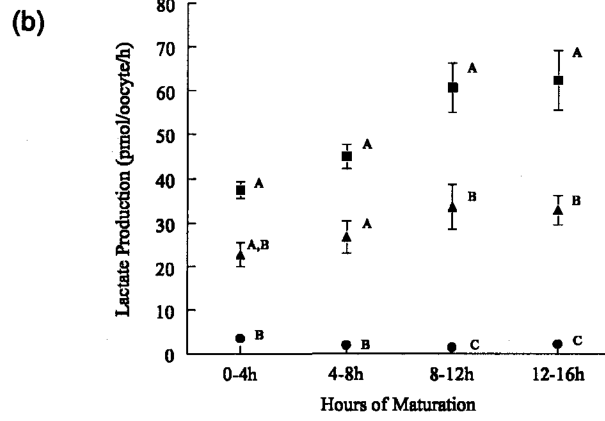
Figure 2 (a)



(b)



(c)

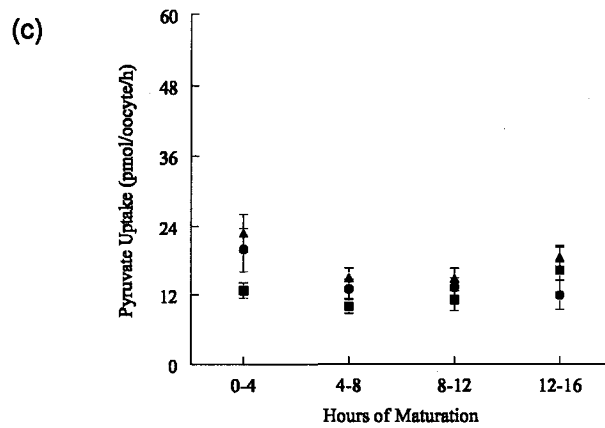


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Figure 3.

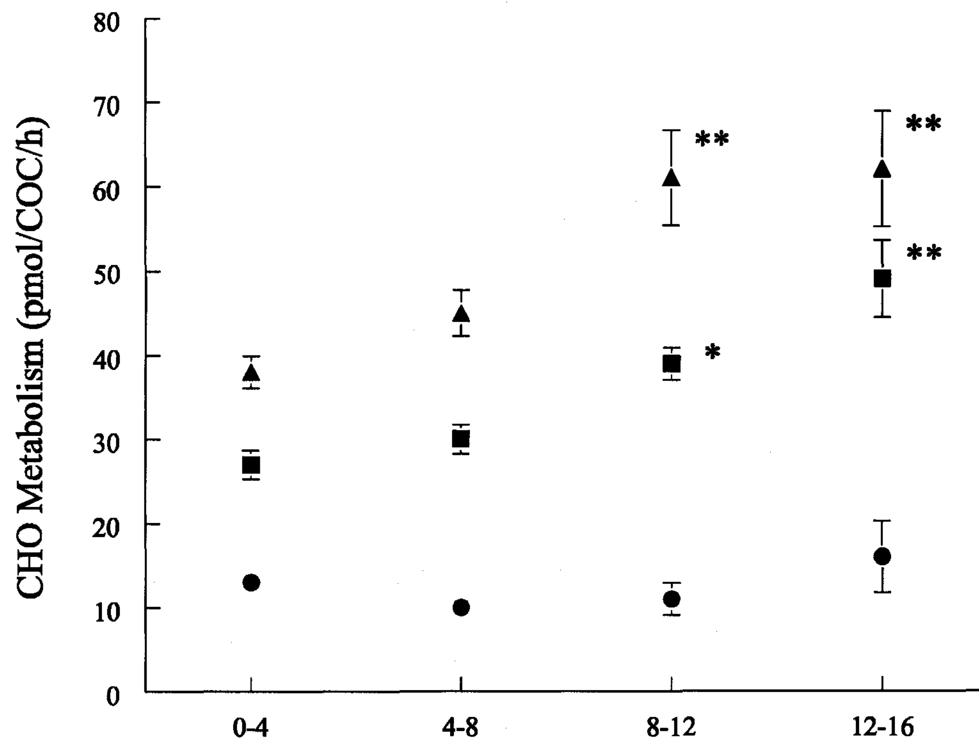

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Figure 4.

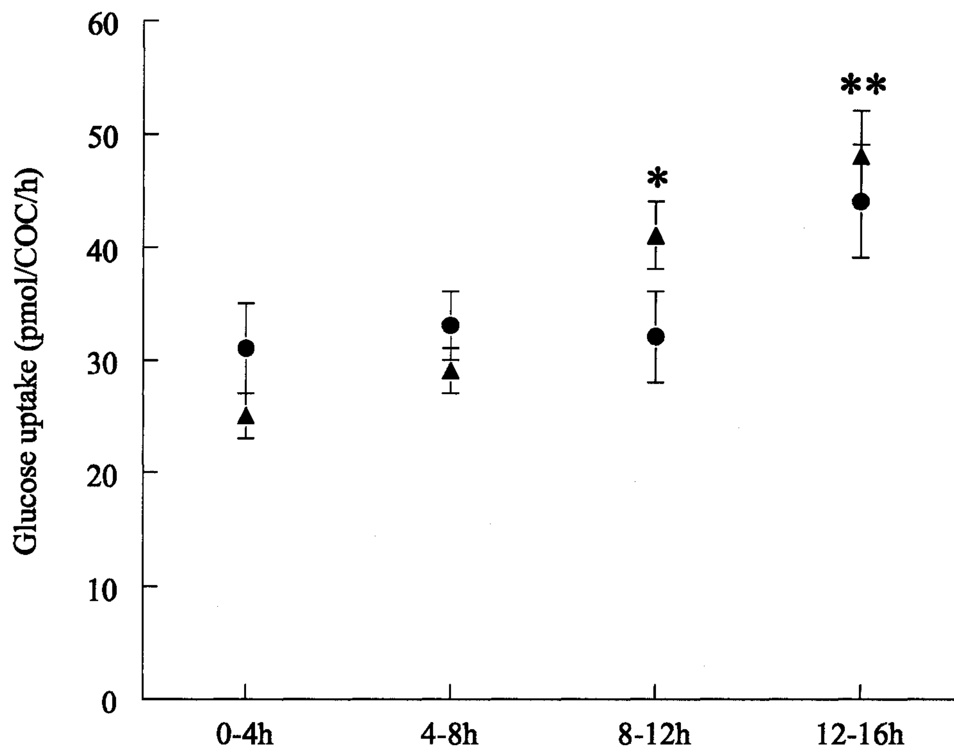

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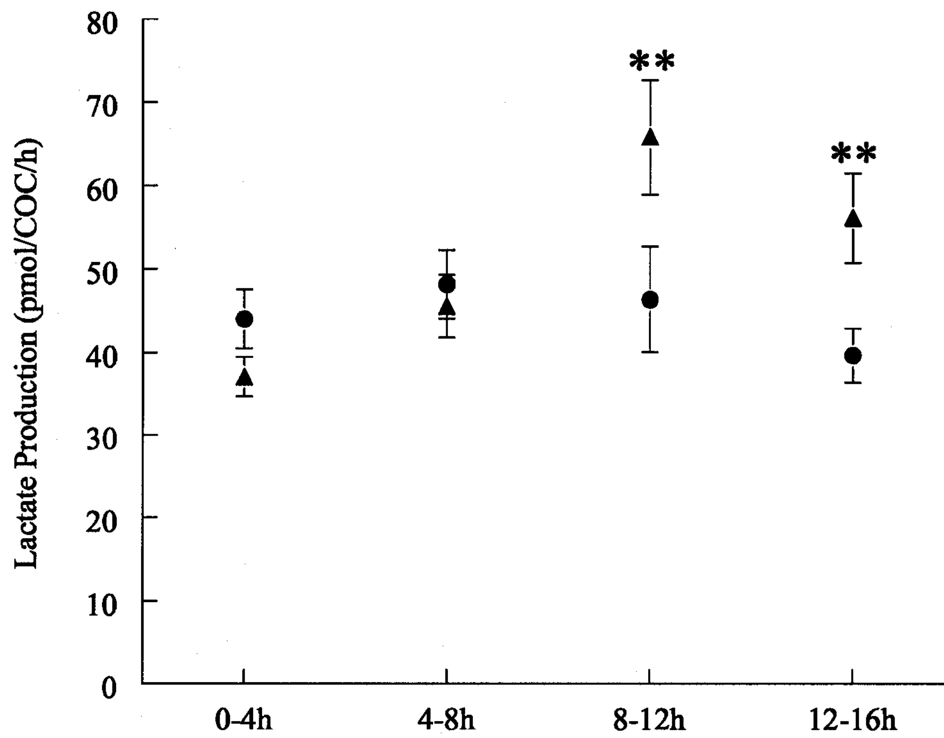
Figure 5.



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CHAPTER V

REDUCED OXYGEN CONCENTRATION IMPROVES THE DEVELOPMENTAL COMPETENCE OF MOUSE OOCYTES FOLLOWING IN VITRO MATURATION

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Accepted for publication: Molecular Reproduction and Development, September 4, 2006

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ABSTRACT

Reduced atmospheric oxygen concentration is beneficial to embryo development; however, optimal oxygen concentration for oocyte maturation remains undetermined. Likewise, there is no consensus of appropriate medium supplementation during maturation. The objective of this study was to determine whether oxygen tension (20% or 5% O_2) and EGF affect oocyte metabolism and subsequent embryo development. Cumulus-oocyte complexes (COCs) were collected from 28-day-old eCG primed or unprimed F1 (C57BL/6 x CBA) mice. COCs were matured in defined medium in one of four groups: 20% O_2, 20% O_2 + EGF, 5% O_2, 5% O_2 + EGF. In vivo matured COCs were also collected for analysis. COCs from unprimed mice, matured in 5% O_2 ± EGF or 20% O_2 + EGF had higher metabolic rates than COCs matured in 20% O_2 ($P<0.05$). COCs from primed mice had higher metabolic rates when matured in the presence of EGF, regardless of oxygen tension ($P<0.01$). Oxygen uptake and mitochondrial membrane potential were higher for in vivo matured oocytes and oocyte matured under 5% O_2 compared to oocyte matured under 20% O_2 ($P<0.05$). Blastocyst formation was not different between maturation groups (primed or unprimed); however, embryo cell numbers were 20-45% higher ($P<0.05$) when COCs were matured at 5% O_2. Results suggest that oocytes matured in physiological concentrations of oxygen have improved development and metabolic activity, more closely resembling in vivo maturation. These finding have implications for oocyte maturation in both clinical and research laboratories.

Short title: Reduced oxygen tension improves IVM.

Key Words: IVM, metabolism, glucose uptake, EGF, mitochondria

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INTRODUCTION

Developmental competence of in vitro matured oocytes has yet to be optimized, as evidenced by low percentages of in vitro matured oocytes developing to the blastocyst stage (30-40% in unprimed bovine, Lechniak, 2002; 40-50% in unprimed mouse, De La Fuente et al., 1999). High proportions of immature oocytes from human patients who have not received exogenous follicle stimulating hormone (FSH) or human chorionic gonadotropin (hCG) are able to mature, fertilize and undergo early cleavage in vitro (Mikkelsen et al., 2001; Chain, 2004); however, only a small percentage of such IVM oocytes (5-8%) develop into viable pregnancies after transfer (Barnes et al., 1996; Mikkelsen et al., 1999). In animal models, oocytes matured in vivo have higher embryo development rates than oocytes matured in vitro (Van de Leemput et al., 1999; Blondin et al., 2002). Similarly, clinical studies using in vivo matured oocytes reported higher rates of implantation (Marek et al., 1999; Gardner et al., 2000) compared to studies using in vitro matured oocytes (Cha et al., 2000; Mikkelsen and Lindenberg, 2001; Soderstrom-Anttila et al., 2005). Reduced developmental competence of oocytes matured in vitro indicates that current oocyte maturation systems do not adequately support nuclear and/or cytoplasmic maturation, which can subsequently affect embryo physiology and viability.

Several factors within the maturation system affect oocyte competence. Interestingly, although a reduced atmospheric oxygen concentration has been shown to have beneficial effects on pre-implantation embryos (Quinn and Harlow, 1978; Batt et al., 1991; Noda et al., 1994; Gardner and Lane, 1996), the optimal oxygen concentration for oocyte maturation has not been thoroughly examined. Maturation of denuded hamster oocytes

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was higher at 5% O_2 than either 0 or 10% O_2 (Gwatkin and Haidri, 1974). In the mouse, oocytes collected from antral follicles showed improved blastocyst formation rates when in vitro maturation (IVM) and in vitro culture (IVC) were conducted under 5% O_2 (Adam et al., 2004). In a separate report, however, fertilization and blastocyst development by COCs recovered from antral follicles were unaffected by maturation under various O_2 atmospheres (5, 10, 15, 20%; Eppig and Wigglesworth 1995). Currently most reports regarding maturation of human oocytes, either for research or clinical procedures, utilize 20% oxygen tension (Wynn et al., 1998; Cha et al., 2000; Soderstrom-Anttila et al., 2005), which is similar to the oxygen tension typically used for human embryo culture (Alves et al., 1998; Rijnders and Jansen, 1998).

Hormonal treatments prior to COC retrieval may also impact developmental potential. Oocytes recovered from mice stimulated with FSH in the form of equine chorionic gonadotropin (eCG) have higher maturation, 2-cell, and blastocyst rates than oocytes from mice that were not primed with eCG (De la Fuente et al., 1999). In humans, exposure to FSH prior to retrieval of immature oocytes increases pregnancy and implantation rates in patients diagnosed with polycystic ovarian syndrome (PCOS; Mikkelsen and Lindenberg, 2001). However, the ability to stimulate women with exogenous gonadotropins has associated risks and may even be contraindicated in certain conditions such as oncology patients with certain steroid dependent tumors. In addition, exogenous stimulation with FSH alters maternal uterine environment in the mouse, leading to reduced implantation and fetal development (Etziod and Storeng, 2001).

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Inclusion of hormones in the medium may also influence oocyte maturation or subsequent development. Follicle stimulating hormone is routinely added to maturation medium for human (Soderstrom-Anttilla et al., 2005; Wynn et al., 1998) and animal oocytes (Eppig, 1979; Ali and Sirard, 2002). In animal models, FSH improved oocyte maturation and subsequent developmental potential (Izadyar et al., 1998); however, human recombinant FSH has now been shown to induce aneuploidy in in vitro matured mouse oocytes (Roberts et al., 2005). An alternative to the use of gonadotropins in the maturation medium is epidermal growth factor (EGF). The physiological relevance of EGF in the maturation process is evidenced by reports of EGF receptors on granulosa cells (Almahbabi et al., 1998; Conti et al., 2006), as well as the presence of EGF in follicular fluid (Franks et al., 1987; Das et al., 1992). EGF-related growth factors are mediators of the LH signal in the follicle (Park et al., 2004). EGF has been shown to promote cumulus expansion and maturation in the mouse (Downs, 1989; De la Fuente et al., 1999); rat (Dekel and Sherizly, 1985); bovine (Lorenzo et al., 1994; Lonergan et al., 1996); sheep (Grazul-Bilska et al., 2003); and human (Das et al., 1991; Gomez et al., 1993; Li et al., 2004). Developmental competence of oocytes from pigs, sheep and cows is also improved when cumulus-enclosed oocytes were matured in the presence of EGF (Harper and Brackett, 1993; Lonergan et al., 1996; Abeydeera et al., 2000; Guler et al., 2000).

In addition to hormones and growth factors, media used for maturation of mammalian oocytes are frequently supplemented with fetal bovine serum (Suikkari et al., 2000; Cha et al., 2000) or 10-20% patient serum (Mikkelsen et al., 1999; Soderstrom-Anttila et al.,

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2005). In animal models, addition of serum to embryo culture medium has been correlated with pathological development, such as damage to organelle ultrastructure, impaired cell function, perturbed metabolic activity, as well as altered gene expression (Thompson et al., 1995; Young et al., 1998; McEvoy et al., 2000; Khosla et al., 2001). In addition to the deleterious effects on the developing embryo, supplementation of medium with serum limits the ability to study the oocyte's requirements during maturation, as serum contains a large number of components, many of which are poorly characterized or unknown. The development of a completely defined maturation system is critically important to determine the effectiveness of specific components in the maturation medium.

The quality of an IVM system is typically assessed by the morphology of the cumulus oocyte complex and nuclear maturation, as well as subsequent fertilization and embryo development. However, oocytes and embryos can have similar morphologies, and yet differ in developmental potential. Therefore, it is important to measure the adequacy of an IVM system using quantifiable parameters. The use of metabolic profiles as an indicator of embryo quality has been established (Gardner and Leese, 1987; Lane and Gardner, 1996), and more recently, metabolic profiles have been correlated with developmental potential of individual oocytes (Preis et al., 2005). Metabolic profiling could therefore be of value in quantifying the efficacy of IVM systems.

In addition to metabolism, an alternative response that may be correlated with oocyte viability is oxygen consumption. Similar to metabolism, measurement of oxygen uptake

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is non-invasive and requires only a short period of incubation, therefore having minimal effects on subsequent development (Thompson et al., 1996). Several groups have measured the oxygen consumption of the preimplantation embryo (Houghton et al., 1996; Sturmey and Leese 2003; Sutton et al., 2003). This technique has been employed to a lesser extent with oocytes, with no information regarding oxygen uptake by oocytes matured under different oxygen tensions.

An additional marker of oocyte viability may be mitochondrial membrane potential. Both nuclear and cytoplasmic maturation are dependent on the organization and metabolic activity of mitochondria (Van Blerkom and Runner, 1984; Hyttel et al., 1986). Oocyte mitochondrial activity subsequently affects development after fertilization (Van Blerkom et al., 1995). Changes in mitochondrial redistribution in oocytes after fertilization were correlated with abnormal embryo development (Barnett et al., 1996). Following evaluation of mitochondrial membrane potential in human embryos, abnormalities were associated with lower membrane potential when compared with normal embryos (Wilding et al., 2003). As membrane potential is indicative of the health of the mitochondria, it therefore could serve as a potential marker of the effectiveness of an IVM system.

The objective of this study was to determine the effects of oxygen tension (20% or 5% O_2) on the metabolism, oxygen uptake, and mitochondrial membrane potential of the individual mouse COC and denuded oocyte. Additionally, the effect of EGF on metabolic activity and subsequent embryo development was determined, as oocytes were matured

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in medium devoid of gonadotropins and serum. In vivo matured COCs served as the control for all experiments.

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MATERIALS AND METHODS

General Experimental Methods

Collection of immature COCs for metabolic analysis

Immature COCs were collected from 28 day old F1 (C57BL/6xCBA) mice and washed a minimum of three times in G-MOPS (Lane and Gardner, 2004), supplemented with 5% fetal calf serum (FCS) to prevent hardening of the zona pellucida. COCs were then washed and incubated in a defined maturation medium (modified G2 (0 mM lactate, 0.5 mM glucose, 0.23 mM pyruvate), supplemented with 2.5 mg/ml human recombinant serum albumin, 0.5 mM citrate, 0.5 mM cysteamine, ITS (insulin 0.5 μg/ml, transferrin 0.275 μg/ml, and selenium 0.25 ng/ ml), and 2 mg/ml fetuin; Preis et al., 2005) under paraffin oil (Ovoil, Vitrolife, Kungsbacka, Sweden). COCs were matured in one of four groups: 20% oxygen, 20% oxygen + 100 ng/ml EGF, 5% oxygen, and 5% oxygen + 100 ng/ml EGF, all including 6% CO_2. Unless otherwise noted, all chemicals and hormones were purchased from Sigma (St. Louis, MO, USA) and prescreened for embryo toxicity using a sequential mouse pronucleate embryo assay (Gardner et al., 2005).

Metabolism was assessed over the entire course of maturation (0-16 h) and over the last 4 h of maturation (12-16 h). During the final hours of maturation, COCs have the highest metabolic activity, and metabolism during this time period has been correlated with subsequent developmental potential (Preis et al., 2005). COCs in the 0-16 h group were individually matured in a 2 μl drop of defined medium for 16 h. COCs in the 12-16 h group were individually matured for 0-12 h in 5 μl drops and then individually placed into a 0.5 μl drop of medium for the remaining 4 h. Upon removal of COCs, medium

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was stored at -80°C for later analysis of glucose uptake and lactate production. At 17 h of maturation, COCs were individually fertilized (Lane and Gardner, 2004) for 8 h, after which presumptive zygotes were placed into individual 5 μl drops of medium G1 supplemented with 5 mg/ml HSA for 48 h, followed by culture in medium G2 supplemented with 5 mg/ml HSA for an additional 48 h. Fertilization and all subsequent embryo cultures were performed at 37°C in 6% CO_2, 5% O_2, and 89% N_2. Blastocyst development was recorded at 5 days post fertilization.

Metabolic analysis of in vivo matured COCs

Twenty eight day old F1 (C57BL/6xCBA) females were administered 5 IU eCG, followed 48 h later by 5 IU hCG. In vivo matured COCs were collected from the follicle 10 h post hCG administration. Individual COCs were placed into 0.5 μl drops of medium for 1.5 h in one of 4 groups: 20% oxygen, 20% oxygen + 100 ng/ml EGF, 5% oxygen, and 5% oxygen + 100 ng/ml EGF, all with 6% CO_2.

Metabolic Measurements

Ultramicrofluorimetry was used to quantify the level of carbohydrate consumption and production from the maturation medium. Fluorometric assays are based on the generation or consumption of the reduced pyridine nucleotides NADH or NADPH, which fluoresce when excited with a wavelength of 340 nm (Leese and Barton, 1984; Gardner and Leese, 1990). Assays were performed as described by Preis et al. (2005).

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Evaluation of quality of blastocysts derived from in vitro matured COCs

To determine the effect of maturation conditions on blastocyst cell number, additional COCs were collected from unprimed and primed mice and placed into the same maturation groups as COCs undergoing metabolic analysis. COCs were matured (5 COCs/ 30 μl) and resultant embryos were cultured (10 embryos/ 20 μl) under the same conditions as oocytes undergoing metabolic analysis. Blastocysts were stained for total cell numbers on day 5 post fertilization.

Analysis of Blastocyst Cell Number

Expanded blastocysts or hatching blastocysts were placed into triton solution for 45 seconds and then into G-MOPS. Washed embryos were transferred into propidium iodide (PI) drops, where they remained for a minimum of ten minutes. Stained embryos were mounted in glycerol on a siliconized glass slide. The cell number was determined by counting the cell nuclei using a fluorescence microscope with an ultraviolet light. The mean number was compared between treatment groups.

Mouse Embryo Transfer

In vitro produced embryos were cultured for 96 h. Blastocysts were transferred to Day 4 pseudo pregnant F1 (C57BL/6xCBA) female mice (-1 day asynchronous). A state of pseudo pregnancy was achieved through the mating of female F1 (C57BL/6xCBA) mice with vasectomized males. Embryos from each treatment were allocated to separate uterine horns. Up to six blastocysts were transferred to each uterine horn. On Day 15 of

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pregnancy, females were sacrificed, and implantation sites and numbers of fetuses were determined. Weights of resultant fetuses were determined.

Experiment 1: Metabolism and developmental potential of in vitro matured COCs recovered from unprimed mice.

COCs (n=231 over four replicates) were recovered from unprimed mice. Metabolism was analyzed following 16 h maturation (n=131) and between 12-16 h maturation (n=100). For blastocyst cell number, 792 COCs were collected over four replicates.

Experiment 2: Metabolism and developmental potential of in vitro and in vivo matured COCs recovered from eCG primed mice.

COCs (n=227 over 4 replicates) were collected from mice 48 h post eCG (5 IU) injection. Metabolism was measured following 16 h maturation (n=118) and between 12-16h maturation (n=109). Two additional groups, matured without EGF, were added at the 12-16 h time period: 20% oxygen + 0.5 IU/ml hCG and 5% oxygen + 0.5 IU/ml hCG. In vivo matured oocytes (n=72 over 2 replicates) served as controls. For blastocyst cell number, 401 COCs were collected over 4 replicates.

Experiment 3: Metabolism of in vitro matured COCs recovered from eCG-primed mice following incubation with glucose transport inhibitors.

Two inhibitors of glucose transport were utilized in this experiment: phloretin and phloridzin. Phloretin binds to GLUT receptors, preventing glucose from entering the cell via facilitative diffusion (Robinson et al., 1990). Phloridzin blocks glucose uptake via

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sodium glucose co-transport (Schultz and Zalusky, 1964). As both phloretin and phloridzin were dissolved in 100% ethanol, a vehicle control was required to determine whether the addition of ethanol alone to the maturation medium affected the metabolism of the COC. COCs were matured in groups (5/ 30 μl) until 12 h at 37°C. 6% CO_2, 20% O_2± 100 ng/ml EGF. At 12 h, COCs were divided into one of 5 groups for metabolic incubation: no EGF, EGF only, EGF + 0.1 mM phloretin, EGF + 0.5 mM phloridzin, and EGF + ethanol vehicle control. For the inhibitor groups and vehicle control, COCs were pre-incubated in their respective treatment for 1 h prior to being placed in a 0.5 μl drop of medium for metabolic analysis. COCs for all groups were individually cultured in 0.5 μl drops of respective media for 3 h. Media were analyzed for glucose uptake.

Experiment 4: Oxygen consumption of in vitro matured COCs recovered from eCG-primed mice.

COCs were recovered 48 h post eCG injection and matured (5 COCs/ 30 μl) in defined maturation medium supplemented with EGF under 5% or 20% O_2 and 6% CO_2 for 17 h. Oxygen uptake was assessed at 17 h maturation for the two in vitro groups or immediately following recovery of the COCs in the vivo matured group. The technique used to measure oxygen uptake was thoroughly described in Houghton et al. (1996). Briefly, 1 μl of oil containing 1mg/ml pyrene was loaded into a glass PCR capillary, followed by 2 μl of G-MOPS (37°C at 20% O_2) containing 2 COCs or 10 denuded oocytes. The capillary was sealed on both ends. The level of pyrene fluorescence was measured every 40 minutes for a total of 160 minutes. Increased pyrene fluorescence

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correlated to increased oxygen uptake. Fluorescence was compared between COCs and denuded oocytes matured under different oxygen tensions and in vivo matured oocytes.

Experiment 5: Mitochondrial membrane potential of oocytes matured under 5 or 20% oxygen tension.

COCs were collected from eCG-primed mice and matured under 5 or 20% O_2 and 6% CO_2 in defined maturation medium supplemented with EGF. Cumulus cell removal occurred at 17 h of maturation for IVM groups or immediately following recovery of COCs from the follicle for the in vivo matured groups. Denuded oocytes were incubated in G maturation medium supplemented with 0.5 µm 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Molecular Probes, Invitrogen) for 30 minutes at either 5% or 20% O_2. Following JC-1 incubation, oocytes were washed in G-MOPS and JC-1 ratio fluorescence was measured on an Eclipse TE2000 (Nikon) with a 20x Plan Fluor objective. Fluorescent images were captured with a CCD camera: Cascade 512B (Roper Scientific) and analyzed using MetaFluor (Universal Imaging) software.

Experiment 6: Blastocyst transfer of embryos derived from oocytes matured under 5 or 20% oxygen tension.

COCs were recovered 48 h post eCG injection and matured (5 COCs/ 30 µl) in defined maturation medium supplemented with EGF under 5% or 20% O_2 and 6% CO_2 for 17 h. Fertilization and all subsequent embryo cultures were performed at 37°C in 6% CO_2, 5% O_2, and 89% N_2. On day 5 of culture, embryos were transferred into recipients (n= 82,

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20% O2; n= 78, 5% O2). Embryos were transferred into 14 recipient mice over seven replicates.

Statistical Analysis

Maturation, fertilization, blastocyst and fetal development were analyzed by Fisher's exact test. Metabolic profiles of COCs were analyzed by Kruskal-Wallis and Dunn's multiple comparisons tests. Blastocyst cell numbers, oxygen uptake and JC-1 ratios were analyzed by one way analysis of variance and Tukey-Kramer multiple comparisons tests.

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RESULTS

Experiment 1: Metabolism and developmental potential of in vitro matured COCs recovered from unprimed mice.

Maturation, fertilization and blastocyst development rates were not different among the four groups; however, total cell numbers were higher when COCs were matured under 5% O_2 ± EGF compared to 20% O_2 ± EGF ($P<0.05$; Table 1).

For metabolism over the entire course of maturation (0-16 h), glucose uptake and lactate production were higher by COCs in 5% O_2 + EGF ($P<0.05$) than the remaining three groups. There was no difference between 5% O_2 and 20% O_2 + EGF, but COCs matured in 20% O_2 had less glucose uptake and lactate production than COCs in the low O_2 groups ($P<0.05$; Table 2). Differences in metabolism were also seen when measured only at the end of maturation (12-16 h). The COCs in the 5% O_2 groups (regardless of EGF) had higher glucose uptake than both of the 20% O_2 groups ($P<0.05$). Differences also appeared between the 20% O_2 groups, in which the COCs matured in the presence of EGF had higher glucose uptake than those matured without EGF ($P<0.05$). Lactate production was similar between the COCs matured in the 5% O_2 groups and those matured in 20% O_2 with EGF; however, COCs matured under 20% O_2 without EGF had reduced lactate production compared to the other three groups ($P<0.05$; Table 2).

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Experiment 2: Metabolism and developmental potential of in vivo and in vitro matured COCs recovered from eCG-primed mice.

Maturation and fertilization rates were lower for COCs matured without EGF, regardless of oxygen tension. Blastocyst development was similar between groups; however, similar to oocytes from non-primed females, blastocysts derived from COCs matured under low oxygen tension had higher total cell numbers than blastocysts derived from COCs matured under high oxygen tension (Table 3).

Over the course of maturation (0-16 h), COCs matured in the presence of EGF, regardless of oxygen tension had higher glucose uptake and lactate production than those matured without EGF ($P<0.05$, Table 4). An identical metabolic pattern was observed during the final hours of maturation; COCs matured in the presence of EGF had higher metabolic activity than oocytes matured in the absence of EGF ($P<0.01$; Fig 1a and b). Of the two groups without EGF, there was a tendency for glucose uptake and lactate production to be higher for COCs matured under 5% O_2 versus 20% O_2; however, the difference was not significant ($P>0.05$). The addition of hCG to the maturation medium devoid of EGF had no effect on glucose uptake (17±2 and 35±7 pmol/COC/h, 20% and 5% O_2, respectively) compared with COCs matured without hCG, regardless of oxygen tension. Additionally, the inclusion of hCG in the medium had no effect on lactate production (14±4 and 36±7 pmol/COC/h, 20% and 5% O_2, respectively).

Glucose uptake and lactate production by in vivo matured COCs did not differ among any treatments ($P>0.05$). COCs matured in the presence of EGF had similar levels of

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lactate production as in vivo matured COCs, whereas the groups matured without EGF had lower lactate production than in vivo matured oocytes ($P<0.01$; Fig. 1b). Glucose uptake by the in vivo groups was higher than all in vitro groups ($P<0.01$), although COCs with EGF were more similar to in vivo matured COCs than COCs matured without EGF (Fig. 1a).

Experiment 3: Metabolism of in vitro matured COCs recovered from eCG-primed mice following incubation with glucose transport inhibitors.

Incubation of COCs in the presence of phloretin resulted in reduced glucose uptake compared to EGF alone (20.9± 2 vs. 46.3± 3 pmol/COC/h, respectively; Fig. 2; $P<0.01$). Likewise, phloridzin resulted in a significant decrease in glucose uptake compared to EGF alone (31.5 ± 1 vs. 46 ± 3 pmol/COC/h, respectively; $P<0.01$). COCs incubated with phloridzin had higher glucose uptake than those incubated with phloretin or the no EGF group ($P<0.01$); however, glucose uptake was similar between the phloretin group and the no EGF group (20.9±2 vs. 16.9 ± 2 pmol/COC/h, respectively). COCs matured without EGF had significantly lower glucose uptake than those matured with EGF ± vehicle control ($P<0.01$; Fig. 2). There was no difference in glucose uptake between COCS matured with EGF or EGF plus the vehicle control.

Experiment 4: Oxygen consumption of in vitro matured COCs recovered from eCG-primed mice.

There was no difference in oxygen uptake between in vitro matured COCs (1.7±0.2 versus 1.04±0.3 nl/COC/h for COCs matured under high and low O_2, respectively; Fig.

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3). However, COCs matured under 5% O_2 had lower oxygen uptake than in vivo matured COCs (1.04±0.3 vs. 2.24± 0.4 nl/COC/h; $p<0.01$). Interestingly, upon removing cumulus from COCs, in vivo matured oocytes and oocytes matured under 5% O_2 tension had higher oxygen uptake (0.159±0.02 and 0.156±0.03 nl/oocyte/h, respectively) compared to oocytes matured under 20% O_2 (0.066±0.018 nl/oocyte/h; $P<0.05$; Fig. 3).

Experiment 5: Mitochondrial membrane potential of oocytes matured under 5 or 20% oxygen tension.

The JC-1 ratio was higher in oocytes matured under 5% O_2 compared to 20% O_2 (1.114±0.005 vs. 1.089±0.003, respectively; $P<0.01$). In vivo matured oocytes (1.114±0.002) had similar JC-1 ratio to oocytes matured under 5% O_2; however, in vivo matured oocytes had significantly higher JC-1 ratio than oocytes matured under 20% O_2 ($P<0.01$).

Experiment 6: Blastocyst transfer of embryos derived from oocytes matured under 5 or 20% oxygen tension.

A total of 82 embryos were transferred from the 20% O_2 group, and 78 embryos were transferred from the 5% O_2 group. There was no difference in the number of implantation sites or fetuses between groups following embryo transfer. Thirty implantation sites (36% of transferred embryos) were observed in the 20% O_2 group, while 37 implantation sites (47% of transferred embryos) were observed in the 5% O_2 group. Seven fetuses (8.5% of transferred embryos) were recovered from the 20% O_2 group, while 12 fetuses (15% of transferred embryos) were recovered from the 5% O_2

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group. Fetal weights were similar between groups (0.2331±0.03g vs. 0.2067±0.02g; 20% and 5% O_2, respectively).

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DISCUSSION

In this study, the metabolism of individual COCs and denuded oocytes matured under different oxygen tensions was assessed via carbohydrate uptake and metabolite release, oxygen uptake and mitochondrial membrane potential. Maturation under 20% oxygen tension resulted in reduced metabolism by COCs recovered from unprimed females compared to maturation under 5% oxygen tension. Interestingly, EGF was able to increase the metabolism of COCs recovered from both primed and unprimed mice matured under 20% or 5% oxygen tension. Although subsequent developmental rates were not different between treatment groups, resulting cell numbers were higher for embryos derived from COCs matured under low oxygen tension, regardless of EGF supplementation. Although an oxygen tension of around 20% is typically used for the maturation of bovine, mouse and human COCs, the non-invasive metabolic analysis used in this study provides evidence that maturation of COCs under high oxygen tension alters oocyte physiology with subsequent and significant downstream effects on the embryo.

Maturation and fertilization rates of COCs collected from unprimed and eCG-primed mice were not affected by oxygen tension, as observed by Eppig and Wigglesworth (1995). However maturation and fertilization rates of COCs from eCG-primed mice were affected by the inclusion of EGF in the maturation medium. EGF may stimulate Ras-Raf-MEK pathways required for improved cytoplasmic maturation, resulting in increased fertilization rates (Fan and Sun, 2004; Huo et al., 2004). In addition LH stimulation results in the expression of EGF related growth factors. These growth factors (amphiregulin, epiregulin, and beta-cellulin) have been proposed to act as paracrine

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regulators of the LH signal within the follicle (Park et al., 2004). After fertilization, neither EGF nor oxygen tension during oocyte maturation affected the percentage of zygotes that developed to the blastocyst stage. Blastocyst formation, however, is not a sensitive indicator of developmental competence. In contrast, cell numbers can provide a more reliable indication of blastocyst quality. Total cell numbers of blastocysts derived from COCs recovered from unprimed and eCG-primed mice were higher for both low oxygen groups versus the high oxygen groups. Therefore, although developmental rates were similar between COCs matured under different oxygen tensions, the number of cells in resulting embryos and plausibly their viability were increased following maturation in 5% oxygen.

Metabolism of the COC was altered by both oxygen tension and EGF supplementation. The degree of metabolic variation depended on previous in vivo exposure to eCG. COCs collected from eCG-primed mice differed from those recovered from unprimed mice, as gonadotropic exposure in vivo promotes cytoplasmic maturation prior to oocyte collection. Differences in developmental rates are generally higher for COCs recovered from eCG-primed mice (De La Fuente et al., 1999). Consistent with differences in developmental potential, results of this study show that metabolic profiles also differed between COCs recovered from unprimed and eCG-primed females, with respect to both oxygen tension and EGF. This is not surprising as the maturation kinetics between these two groups of oocytes differ, with the COCs recovered from eCG-primed mice maturing more rapidly. Clinical studies that exposed PCOS patients to FSH prior to oocyte retrieval resulted in enhanced oocyte competence (Mikkelsen and Lindenberg, 2001).

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However, while some reports indicate there is no benefit of FSH priming in the normal cycling patient (Mikkelsen et al., 1999), other reports have shown an improvement in oocyte quality following FSH stimulation (Wynn et al., 1998). As there are differences in methodology between reports, this area requires further investigation. In the present study, oxygen tension and EGF had an affect in both the primed and unprimed mouse model; therefore, these two factors are likely to have an impact on the human oocyte, regardless of exposure to exogenous FSH.

For unprimed mice, metabolism (glucose uptake and lactate production) differed with respect to EGF supplementation and oxygen tension. Under low oxygen, COCs matured with EGF had higher glucose uptake and lactate production over the course of maturation than COCs matured without EGF. During the final hours of maturation however, metabolism between low O_2 groups was similar, indicating that EGF has a more significant impact earlier in the maturation process. For COCs matured under 20% O_2, inclusion of EGF enhanced metabolic rates. EGF was able to modify metabolism of COCs matured under 20% O_2 to levels equivalent to low O_2 without EGF. These differences were similar over the course of maturation, as well as during the final hours, suggesting that EGF is actively affecting metabolism throughout the course of maturation.

For COCs collected from eCG-primed mice, EGF clearly increased the metabolic response. Metabolism of COCs matured with EGF, regardless of oxygen tension, was significantly higher than COCs matured without EGF. It appears that EGF has a larger

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impact on the metabolism of COCs recovered from eCG-primed mice. As cytoplasmic maturation had already begun following eCG administration, it is plausible that EGF was able to expedite a cascade of events, leading to an increase in the number of enzymes or substrates required in metabolic pathways. The presence of EGF in the maturation medium increased the metabolic activity of the COC; therefore, resulting in metabolic profiles that more closely resembled those of in vivo matured COCs. However, all in vitro groups, regardless of EGF supplementation, had significantly lower glucose uptake than in vivo groups, suggesting that altered metabolism may reflect the decreased developmental potential of IVM oocytes relative to their in vivo counterparts. It has been reported that metabolic activity of cumulus cells is regulated by the oocyte (Sugiura et al., 2005). Therefore, as the majority of the glucose consumption was a reflection of cumulus cell metabolism, the reduced glucose uptake by cumulus cells of IVM COCs may reflect impaired signaling between the oocyte and surrounding cumulus cells or impaired oocyte quality.

As in vivo matured COCs were exposed to eCG and hCG, two additional in vitro groups were added to determine if hCG exposure during in vitro maturation increased metabolism. COCs recovered from eCG-primed mice subsequently matured in medium devoid of EGF had significantly diminished metabolism compared to COCs matured in the presence of EGF; therefore, hCG was added to the EGF devoid groups (5% and 20% O_2). However, addition of hCG had no significant effect on metabolism; therefore, the metabolic effects of EGF were examined more closely.

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Incubation with glucose uptake inhibitors suggested that EGF increased glucose metabolism by enhancing the activity of both GLUTs (facilitative) and SGLT (sodium dependent active transport). The addition of ethanol alone to the metabolic medium resulted in no metabolic shift in glucose uptake. Therefore, the altered metabolism of COCs incubated with phloretin or phloridzin can not be attributed to ethanol present in the medium. As the addition of phloretin to EGF-supplemented medium resulted in a similar glucose uptake to the no EGF group, the upregulation of GLUTs by EGF in the cumulus cells was evident. Sodium dependent glucose transport was also present in the cumulus and/ or oocyte, as the addition of phloridzin resulted in a significant decrease in glucose uptake compared to the COCs matured with EGF alone; however, COCs cultured with phloridzin still had significantly higher glucose uptake than the no EGF group, suggesting that glucose uptake is more highly regulated by facilitative transport. The significant decrease in glucose uptake by COCs incubated with either phloretin or phloridzin suggests that EGF enhanced both facilitative and active transport of glucose. Previous reports have shown the presence of GLUT 1 (Williams et al., 2001) and GLUT 4 (Williams et al., 2001; Roberts et al., 2004) on granulosa cells; however this is the first report implicating SGLT in the cumulus-oocyte complex. GLUT 4 is upregulated by the inclusion of FSH in the medium (Roberts et al., 2004). However, there are no reports regarding the effect of EGF on glucose transport with respect to cumulus cells.

As oxygen concentration increases, there is a subsequent increase in production of intracellular reactive oxygen species (ROS) in both mouse (Gotto et al., 1993) and bovine (Nagao et al., 1994) embryos. Addition of H_2O_2 to embryo culture medium devoid of

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EGF resulted in decreased inner cell mass and trophectoderm, as well as an increase in the number of apoptotic cells, when compared to embryos cultured in the same medium supplemented with EGF (Kurzawa et al., 2004). Addition of EGF to maturation medium in the present study altered the metabolism of COCs matured under high oxygen tension; however, its effects as an antioxidant were not studied. However, other antioxidants (cysteamine, pyruvate) were present in the maturation medium for all groups of COCs. Cysteamine has been shown to break down into a precursor of the reduced form of glutathione (GSH), which then actively converts H_2O_2 into H_2O (Kurzawa et al., 2004). Additionally pyruvate, has been shown to reduce intracellular H_2O_2 in the embryo (Gardner and Lane, 1993; O'Fallon and Wright, 1995). The presence of these antioxidants in the maturation medium may explain why COCs matured under 20% O_2 were able to cope with the increased oxidative stress and develop to the blastocyst stage. Therefore, the antioxidant sequelae of EGF during oocyte maturation require further investigation.

Mitochondrial respiration involves the pumping of protons across the inner mitochondrial membrane, creating an inner membrane potential. The energy created from this process is utilized to drive the conversion of ADP to ATP for cellular functions. Higher mitochondrial membrane potential has been correlated with increased developmental competence in oocytes (Wilding et al., 2001) and embryos (Ahn et al., 2002; Jones et al., 2004; Wilding et al., 2003). In the present study, mitochondrial membrane potential was higher in oocytes matured under 5% O_2 compared to oocytes matured under 20% O_2. Additionally, there was no difference between oocytes matured under 5% O_2 and in vivo

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matured oocytes, suggesting increased oocyte competence following in vitro maturation under 5% O_2, which was further reflected by higher cell numbers in embryos produced from oocytes matured under 5% O_2.

The relationship between mitochondrial membrane potential and oxidative metabolism is well established (Mitchell and Moyle, 1967). In human embryos, JC-1 ratios appeared to correlate with the level of oxidative metabolism (Wilding et al., 2002). In the present study, oxidative metabolism may have been negatively affected in oocytes matured under 20% O_2, resulting in the production of less ATP and impaired cytoplasmic maturation. As mitochondria are oocyte derived, alterations in mitochondrial functioning resulting from maturation conditions will plausibly be reflected in subsequent embryo viability.

In addition to mitochondrial activity, oxidative metabolism and ATP production are also associated with oxygen uptake. Oxygen is required for oxidative metabolism and increased oxygen uptake in embryos was correlated with increase ATP production (Thompson et al., 1996). In the present study the oxygen consumption was similar between in vivo oocytes and oocytes matured under 5% O_2. Oocytes matured under 20% O_2 had significantly lower oxygen uptake. These findings mimic that of the mitochondrial membrane potential, further suggesting a decrease in ATP production by oocytes matured under 20% O_2, resulting in decreased competence of subsequent embryos.

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The mitochondrial membrane potential and oxygen uptake data suggest that oocytes matured under 20% O_2 have reduced mitochondrial respiration. The utilization of substrates involved in oxidative phosphorylation increases throughout the course of maturation, suggesting the importance of this pathway in oocyte development (Rieger and Lostutoff, 1994; Steeves and Gardner, 1999). Additionally, when inhibitors of oxidative phosphorylation were present, rat oocytes arrested at the germinal vesicle stage (Zeilmaker and Verhomme, 1974). Higher oxygen concentration during oocyte maturation may increase the production of ROS, resulting in impaired mitochondrial function and metabolic activity.

Measurements of oocyte markers assist in the prediction of viability; however, ultimately oocyte and embryo competence is determined by the development of offspring following transfer. In the present study, there was no significant difference following transfer of embryos derived from oocytes matured under 20 or 5% O_2; however, there was a tendency for increased implantation and fetal development following maturation under 5% O_2. Larger number embryos will need to be transferred in order to determine if maturation under 5% O_2 results in improved fetal development.

In conclusion, maturation of COCs under 20% O_2 affected the quality of the oocyte and subsequent embryo. Although subsequent embryo developmental rates were not affected by maturation of COCs under 20% O_2, the numbers of cells per blastocyst were reduced. For COCs recovered from unprimed females, the use of EGF appeared to moderate some negative metabolic effects of maturation under 20% O_2. Carbohydrate metabolism of

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COCs recovered from eCG-primed mice was significantly improved in the presence of EGF regardless of oxygen tension; however, oxygen uptake and mitochondrial membrane potential of these oocytes were negatively affected by 20% O_2. All three of these markers are involved in ATP production, thus suggesting an overall decrease in energy available to oocytes matured under 20% O_2. As many laboratories, both clinical and research, currently utilize 20% O_2 for both oocyte maturation and embryo culture, further studies are required to determine any additional nuclear or cytoplasmic events that may be negatively impacted when maturation occurs under 20% O_2, subsequently leading to lower implantation rates and fewer live births of offspring from in vitro matured oocytes. The findings of the present study suggest that maturation under 5% O_2 results in oocytes of higher developmental potential than oocytes matured under 20% O_2.

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Acknowledgements

The authors would like to thank Dr. Mark Johnson for providing valuable advice and comments on the manuscript and Vitrolife for support of this study.

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Table 1. Developmental rates and cell numbers of COCs collected from unprimed donors.

	No. oocytes	No. MII	No. Fert/ oocyte	% Fert/ MII	No. Blast	% Blast/ Fert	Mean Cell No.
20% O_2	168	139 (83%)	132 (79%)	95	49 (29%)	37%	51 ± 2^a
20% O_2 +EGF	217	182 (84%)	167 (77%)	92	50 (23%)	30%	52 ± 2^a
5% O_2	200	160 (80%)	154 (77%)	96	50 (25%)	32%	74 ± 3^b
5% O_2 +EGF	207	171 (83%)	161 (78%)	94	56 (27%)	35%	69 ± 3^b

[a,b] Means with different superscripts within columns differ (P<0.05).

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Table 2. Glucose uptake and lactate production by COCs recovered from unprimed donors.

	0-16 h			12-16 h	
	Glucose (pmol/COC/h)	Lactate (pmol/COC/h)		Glucose (pmol/COC/h)	Lactate (pmol/COC/h)
20% O_2	24±1[a]	14±1[a]		22±2[a]	13±2[a]
20 % O_2 + 100 ng EGF	31±3[a,b]	28±2[b]		36±3[b]	43±4[b]
5% O_2	34±2[b]	30±3[b]		50±3[c]	56±4[b]
5% O_2 + 100 ng EGF	46±2[c]	49±3[c]		53±5[c]	61±5[b]

[a,b,c] Means within columns without common superscripts differ ($P<0.05$). Values are means±SEM.

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Table 3. Developmental rates and cell numbers of blastocysts derived from COCs collected from eCG-primed donors.

	No. oocytes	No. MII	No. Fert/ oocyte	%Fert/ MII	No. Blast/ oocyte	% Blast/ Fert	Mean Cell No.
20% O_2	110	90 (82%)^{a}	90 (82%)^{a}	100	62 (56%)	69	55±2^{a,b}
20% O_2 +EGF	114	108 (95%)^{b,c}	108 (95%)^{b,c}	100	64 (56%)	59	51±2^{a}
5% O_2	89	79(89%)^{a,b}	79 (89%)^{a,b}	100	48 (61%)	69	66±3^{b,c}
5% O_2 +EGF	98	95 (97%)^{c}	95 (97%)^{c}	100	60 (61%)	63	69±3^{c}

^{a,b,c} Mean within columns without common superscripts differ ($P<0.05$).

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Table 4. Glucose uptake and lactate production over the course of maturation (0-16 h) by COCs recovered from eCG-primed females.

	Glucose Uptake (pmol/COC/h)	**Lactate Production (pmol/COC/h)**
20% O_2	16±2[a]	19±2[a]
20% O_2 + 100ng EGF	35±2[b]	46±2[b]
5% O_2	23±2[a]	24±2[a]
5% O_2 + 100ng EGF	39±2[b]	51±3[b]

[a,b] Means within rows with different superscripts differ ($P<0.001$). Values are means±SEM.

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FIGURE LEGENDS

Figure 1a. Glucose uptake (12-16 h) by individual COCs recovered from eCG-primed mice and matured under 20% O_2, 20% O_2 + EGF, 5% O_2, or 5% O_2 + EGF. Metabolism of in vivo matured COCs was analyzed following recovery from follicle. Values are means±SEM. Groups without common letters differ ($P<0.05$).

Figure 1b. Lactate production (12-16 h) by individual COCs recovered from eCG-primed mice and matured under 20% O_2, 20% O_2 + EGF, 5% O_2, or 5% O_2 + EGF. Metabolism of in vivo matured COCs was analyzed following recovery from follicle. Values are means±SEM. Treatment without common letters differ ($P<0.05$).

Figure 2. Glucose uptake (13-16 h) by COCs recovered from eCG-primed mice following exposure to phloretin, phloridzin or ethanol. Values are means ± SEM. [a,b]Treaments without common letters differ ($P<0.05$).

Figure 3a. Oxygen uptake of COCs recovered from eCG-primed mice following 17 h maturation. Oxygen uptake of in vivo matured COCs was analyzed following recovery from follicle. Values are means ± SEM. [a,b] Treatments without common letters differ ($P<0.05$).

Figure 3b. Oxygen uptake of denuded oocytes recovered from eCG-primed mice following 17 h maturation. Oxygen uptake of in vivo matured oocytes was analyzed

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following recovery from follicle. Values are means ± SEM. [a,b] Treatments without common letters differ ($P<0.05$).

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Figure 1a.

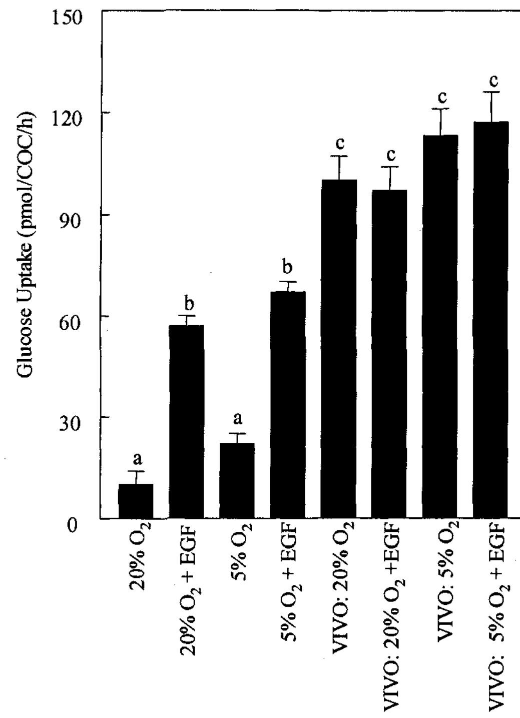

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Figure 1b.

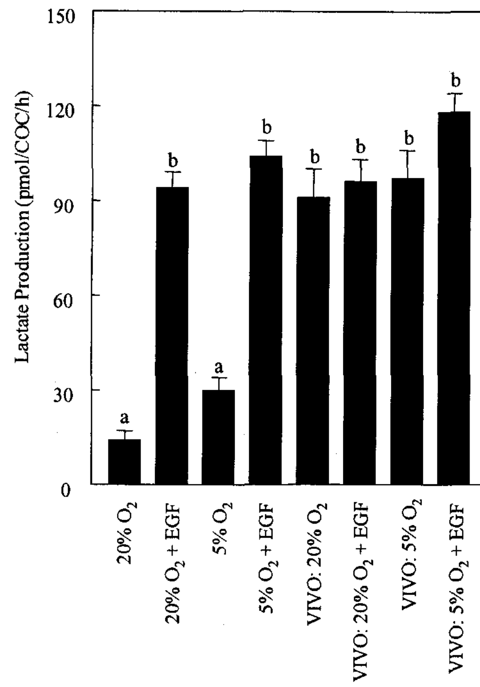

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Figure 2.

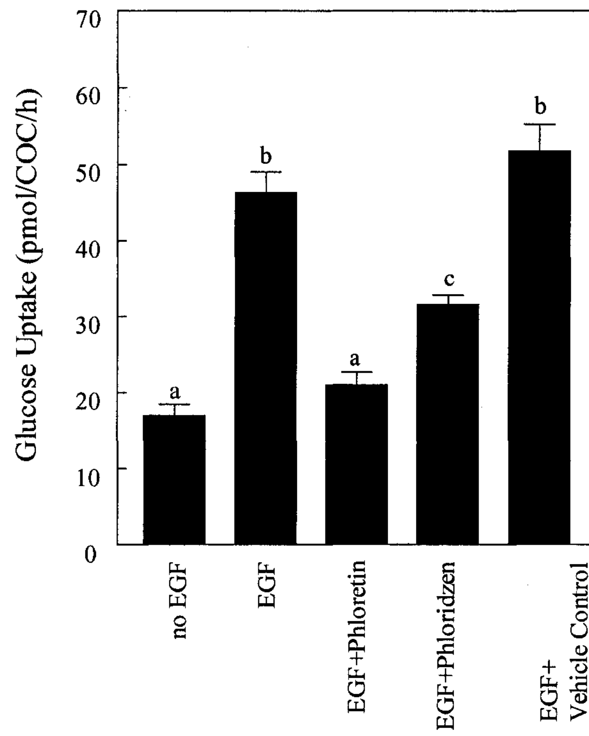

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Figure 3a.

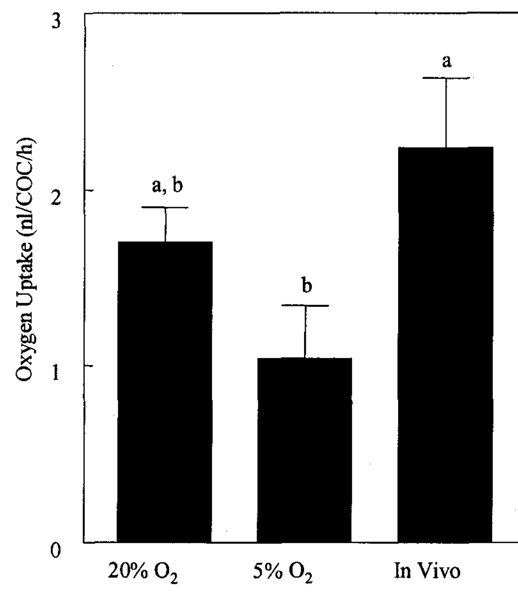

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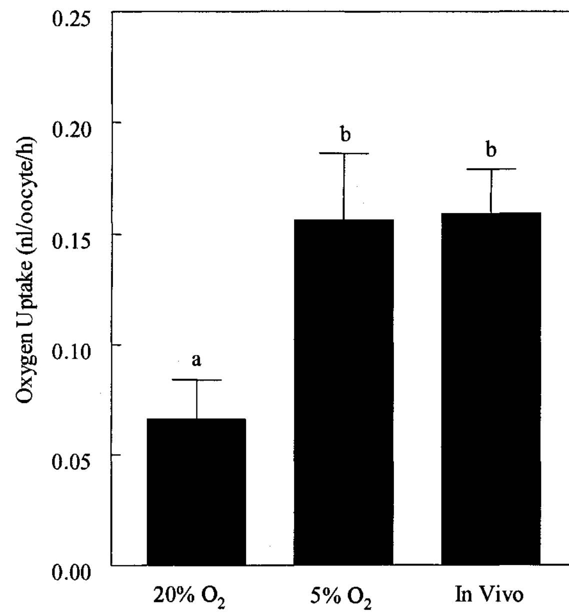
Figure 3b.



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CHAPTER VI

EFFECT OF MEDIUM SUPPLEMENTS DURING BOVINE OOCYTE MATURATION AND SUBSEQUENT EMBRYO DEVELOPMENT

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ABSTRACT

In vitro production of bovine blastocysts is limited by the initial suboptimal quality of the immature oocytes, which is exacerbated by suboptimal in vitro maturation procedures. The composition of maturation medium greatly affects oocyte quality as well as the quality of the resulting embryos. The objective of this study was to evaluate the quantity and quality of bovine embryos produced from oocytes matured in defined or serum-supplemented G maturation medium with one of six combinations of supplements: A) +EGF; B) +EGF, IGF-I, ITS; C) +EGF, IGF-I, ITS, FSH, LH; D) +EGF, FSH, LH; E) +EGF, 5% FCS; F) +EGF, 20% FCS. All groups were matured at 38.5˚C, 6% CO_2 in air for 24 h. Oocytes were fertilized, and the metabolism of subsequent embryos was evaluated at the 8-cell, morula, and blastocyst stages. All embryos were cultured in sequential G1/G2 medium at 38.5°C under 6% CO_2, 5% O_2, and 89% N_2. Blastocyst rates and total cell numbers were evaluated on day 7.5 post IVF. The percentage of oocytes developing to blastocysts was higher for group A (33%) than for groups B and C (25%; $P<0.05$), while other treatments were intermediate. Cell numbers were significantly higher for the 20% serum group than for groups C and D ($P<0.05$), but not significantly different from other treatments. Glucose metabolism and lactate production were elevated in the 20% serum group compared to all other treatment groups ($P<0.05$). These data indicate that maturation conditions affect the quality of subsequent embryos, and that a defined maturation medium supplemented with EGF alone was able to support development, while maintaining the quality of blastocysts.

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INTRODUCTION

Current procedures for in vitro production of bovine embryos from oocytes from small follicles were established over 20 years ago; however, blastocyst rates per oocyte remain between 30-40% (Lechniak, 2002). A higher proportion of in vivo matured oocytes develop to blastocyst (Van de Leemput et al., 1999; Blondin et al., 2002), in part suggesting that oocytes from small follicles are less viable, and that the current bovine oocyte maturation system is less than optimal.

The quality of the oocyte is thought to determine the percentage of blastocyst development (Blondin and Sirard, 1995), while embryo culture conditions determine the quality of the embryo. Many cellular events must occur in the oocyte to support both nuclear and cytoplasmic maturation. In vitro, the majority of bovine oocytes achieve nuclear maturation (Sirard et al., 1989), as evidenced by the extrusion of the first polar body; however, assessment of cytoplasmic maturation is more difficult. Maturation in vitro may alter a number of cellular processes involved in cytoplasmic maturation, thus potentially impacting the subsequent quality of the embryo.

Many factors will influence the maturation process including media, temperature, pH, as well as oxygen tension. The type of medium used is critical to success. Similar base media have been used for different species, typically including salts, amino acids, buffers, vitamins and carbohydrates. Many different supplements have been added to maturation media; however, optimum supplementation for subsequent developmental potential of oocytes remains unknown. Epidermal growth factor (EGF) has been shown to increase

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both maturation, and subsequent bovine embryo development (Lonergan et al., 1996). Follicle stimulating hormone (FSH) and luteinizing hormone (LH) have both been shown to increase the developmental potential of bovine oocytes (Ali et al., 2005; Zuelke and Bracket, 1990). Other additives, such as insulin-like growth factor I (IGF I) and insulin transferrin and selenium (ITS) have been reported to have beneficial effects when included in bovine oocyte maturation medium (Lorenzo et al., 1994).

It is unclear to what extent medium supplements work in concert, or individually. Unfortunately, many reports examining bovine oocyte maturation utilized serum in the maturation medium. Although serum contains growth factors and hormones that are beneficial to oocyte maturation, it also contains undefined compounds and contaminants that make it difficult to determine the impact of individual medium additives (Barnes and Sato, 1980). Inclusion of serum not only makes it difficult to determine the requirements of the oocytes during maturation, but serum has been correlated with pathological development when included in embryo culture media (Thompson et al., 1995; Young et al., 1998; McEvoy et al., 2000; Khosla et al., 2001). The use of a defined maturation system will allow assessment of each additive to the maturation medium, as all components of the medium are known.

Current techniques of assessing embryo quality are largely morphological, and therefore inherently subjective. Quantifiable markers of embryo viability would increase the selection of healthy embryos for transfer, as well as provide more information regarding the quality of the in vitro production (IVP) system. Assessment of embryo metabolism is

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an attractive quantitative marker of embryo health, as it is a non-invasive procedure that is sensitive enough to evaluate a single embryo and can provide results relatively quickly. The metabolic patterns of embryos have been studied in some detail. Pyruvate is the primary substrate used by early cleavage stage bovine embryos, while blastocysts prefer to utilize glucose (Steeves et al., 1999). Metabolism of bovine embryos has also been correlated with subsequent viability post transfer (Renard et al., 1980).

The objective of this experiment was to evaluate the quantity and quality of bovine embryos produced from slaughterhouse-derived oocytes matured in one of four defined systems or systems supplemented with fetal calf serum (FCS). Metabolism of embryos was assessed at three developmental stages: 8 cell, morula, and blastocyst. It was hypothesized that embryo quality would be affected by the type of medium used during in vitro maturation, with oocytes matured in serum producing embryos of reduced viability.

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MATERIALS AND METHODS

Immature cumulus oocyte complexes (COCs) were collected from 3-6 mm follicles of slaughterhouse ovaries and matured in G maturation medium at 38.5 °C, 6% O_2 in air. COCs were matured in groups of 33/500 µl of maturation medium with a paraffin oil overlay (Ovoil, Gothenburg, Sweden) in four well dishes (Nunc, Denmark). Treatment groups varied according to medium supplementation:

A) G maturation medium + EGF

B) G maturation medium + EGF+ IGF-I+ ITS

C) G maturation medium + EGF + IGF-I + ITS + FSH + LH

D) G maturation medium + EGF + FSH + LH

E) G maturation medium + EGF + 5% FCS

F) G maturation medium + EGF + 20% FCS

Group F was done with different batches of oocytes from the other treatments. Maturation media in groups A-D were supplemented with 2.5 mg/ml recombinant human serum albumin, 0.5 mM sodium citrate, and 0.5 mM cysteamine. Concentrations of growth factors and gonadotropins were as follows: 100 ng/ml EGF, 100 ng/ml IGF-I, 15 ng/ml oFSH (NIDDK-oFSH-20), 1µg/ml bLH (NIH-LH-S1), ITS (insulin 0.5 µg/ml, transferrin 0.275 µg/ml, and selenium 0.25 ng/ml). Unless otherwise noted, all chemicals and hormones were purchased from Sigma (St. Louis, MO, USA).

Fertilization and embryo culture were identical for all maturation groups. COCs were transferred to fertilization medium (G-Fert; 50 COC/ 450 µl) at 24 h of maturation. A

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45/90% puresperm gradient was used to separate motile sperm. Sperm concentration was adjusted to $5x10^6$ sperm/ml by adding the pertinent amount of G-Fert. Sperm suspension (50µl) was then added to the fertilization dish already containing the COCs (50 COCs/ 450 µl), for a final concentration of 5×10^5 sperm/ml. Gametes were co-incubated for 18 h, at which time presumptive zygotes were vortexed for 2 min to remove cumulus cells. Once zygotes were free of cumulus cells and sperm, they were placed into G1.3 + 5mg/ml HSA (50 embryos/500 µl). After 72 h of culture, embryos were moved to G2.3 + 5 mg/ml HSA until 180 h. Blastocyst development was recorded on day 7.5 post insemination. Blastocysts were stained, and total cell numbers were recorded.

Metabolic Analysis

Metabolism was assessed at three separate developmental stages: 8 cell, morula, and blastocyst. Embryos were placed in a 0.5 µl drop of metabolic medium (modified G2 (0.5 mM glucose, 0.23 mM sodium pyruvate, 0 mM sodium lactate) supplemented with 0.5 mM sodium citrate and 2.5 mg/ml recombinant human serum albumin) for 3-4 h, depending on the developmental stage. A 4 h incubation was used for both 8 cell and morula while blastocysts were incubated for 3 h. As metabolic assessments were non-invasive, embryos were placed back into culture following metabolic incubation. Once embryos were removed from the metabolism medium, fluorometric assays were performed to determine glucose and pyruvate uptake and lactate production. Fluorometric assays were based on the generation or consumption of the reduced pyridine nucleotides NADH or NADPH, which fluoresce when excited with a wavelength of 340 nm (Leese

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and Barton, 1984; Gardner and Leese, 1990). Assays were performed as described by Preis et al. (2005).

Analysis of Blastocyst Cell Number

Expanded blastocysts or hatching blastocysts were placed into Triton x100 solution (20 μl in 100 ml G-MOPS) for 45 sec and then into G-MOPS + 5mg/ml HSA (Lane and Gardner, 2004). Embryos were then moved into a wash dish containing G-MOPS and transferred into 20 μl propidium iodide (PI; 0.2 mg/ml PI in G-MOPS) drops under paraffin oil, where they remained for a minimum of 10 min. Stained embryos were mounted in glycerol on a siliconized glass slide. The cell number was determined by counting the cell nuclei using a fluorescence microscope with an ultraviolet filter.

Statistical Analysis

Embryo metabolism between and within groups, as well as blastocyst cell numbers were analysized by ANOVA and Tukey's hsd test. Blastocyst rates were analyzed with Fisher's Exact Test.

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RESULTS

The developmental competence of in vitro matured oocytes was affected by the supplements included in the maturation medium (Table 1). Higher rates of blastocyst development were achieved when oocytes were matured in a defined medium with the inclusion of EGF alone, compared to the two groups containing IGF-I and ITS ($P<0.05$). Total cell numbers were increased for the 20% FCS group compared to the two groups containing gonadotropins (C+D; $P<0.05$). There was no difference in cell numbers among remaining groups.

Metabolism was moderately affected by conditions of maturation. Glucose uptake by embryos from all treatment groups increased from the 8 cell to the blastocyst stage ($P<0.01$); however, only embryos matured in the presence of serum (5 and 20%) had increased glucose uptake between the 8 cell and morula stage ($P<0.01$). At all time points, embryos derived from oocytes matured in the presence of 20% serum had higher glucose uptake than all other groups ($P<0.05$; Table 2).

Similar to glucose uptake, lactate production also increased as embryos developed from 8 cell to the blastocyst stage ($P<0.05$). Lactate production was similar at the 8 cell and morula stage for groups B and C; however, for the remaining groups lactate was higher at the 8 cell than the morula stage ($P<0.05$). There was no difference in lactate production between any groups at both the 8 cell and morula stage (Table 3). However, by the blastocyst stage, lactate production was higher for embryos in group F relative to all treatments except C ($P<0.05$).

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To indirectly assess the percentage of glucose utilized by the glycolytic pathway, both glucose and lactate metabolism were analyzed (Table 4). With the exception of treatment C, E and F, the percentage of glucose utilized in the glycolytic pathway decreased as the embryo developed. At the morula stage, group B had higher glycolytic rates than other groups ($P<0.05$), while group C had a highest glycolytic rate at the blastocyst stage ($P<0.05$).

Pyruvate uptake decreased between the 8 cell and morula stage for all groups ($P<0.01$) except group A and the two serum groups (E and F; Table 5). Pyruvate uptake increased from the 8 cell to blastocyst and morula to blastocyst stages for all groups ($P<0.05$).

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DISCUSSION

The objective of this study was to determine the effect of oocyte maturation medium on the quantity and quality of subsequent bovine embryos produced. An additional goal was development of a defined maturation system capable of producing developmental rates equivalent to or higher than those of oocytes matured in systems containing serum. Although serum contains a wide variety of amino acids, hormones, growth factors, cytokines, and vitamins, the effectiveness of serum on in vitro maturation and in vitro fertilization varies considerably from batch to batch, even from the same supplier (Sirard and Lambert, 1985). This variability leads to poor reproducibility of results between and within laboratories. Additionally, a quantitative understanding of cellular physiology can not be obtained with ill defined and variable conditions. Defined systems, such as those in this study allow for greater understanding of individual medium components on oocyte maturation.

Developmental competence of oocytes was altered by the maturation medium. Previous studies have linked blastocyst development with oocyte quality (Blondin and Sirard, 1995; Rizos et al., 2002); therefore, maturation medium yielding oocytes with higher developmental competence may provide better support of the cellular processes of oocyte maturation, resulting in higher quality oocytes. All maturation groups supplemented with recombinant human serum albumin resulted in similar developmental rates as serum groups, 5 and 20%. Interestingly, the two groups matured with IGF-I and ITS had lower blastocyst development than the EGF only group. There was no synergistic effect between growth factors and gonadotropins, as oocytes matured under these conditions

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had lower developmental rates than EGF alone. Previous studies have reported increased developmental competence following maturation with combinations of gonadotropins and growth factors (Harper and Brackett, 1993); however, comparisons between studies are difficult, as there are differences in base medium for both maturation and embryo culture. Under the conditions of the present study, the least complicated maturation system, supplementation with EGF only, resulted in equivalent blastocyst development to medium supplemented with serum.

The present study not only assessed the affect of maturation medium on embryo quantity, but also embryo quality. Oocytes were matured under different maturation conditions; however, fertilization and embryo culture were identical for all six groups. Therefore, any differences in embryo quality were the result of treatment during maturation. Embryo cell number is a frequently used marker of embryo quality (De La Fuente and King, 1997). Interestingly, both groups that were matured with gonadotropins (C+D) had lower cell numbers than embryos derived from oocytes matured with 20% serum, while the other defined groups without gonadotropins and the 5% serum group did not have significantly lower cell numbers than the 20% serum group. Although addition of IGF-I and ITS (group B) resulted in a lower percentage of blastocysts, the resulting embryos were of equal quality to the serum groups and EGF alone.

Metabolic profiles of mammalian embryos have been characterized by several groups (Biggers et al., 1967; Leese et al., 1986; Thompson et al., 1996). Early embryos utilize pyruvate as the primary energy source, later switching to glucose metabolism (Gardner

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and Leese, 1986). In contrast to mouse embryos, in which pyruvate uptake decreased after the 8-cell stage in the presence of lactate (Gardner and Leese, 1986), bovine embryos had increased pyruvate uptake with development, especially following compaction (Thompson et al., 1996). This observation was supported by the present study, with pyruvate uptake increasing from the 8-cell to the blastocyst stage. Pyruvate consumption by early embryos was similar between groups. However, the 20% serum group consumed less pyruvate than the amount of glucose taken up at the 8-cell stage.

There is some evidence that serum has a detrimental effect on mitochondria. Compact morula produced in the presence of serum had fewer mitochondria (Crosier et al., 2000), and blastocysts cultured in serum showed increased mitochondrial degeneration (Dorland et al., 1994), when compared to embryos that were not cultured in serum. Increased glycolytic activity observed in embryos cultured in serum was proposed to reflect this mitochondrial damage (Leese et al., 1998). No studies have examined the effect of serum during maturation on mitochondria, with respect to subsequent embryo health. The metabolic data in this study suggest that the inclusion of serum in maturation medium may have negatively impacted the mitochondria within the oocyte, which is reflected by decreased pyruvate uptake during early cleavage.

In addition to reduced pyruvate uptake, the metabolism of glucose increased between the 8 cell and morula stage for the serum groups only, perhaps indicating a shift in kinetics of the metabolic transition from pyruvate to glucose in the serum groups. In a previous report, glucose uptake increased between the 8 cell and morula stages when serum was

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present in the maturation medium (Rieger et al., 1992). This observation may also be a reflection of mitochondrial damage, with the early embryo's having a decreased reliance on the oxidation of pyruvate. As the maturation and culture of bovine oocytes and embryos in serum is correlated with altered physiology, an early increase in glucose uptake may be indicative of embryonic stress.

Lactate production by embryos in all groups increased over the course of development as glucose uptake increased, confirming previous reports (Thompson et al., 1996). A functional purpose of higher lactate levels in later stages of development may be in preparation for the reduced oxygen tension of the uterine cavity, relative to the oviduct (Brown and Mattner, 1984). Aerobic glycolysis generates energy for blastocoel expansion and mitosis, and additionally provides substrates for biosynthetic pathways involved in DNA replication and RNA transcription (Newsholme et al., 1985). However, in vitro-derived embryos produce significantly higher levels of lactate than their in vivo counterparts; therefore reduced lactate production may be indicative of healthier embryos.

Lactate production was similar between all groups at both the 8 cell and morula stages; however by the blastocyst stage, lactate production was higher in the 20% serum group and group C (gonadotropins and growth factors). Group C had lower blastocyst development and cell numbers; however there was no negative effect on blastocyst rate or number of cells per embryo in the 20% serum group. The increase in lactate production in the 20% serum group probably is related to the significantly higher amount

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of glucose consumption by this group. At the blastocyst stage there was roughly a 1:1 ratio of glucose uptake to lactate production in the 20% group, as well as in groups A, B, and D, but this ratio was closer to 1:2 for group C. As blastocyst rates and number of cells per blastocyst were lower in group C, lactate production may be a useful indicator of embryonic health when assessed in relative, rather than absolute terms.

Glycolytic activity estimated through glucose and lactate metabolism was lower at the 8 cell and morula stage for the 20% serum treatment and the morula and blastocyst stage for the 5% serum treatment, compared to the defined groups. Previous research has reported that 90% of ATP generated by pre-compaction bovine embryos is derived from oxidation, while the post compaction embryo shifts to increased glycolysis to meet the increased demand for ATP (Thompson et al., 1996). The results of this research suggest that the embryos generated from the defined groups are generating more energy from glycolysis during the pre-compaction stage, compared to the serum groups. However, embryos in the defined treatments also tended to utilize more pyruvate than the serum groups, suggesting increased oxidation during this same time period, and therefore perhaps an overall increase in energy production relative to serum groups.

In addition, an increase in glucose oxidation has also been reported following the 16 cell stage of bovine embryos (Khurana and Nieman, 2000), which would explain the decrease in glycolysis in all groups over the course of maturation with the exception of group C, which had an increase. Results of this study differ from previous bovine embryo metabolic data indicating that nearly 100% of glucose is converted to lactate by the

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bovine blastocyst (Thompson et al., 1996; Thompson et al., 2000). Comparisons between metabolic studies are difficult as conditions of maturation, especially the formulation of maturation medium, greatly affect the metabolic behavior of embryos. Interestingly, maturation medium in the studies by Thompson et al. (1996, 2000) was supplemented with 10% FCS, FSH, LH and E2. As FCS is thought to contain growth factors, this medium was similar to the maturation medium of group C (growth factors and gonadotropins) in the present study, which may explain the similarity in subsequent embryo metabolism. Under the conditions of the present study, glycolytic activity decreased over the course of development; however, glycolysis by embryos in group C remained elevated at the blastocyst stage. Perhaps the elevated glycolytic activity of embryos in group C was indicative of reduced embryo heath, as evidenced by fewer blastocysts per oocyte and reduced number of cells per embryo.

Embryos use glucose in several pathways, such as glycolysis, the pentose phosphate pathway (PPP), and oxidative phosphorylation. A shift in glucose metabolic pathways during embryonic development is indicative of an increased need for ATP production via oxidation, as well as the need for the production of ribose moieties for DNA and RNA synthesis through the PPP (Khurana and Wales, 1989; Sutten et al., 2003). The activity of these metabolic pathways is indicative of embryo health; therefore, future studies evaluating maturation conditions should examine the metabolic pathways that the resulting embryos utilize.

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In conclusion, maturation conditions affect the quantity and quality of blastocysts produced in IVP systems. Cell number may be indicative of embryo health and was negatively affected by gonadotropins in the maturation medium. Supplementation of maturation medium with serum altered embryo metabolism. As oocytes matured in serum resulted in blastocysts with more cells, effects of altered metabolism may not materialize until later in fetal development. It is clear from this study that a defined system of bovine IVP results in similar embryo production to a system that utilizes serum. Defined medium supplemented with EGF alone resulted in high blastocyst production per oocyte and a normal number of cells per blastocyst, as well as normal embryonic metabolism.

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Table1. Developmental competence of oocytes matured in different in vitro maturation systems.

Maturation Treatment	Blastocyst Development per Oocyte	Cell Number
A	128/383 (33%)^{b}	108±5 ^{ab}
B	84/339 (25%)^{a}	106±7 ^{ab}
C	82/328 (25%)^{a}	98±6 ^{b}
D	104/374 (28%)^{a,b}	100±5 ^{b}
E	125/400 (31%)^{a,b}	110±6 ^{ab}
F	76/276 (28%) ^{a,b}	130±7^{a}

^{a,b} Means within a column without common superscripts differ, $P<0.05$.

A= EGF; B= EGF, ITS, IGF; C= EGF, ITS, IGF, FSH, LH; D= EGF, LH, FSH; E= EGF, 5% FCS; F= EGF, 20% FCS

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Table 2. Glucose uptake (pmol/embryo/h) by bovine embryos at different developmental stages.

Developmental Stage	Treatment A	Treatment B	Treatment C	Treatment D	Treatment E	Treatment F
8 Cell	9±1.8 [a]	9±1.4[a]	13.±2.5 [a]	6±1.5 [a]	8±1.2 [a]	17±1.6 [b]
Morula	12±1.2 [a]	10±1 [a]	11±1.3 [a]	10±1.3 [a]	16±1.6 [a]	29.2±3 [b]
Blatocyst	29±2.4 [a]	28±1.5 [a]	24±3.4[a]	28±1.7 [a]	35±1.2 [a]	52±4.1 [b]

[a,b] Means with different superscripts within rows differ ($P<0.05$).
A= EGF; B= EGF, ITS, IGF; C= EGF, ITS, IGF, FSH, LH; D= EGF, LH, FSH; E= EGF, 5% FCS; F= EGF, 20% FCS

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Table 3. Lactate production (pmol/embryo/h) by bovine embryos at different stages of development.

Developmental Stage	Treatment A	Treatment B	Treatment C	Treatment D	Treatment E	Treatment F
8 Cell	21±1.6 [a]	19±1.8 [a]	18±2.3 [a]	16±2.3 [a]	3±0.6 [b]	17±3.2 [a]
Morula	13±1.4	15±1.2	12±1.7	10±1.3	11±1.6	10±3
Blastocyst	29±2.4 [a]	31±3.4 [a]	42±3.7 [a,b]	31±2.8 [a]	26±2.9 [a]	52±8 [b]

[a,b]Means without common superscripts within rows differ ($P<0.05$).

A= EGF; B= EGF, ITS, IGF; C= EGF, ITS, IGF, FSH, LH; D= EGF, LH, FSH; E= EGF, 5% FCS; F= EGF, 20% FCS

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Table 4. Percentage of glucose utilized in the glycolytic pathway.

	A	B	C	D	E	F
8 Cell	100%[a]	100%[a]	67%[b]	100%[a]	19%[c]	49%[d]
Morula	55%[a]	76%[b]	52%[a]	53%[a]	35%[c]	17%[d]
Blastocyst	49%[a]	55%[a]	87%[b]	56%[a]	37%[c]	50%[a]

[a,b,c,d]Means without common superscripts within rows differ ($P<0.05$).

A= EGF; B= EGF, ITS, IGF; C= EGF, ITS, IGF, FSH, LH; D= EGF, LH, FSH; E= EGF, 5% FCS; F= EGF, 20% FCS

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Table 5. Pyruvate uptake (pmol/embryo/h) by bovine embryos at different stages of development.

Developmental Stage	Treatment A	Treatment B	Treatment C	Treatment D	Treatment E	Treatment F
8 Cell	13±1.3 ^{a,b}	17±1.1 ^{a}	14±0.9 ^{a,b}	15±1.3 ^{a,b}	10±1 ^{b}	11±2.2 ^{b}
Morula	10±1.4	9±0.9	8±1.7	6±1.3	7±1	7±1.2
Blastocyst	20±1.6 ^{a,b}	26±1.7 ^{a}	21±1.6 ^{a,b}	19±1.8 ^{a,b}	15±1.8 ^{b}	18±2.9 ^{a,b}

^{a,b} Means without common superscripts within rows differ (P<0.05).
A= EGF; B= EGF, ITS, IGF; C= EGF, ITS, IGF, FSH, LH; D= EGF, LH, FSH; E= EGF, 5% FCS; F= EGF, 20% FCS

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CHAPTER VII

IN VITRO MATURATION AFFECTS METABOLISM AND GENE EXPRESSION OF BOVINE OOCYTES

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ABSTRACT

Current in vitro maturation systems (IVM) are inadequate, as evidenced by low blastocyst production from in vitro matured bovine oocytes compared to in vivo matured oocytes. The objective of this study was to compare metabolism and gene expression of in vivo matured oocytes with oocytes matured under different in vitro maturation conditions. Immature and mature oocytes were recovered transvaginally from FSH-treated cattle. Immature oocytes were matured in maturation medium supplemented with recombinant human serum albumin or 20% FCS. Oocytes for additional experiments were recovered from 3-6 mm follicles from abattoir ovaries, and maturation occurred under 20% or 5% O_2. Mature COCs from all experiments were individually incubated in metabolic medium, which was later analyzed for glucose uptake and lactate production. RNA was isolated from in vivo and in vitro matured oocytes recovered from FSH-treated cows, and amplified and hybridized to a bovine-specific microarray. The only metabolic difference observed was in COCs recovered from FSH-treated cows in which lactate production by COCs matured with 20% serum was lower than that produced by in vivo matured oocytes ($P<0.05$). There was no statistically significant difference in gene expression between in vivo and in vitro matured oocytes, although the amount of mRNA for several genes was altered by more than 2 fold. It is concluded that oocyte physiology is altered following IVM. The use of metabolic and gene markers should aid in identification of physiological anomalies and improvement of IVM systems.

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INTRODUCTION

In vitro production of bovine blastocysts is less than optimal, as evidenced by low blastocyst development (30-40%; Lechniak, 2002). It is thought that the intrinsic quality of the oocyte determines the proportion of oocytes that develop to the blastocyst stage (Rizos et al., 2002). For example, oocytes recovered from larger follicles are more competent than oocytes recovered from small follicles (Pavlok et al., 1992; Lonergan et al., 1994). Also, in vivo matured oocytes are more viable than in vitro matured oocytes (Blondin et al., 2002). Meiotic resumption of oocytes occurs spontaneously upon removal from the follicle (Pincus and Enzmann, 1935); however, oocytes matured in vitro resume meiosis earlier than would occur in vivo; thus the entire process of maturation is accelerated in vitro compared to in vivo. Events of nuclear maturation have similar kinetic rates in vitro as in vivo, if removal from the follicle is compared with time of LH surge (Sirard et al., 1989); however, immature oocytes recovered from donors have reduced cytoplasmic maturation compared to in vivo counterparts from pre-ovulatory size follicles exposed to an LH surge. Therefore, the optimal maturation system must be able to not only support nuclear maturation, but also be able to support cytoplasmic maturation of in vitro matured oocytes effectively.

The composition of maturation medium for bovine oocytes has been extensively examined. Supplements to bovine maturation medium commonly include gonadotropins and growth factors. LH included in bovine maturation medium without serum enhanced maturation, fertilization and blastocyst development (Younis et al., 1989; Zuelke and Brackett, 1990; Saeki et al., 1991). FSH increased in vitro fertilization (IVF) and

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subsequent embryo development of bovine oocytes (Ali et al., 2005). Inclusion of EGF in maturation medium resulted in increased cumulus expansion and maturation in bovine oocytes (Lorenzo et al., 1994; Lonergan et al., 1996); as well as enhanced developmental competence (Harper and Brackett, 1993; Lonergan et al., 1996). Improved fertilization and blastocyst development suggests that gonadotropins and growth factors enhance both nuclear and cytoplasmic maturation of in vitro matured oocytes.

Unfortunately, many studies of bovine IVP have included blood serum in the maturation medium. The inclusion of serum in IVM and/ or embryo culture medium generally results in high developmental rates; however, serum contains unknown substances and possible contaminants, making it difficult to replicate findings between laboratories. As the medium is ill defined, it is difficult to understand the impact of individual medium components on oocyte physiology. In addition to batch variability and limitations regarding the understanding oocyte physiology, the inclusion of serum in ruminant embryo culture medium has been correlated with pathological development (Thompson et al., 1995; Young et al., 1998; McEvoy et al., 2000; Khosla et al., 2001). Therefore, it is important to develop a bovine maturation system devoid of serum that is capable of supporting both nuclear and cytoplasmic maturation.

In addition to medium composition, conditions of maturation, such as oxygen concentration, impact oocyte development. In vitro maturation and culture under 20% O_2 is speculated to increase the production of reactive oxygen species (ROS) within the mouse oocyte and embryo, resulting in developmental arrest (Nasr-Esfari and Johnson,

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1991; Kwon et al., 1999). An increase in H_2O_2 production in the bovine oocyte was reported following maturation under 20% O_2 (Hashimoto et al., 2000). However, in the majority of reports on bovine IVP, oocytes were matured under 20% O_2 (Harper and Brackett, 1993; Lonergan et al., 1996; Ali et al., 2005). Maturation of bovine oocytes under low oxygen tension resulted in improved blastocyst development (28%) compared to oocytes matured under 20% O_2 (16%; Hashimoto et al., 2000). In addition, increasing glucose concentration during maturation improved blastocyst development following IVM under low oxygen tension (Oyamada et al., 2004). As oocytes in the follicular environment are exposed to oxygen tension significantly lower than atmospheric oxygen and maturation of oocytes in atmospheric oxygen is associated with increased ROS production, more research is required that examines oxygen tension during maturation. Maturation under physiological conditions may require modification of maturation medium, but it potentially offers benefits to IVM oocytes.

The development of a successful in vitro maturation system will be expedited when reliable methods to assess oocyte quality are determined. Many laboratories use the morphology of cumulus cells surrounding the oocyte as a selection criterion prior to in vitro maturation (Lonergan et al., 1994; Goud et al., 1998) and the degree of expansion as a morphological indicator of oocyte quality after maturation. However, it is unclear as to whether cumulus expansion directly relates to developmental capacity of the oocyte (Ali and Sirard, 2002).

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Understanding energy substrate metabolism of the cumulus oocyte complex (COC) throughout in vitro maturation may aid in optimizing maturation conditions and serve as a potential viability marker of developmental competence. A critical aspect of oocyte maturation involves development and activation of appropriate metabolic pathways. As metabolic measurements are non-invasive, this approach provides the ability to link a quantitative measurement of a single oocyte with subsequent developmental competence. A few studies have correlated increased glycolytic activity in denuded oocytes with subsequent developmental competence (Krisher and Bavister, 1999; Spindler et al., 2000); however, few groups have examined the relationship between the COC and developmental competence (Preis et al., 2005). Removal of the cumulus may inherently change the physiology of the oocyte; therefore, an accurate understanding of oocyte physiology requires examining the oocyte and cumulus as an intact complex.

In addition to carbohydrate metabolism, oocyte quality may be linked to oxygen consumption. Oxygen consumption provides a method of determining total ATP production by the oocyte. ATP is produced by two pathways in the cell: oxidative phosphorylation and glycolysis. ATP production can be determined through the measurement of oxygen consumption, glucose uptake and lactate production (Thompson et al., 1996). The measurement of oxygen uptake is non-invasive, and the short period of incubation required for the technique has little detrimental effect on subsequent development (Thompson et al. 1996). Several groups have measured oxygen consumption of preimplantation embryos (Haughton et al., 1996; Thompson et al., 1996);

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however, less information is available regarding oxygen uptake of in vitro matured bovine COCs and denuded oocytes.

During oocyte growth and maturation, mRNA and protein molecules are being synthesized, which contribute to early development prior to embryonic genome activation (Hyttel et al., 1996). Lack of developmental competence in the majority of in vitro matured oocytes may reflect the composition and abundance of specific mRNA transcripts in the oocyte (De Sousa et al., 1998). Comparison of highly competent in vivo matured oocytes to less competent in vitro matured oocytes may provide valuable information regarding particular pathways that are up or down regulated under in vitro conditions. Although there is a large amount of data regarding gene expression of oocytes during different stages of oocyte growth and maturation (Bachvarova, 1985; Marello et al. 1992; Robert et al., 2000), there is a paucity of information regarding the level of gene expression in oocytes matured under varying maturation conditions. In one study, differences in maturation medium affected transcript abundance of two out of six gene transcripts (Watson et al., 2000), while ten out of twelve gene transcripts were shown to differ in abundance between in vivo matured and in vitro matured oocytes (Lonergan et al., 2003). Using heterologous hybridization onto a human cDNA array, 70 transcripts underwent significant differential regulation during in vitro maturation of bovine oocytes (Dalbies-Tran and Mermillod, 2003). The use of DNA microarray technology will aid in identifying candidate genes that are altered during in vitro maturation and will increase our knowledge of genes and families of genes that are involved in oocyte maturation and subsequent developmental competence.

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The first objective of the present study was to determine differences in metabolism and gene expression between in vitro matured and in vivo matured oocytes recovered from FSH-treated cows. In addition, the effects of oxygen tension during maturation on metabolism and gene expression were evaluated for oocytes recovered from slaughterhouse ovaries.

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MATERIALS AND METHODS

General Procedures

<u>Collection of COCs from FSH-treated cattle.</u>

For collection of immature oocytes, cattle were administered 6 injections of FSH i.m. (50mg; Folltropin-V, Bioniche, Belleville, Ontario), twice daily for 3 days. For collection of in vivo matured oocytes, cows received a single injection of 25 mg prostaglandin F-2-alpha i.m.(PG; Lutalyse, Pfizer, New York, USA) simultaneously with the last FSH injection. GnRH (50 μg; Cystorelin, Merial Limited, Duluth, USA) was administered i.m. 37 h post PG, and transvaginal aspiration (TVA) was performed 24 h post GnRH from follicles 8 mm in diameter or larger.

<u>Oocyte Recovery and Maturation from Abattoir Ovaries</u>

Immature COCs were aspirated from slaughterhouse ovaries. Ovaries were transported at room temperature for ≤2 h in saline. Follicles (3-6 mm) were aspirated, and COCs were recovered from the follicular aspirate. COCs were washed (x3) in G-MOPS + 5mg/ml HSA and placed into G maturation medium supplemented with 100 ng/ml EGF, 0.5 mM cysteamine, 0.5 mM sodium citrate, and 2.5 mg/ml recombinant human serum albumin (Recombumin). COCs were matured in groups (10/100 μl) for 23 h at 38.5°C, under 6% CO_2 and either 5% O_2 or 20% O_2.

<u>Metabolic Analysis</u>

For in vitro matured COCs, metabolism was measured following 23 h maturation; for in vivo matured COCs, metabolic measurements were taken immediately following

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recovery from the follicle. COCs were thoroughly washed in metabolic medium, modified G2 (0.5mM glucose, 0.23 mM sodium pyruvate, 0.0 mM sodium lactate) supplemented with 2.5 mg/ml Recombumin and 0.5 mM sodium citrate. Following metabolic incubation, COCs were removed from metabolic medium and the medium was stored at -80°C for later analysis of glucose uptake and lactate production. Ultramicrofluorimetry was used to quantify the level of carbohydrate consumption and production from the maturation medium. Fluorometric assays were based on the generation or consumption of the reduced pyridine nucleotides NADH or NADPH, which fluoresce when excited at a wavelength of 340 nm (Leese and Barton, 1984; Gardner and Leese, 1990). Assays were performed as described by Preis et al. (2005).

RNA Isolation and Extraction

COCs were denuded by pipetting in G-MOPS with 1 mg/ml hyaluronidase. Denuded MII oocytes were placed into pronase until the zona disappeared. Upon removal of the zona, oocytes were washed in phosphate-buffered solution (PBS) supplemented with 10% molecular grade BSA. With a clean pipette, oocytes (single or groups of $\leq$9) were transferred with minimal PBS/BSA into a 0.65 ml tube containing 10 μl of extraction buffer (Arcturus, CA, USA). Contents of the tube were mixed by pipetting and then snap frozen and stored at -80°C until extraction. RNA extraction was performed using the Arcturus PicoPure RNA isolation kit with modifications (Arcturus, CA, USA). Each purification column extracted RNA from a combined ten oocytes of the same treatment group or single oocytes. Extracted RNA was stored at -80°C until further processing.

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RT-PCR and Quantitative real-time PCR

Samples for real-time PCR included the amplified RNA generated from the microarray experiment and/or total RNA extracted from single oocytes in each treatment group. RNA was converted to cDNA with the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics), according to manufacturer's instructions. Relative quantification was performed generating Ct values and normalized ratios, using light cycler software (Roche Diagnostics).

Experiment 1: Metabolism of in vivo and in vitro matured COCs recovered from FSH-treated cattle.

Forty six in vivo matured COCs and 92 immature COCs were collected from FSH-treated cattle by transvaginal aspiration. Recovered COCs were washed three times in G-MOPS + 5mg/ml HSA and placed into maturation medium (immature) or metabolic medium (in vivo matured). Immature COCs were matured in G maturation medium supplemented with 100 ng/ml EGF and 0.5 mM cysteamine. COCs were divided into two maturation groups: G maturation supplemented with 2.5 mg/ml Recombumin or G maturation supplemented with 20% fetal calf serum (FCS). All immature COCs were matured for 23 h at 38.5°C, 6% CO_2 in air in groups of ≤10 COCs/ 100 µl medium.

Metabolism of individual COCs was measured at 23 h maturation or immediately following recovery from the follicle for in vivo matured oocytes. For metabolic measurements, individual COCs from FSH-treated cows were incubated in a 1 µl drop of metabolic medium for 1 h, at 38.5°C, under 6% CO_2 in air.

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Experiment 2: Gene Expression of in vivo and in vitro matured COCs recovered from FSH-treated cattle.

Following metabolic analysis, mature COCs from experiment 1 were denuded of cumulus cells. RNA was extracted and amplified from single oocytes for real time PCR or groups of 10 oocytes for microarray analysis within each treatment group (in vivo; IVM: Recombumin; IVM: 20% FCS). Three replicates were performed for each treatment.

Microarray Analysis

Extracted RNA samples were sent to a core Affymetrix laboratory for microarray analysis (Health Sciences Center, University of Colorado). RNA was quantified using a nanodrop spectrophotometer, and RNA integrity was measured on an Agilent 2100 Bioanalyzer. Two rounds of RNA amplification, incorporating a T7 polymerase promoter, were performed followed by labeling and hybridization to a bovine-specific microarray according to manufacturer's instructions (GeneChip Bovine Genome Array; Affymetrix, Santa Clara, CA). As a quality control step, microarrays were validated with two genes using real time PCR.

Experiment 3: Metabolism and oxygen uptake of oocytes following maturation under 5% or 20% oxygen tension.

COCs were recovered from abattoir ovaries and matured for 23 h at 38.5°C, 6% CO_2, and either 5 or 20% O_2. Following in vitro maturation, metabolism of individual COCs and oxygen uptake of groups of COCs or denuded oocytes was assessed. For metabolic

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measurements, COCs recovered from abattoir ovaries were individually incubated in a 2 µl drop of metabolic medium for 1 h, at 38.5°C, under 6% CO_2 and either 5 or 20% O_2.

Measurement of oxygen uptake

Oxygen uptake was evaluated by COCs and denuded oocytes. The technique used to measure oxygen uptake was thoroughly described in Houghton et al. (1996). Briefly, 1 µl of oil containing 1mg/ml pyrene was loaded into a glass PCR capillary, followed by 2 µl of G-MOPS (37°C at 20% O_2) containing 2 COCs or 6 denuded oocytes. The capillary was sealed on both ends. The level of pyrene fluorescence was measured every 40 min for 160 min. Increased pyrene fluorescence correlated to increased oxygen uptake. Fluorescence was compared between COCs and denuded oocytes matured under different oxygen tensions.

Experiment 4: Gene expression of oocytes following maturation under 5% or 20% oxygen tension.

COCs were recovered from abattoir ovaries and matured for 23 h at 38.5°C under 5% O_2 or 20% O_2. Following maturation, cumulus cells were removed from oocytes. RNA was extracted from single oocytes (n=10) in each group and converted to cDNA. Two genes from the oxidative phosphorylation pathway showing differential gene expression were selected from experiment 2 (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the housekeeping gene. Primers were designed using the Roche Probe Design Software 2.0 (Roche Diagnostics). Real time PCR was performed on cDNA using Roche Faststart DNA master kit (Roche Diagnostics), according to

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manufacturer's instructions. Standard curves were made for each primer using dilutions of bovine reference RNA, generated from bovine ovaries.

Statistical Analysis

Metabolic data were analyzed by a one way ANOVA, and maturation rates were analyzed by Fisher's Exact test. Microarray data were analyzed by ANOVA and K cluster analysis. Real time PCR data were analyzed by normalized ratios.

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RESULTS

Experiment 1: Metabolism of in vivo and in vitro matured COCs recovered from FSH-treated cattle.

There was no difference between treatments in maturation rates of immature oocytes recovered from FSH-treated cattle: 72% versus 69%, Recombumin and 20% FCS, respectively. Glucose uptake was similar between both in vitro groups and the in vivo group; however, lactate production was lower for COCs matured in 20% FCS compared to in vivo matured COCs ($P<0.05$; Table 2). There was no difference in lactate production by COCs matured in defined maturation medium and in vivo matured COCs.

Experiment 2: Gene Expression of in vivo and in vitro matured COCs recovered from FSH-treated cattle.

Analysis of microarray data indicated that bovine oocytes expressed 11,561 transcripts out of the 24,128 total transcripts on the array. Analysis of variance revealed no statistical differences in gene expression between in vivo matured oocytes and either in vitro group (Recombumin or 20% FCS). Cluster analysis identified many genes having a ≥ 1.3 fold differential expression. When both IVM groups were compared to the in vivo group, 118 genes were upregulated and 160 were down regulated. When the 20% FCS group was compared to the in vivo group, 279 genes were upregulated and 272 genes were down regulated. From the cluster analysis, two pathways had multiple gene transcripts affected. In the oxidative phosphorylation pathway, one transcript was upregulated (succinate dehydrogenase flavoprotein) and two transcripts were down regulated (NADH dehydrogenase and cytochrome C oxidase) in IVM oocytes compared

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to in vivo oocytes (Table 3). An additional 3 transcripts were down regulated in the 20% FCS group (ESSS, ubiquinol cytochrome C reductase, and ATP synthase H+ transporting FO complex). In the MAPK pathway, 3 genes were upregulated (Rheb, PP1, and 14-3-3) and 2 genes were down regulated (MAPK1 and HSP70) in IVM oocytes compared to in vivo oocytes (Table 4). One additional transcript was upregulated (RGS3) and one transcript was down regulated (Tau) in the 20% FCS group. Microarray results were validated by analysis of two separate candidate genes using RT-PCR and real time PCR.

Experiment 3: Metabolism and oxygen uptake of oocytes following maturation under 5% or 20% oxygen tension.

Glucose uptake and lactate production were similar between COCs matured under 20% and 5% O_2 ($P>0.05$; Table 5). Oxygen consumption by COCs matured under 20% O_2 (n=54) was significantly higher than COCs matured under 5% O_2 (n=54; 2.19±0.2 vs. 1.23±0.2 nl/COC/h, respectively; $P<0.01$). However, there was no difference between the oxygen uptake by denuded oocytes matured under 20% (n=84) or 5% O_2 (n=84; 0.396±0.05 vs. 0.415±0.04, respectively).

Experiment 4: Gene expression of oocytes following maturation under 5% or 20% oxygen tension.

There was no difference in relative abundance of succinate dehydrogenase flavoprotein or NADH dehydrogenase between oocytes matured under 5 or 20% O_2.

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DISCUSSION

This study examined differences in the metabolism and gene expression between oocytes matured in vivo and oocytes matured under different in vitro conditions. Many factors in vitro are known to alter oocyte physiology; therefore, this study examined the effects of different components of maturation medium, as well as oxygen tension during maturation on oocyte physiology. Although differences were not noted in metabolism, gene expression was altered by in vitro maturation, which may explain the reduced developmental competence of in vitro matured oocytes relative to in vivo matured oocytes.

Maturation rates between COCs matured in the defined system supplemented with EGF and COCs matured in 20% FCS were similar, indicating that the defined system used in the present study was equally capable of supporting nuclear maturation as systems that utilize serum. It is perceived that inclusion of serum enhances maturation and subsequent development of bovine oocytes; however, inclusion of serum creates poorly defined conditions making it difficult to understand oocyte physiology clearly. In addition, pathological abnormalities have been associated with the use of serum (Thompson et al., 1995; Young et al., 1998; McEvoy et al., 2000; Khosla et al., 2001); therefore, it is imperative to develop and shift to a defined system capable of supporting both nuclear and cytoplasmic maturation.

There was no difference in glucose uptake between the two in vitro groups and the in vivo matured COCs; however, COCs matured in serum produced less lactate compared to

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in vivo COCs. Interestingly, in vivo matured oocytes converted 100% of glucose to lactate, whereas the two in vitro groups only converted approximately 70% of glucose to lactate. In vitro maturation may result in diverting glucose from glycolysis to alternative pathways, such as the pentose phosphate pathway (PPP) or oxidation. The majority of glucose uptake by the COC is via the cumulus, which metabolizes glucose into substrates that can be utilized by the oocyte. It is plausible that under in vitro conditions, cumulus cells convert more glucose to pyruvate, which then is directly transferred to the oocyte via gap junctions. Metabolic measurements taken in this study are indicative of cumulus physiology and suggest down regulation of glycolysis or a general reduction in metabolic activity by in vitro matured COCs.

Hundreds of genes were differentially expressed greater than 1.5 fold by in vitro matured oocytes compared to in vivo matured oocytes. Expression differences were not statistically significant, which may be a result of too few replicates. Though not statistically different, a doubling of transcript level may result in significant physiological effects within the oocyte. Two predominant pathways (oxidative phosphorylation and MAPK) that are critical to oocyte health appear to have been affected, as several genes in these pathways had altered expression compared to in vivo matured oocytes.

Oxidative phosphorylation is an important metabolic pathway for ATP production in the maturing oocyte. Over the course of maturation, pyruvate, glutamine and glycine metabolism increase in the bovine oocyte (Rieger and Loskutoff, 1994; Gandolfi et al., 1998). All three of these substrates are involved in oxidative metabolism, suggesting the

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importance of this pathway during maturation. Metabolic enzymes involved in oxidative phosphorylation, such as malate dehydrogenase, also increased during maturation in the rat (Tsutsumi et al., 1992). Furthermore, when inhibitors of oxidative phosphorylation are present, denuded rat oocytes arrested at the GV stage (Zeilmaker and Verhamme, 1974). During the process of maturation, the cytoplasm of the oocyte must provide enough cellular energy to support maturation, fertilization and early embryo development. The oocyte is able to achieve these high levels of energy production via oxidative phosphorylation. In addition, early embryos also rely on oxidative phosphorylation. Pyruvate is the dominant energy substrate until the 8-16 cell stage in the cow (Rieger et al., 1992; Tiffen et al., 1991). Therefore, if transcripts are down regulated following IVM, genes corresponding to these transcripts will also decrease during early embryonic development, when oxidative phosphorylation is critical.

Results of microarray analysis indicate that oxidative phosphorylation may be down regulated in oocytes matured in vitro with serum. The electron transport chain is composed of 4 complexes, plus ATP synthase. COCs matured in vitro had altered gene expression in all complexes, as well as ATP synthase, compared to in vivo matured oocytes (Figure 1). In complex I, NADH dehydrogenase was down regulated in both IVM groups. Additionally, the serum group had a down-regulation in ESSS. The absence of this recently identified protein in complex I results in inactivation of complex I (Potluri et al., 2004). In IVM oocytes succinate dehydrogenase flavoprotein was upregulated, while cytochrome C oxidase (Complex III and IV) was down regulated. In the serum groups an additional two genes were down regulated, ubiqinol cytochrome c

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reductase (complex III and IV) and ATP synthase H+ transporting FO complex. The down regulation of this pathway may indicate that less ATP is available to these oocytes for critical cellular functions. A possible cause of altered transcript level may be increased mitochondrial damage in IVM oocytes. Mitochondrial degeneration has been reported in embryos cultured with serum (Dorland et al., 1994), which would result in reduced oxidation and diminished requirement for transcripts involved in this pathway.

In addition to the oxidative phosphorylation pathway, alterations in gene expression were observed in the MAPK pathway. This pathway is essential for oocyte maturation, as well as subsequent fertilization and development (Liu and Yang, 1999). The MAPK pathway is responsible for activating factors involved in metaphase II arrest, as well as transcription factors required for gene activation and cell proliferation (Fissore et al., 1996). Three transcripts were upregulated in the IVM oocytes, with an additional transcript upregulated in the serum group (Figure 2). Three of the upregulated genes (RGS3, RHeb, and PP1) function to inhibit genes in the MAPK pathway; therefore, overexpression of these genes implies suppression of the pathway. The transcript for mitogen activated protein kinase (MAPK) was down regulated in both IVM groups. As MAPK is a major kinase in this pathway, its down regulation likely causes a decrease in activity of the pathway. The down regulation of this pathway did not prevent nuclear maturation of 70% of the IVM oocytes; however, the 30% of oocytes that failed to mature may have had more severe alterations in gene expression of the MAPK or other pathways. The MAPK pathway also is involved in both fertilization and embryo

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development; therefore, a reduction at the oocyte level would lead to reduced developmental competence, which is observed in IVM oocytes.

The second half of the study examined characteristics of COCs recovered from abattoir ovaries not exposed to exogenous FSH. Interestingly, metabolism of COCs recovered from abattoir ovaries was significantly higher than the metabolism of COCs recovered from superovulated cattle. FSH priming could potentially account for the difference between metabolism of COCs recovered from FSH-treated cows and abattoir ovaries. However, oocytes exposed to FSH in vivo are fundamentally different than oocytes that are recovered from the smaller follicles of abattoir ovaries containing immature oocytes. Another explanation may be the different number of cumulus cells between the two populations of COCs. Though cumulus cells were not counted, a greater number of cumulus layers were observed surrounding oocytes recovered from abattoir ovaries, than oocytes recovered from superovulated cows. As the majority of metabolic activity measured was likely due to the cumulus cells, higher numbers of cells would explain the discrepancy in carbohydrate metabolism.

There was no difference in metabolism between COCs matured under different oxygen tensions. Cumulus cells may be less affected by atmospheric oxygen than the oocyte. Additional studies are required to determine effects of oxygen on metabolism of the denuded oocyte. However, similar to in vivo matured oocytes in experiment 1, all glucose was accounted for by lactate production in COCs matured under either 20 or 5%

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O_2, indicating that the metabolic activity of the cumulus cells was similar to in vivo cumulus cells.

Oxygen consumption is required for ATP production via oxidative phosphorylation; however, excess oxygen leads to production of ROS, which is detrimental to cellular viability. There was no difference in oxygen uptake between denuded oocytes matured under 20 or 5% O_2, indicating similar capability within the oocyte for ATP production. However, COCs matured under 20% O_2 consumed significantly larger quantities of oxygen than COCs matured under 5% O_2. COCs matured under reduced oxygen tension were observed to have less cumulus expansion than COCs matured under atmospheric oxygen; therefore, the reduction in oxygen uptake may result from poor oxygen profusion through compacted cumulus cells. Reduced oxygen profusion in COCs matured under 5% O_2 would suggest that the oocyte is not receiving enough O_2, resulting in reduced respiration and developmental competence. Alternatively, as oocytes in both groups consumed similar levels of oxygen, the increased oxygen uptake by COCs in the 20% O_2 group may lead to the generation of ROS. Maturation medium is supplemented with antioxidants, and oocytes have intracellular stores of antioxidants, which serve to protect the oocyte from ROS; therefore, negative effects of maturation under atmospheric O_2 may not be immediately evident. However, maturation conditions that reduce ROS production will decrease the cellular energy required to respond to oxidative stress.

The expression level of succinate dehydrogenase flavoprotein and NADH dehydrogenase were analyzed in oocytes matured under different oxygen tensions. There was no

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difference in relative abundance of these two transcripts in oocytes matured at 20 or 5% O_2. However, as the study was limited to the examination of only two transcripts, it is unclear if this pathway was altered by maturation under different oxygen tensions. Further studies will be required to evaluate large scale differential gene expression between these two populations of oocytes.

In conclusion, COC and oocyte physiology were altered by in vitro maturation. The defined maturation system showed more similarities to in vivo matured oocytes, both in terms of metabolism and gene expression, than oocytes matured in 20% FCS. Differential gene expression in the oxidative phosphorylation and MAPK pathways provides some insight into the lower developmental competence of in vitro matured oocytes. Future experiments might examine gene transcripts from these pathways to determine effects of different IVM systems. It is evident that conditions of maturation alter oocyte physiology, and the development of an optimal maturation system will be facilitated by utilizing metabolic and gene expression markers of oocyte quality.

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Table 1. Forward and reverse primer sequences for genes quantified in experiment 4.

Gene	Primer	Sequence
GAPDH	Forward	ATGACCACTTTGGCATCG
	Reverse	CCATCCACAGTCTTCTGGG
Succinate Dehydrogenase Flavoprotein	Forward	TGCTCAGTACAAGTCCATCG
	Reverse	CGTCCAGTTTCTCACGG
NADH Dehydrogenase	Forward	CCGAGTTCTTCAGTGCG
	Reverse	TACCCAATTCCTCATCTGTCC

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Table 2. Metabolism of COCs following in vitro or in vivo maturation.

	Glucose Uptake pmol/COC/h	Lactate Production pmol/COC/h
Recombumin (n=40)	234±20	323±31 [a,b]
Serum (n=40)	188±17	269±26 [b]
In Vivo (n=40)	186±17	375±32 [a]

[a,b] Means within columns without common superscripts differ ($P<0.05$). Values are means±SEM.

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Table 3. Differential expression of genes involved in the oxidative phosphorylation pathway between in vitro matured bovine oocytes and in vivo matured bovine oocytes.

Gene	**Mean Expression Ratio**	
NADH Dehydrogenase	2.7	Downregulated in IVM compared to in vivo
succinate dehydrogenase flavoprotein	1.5	Upregulated in IVM compared to in vivo
ESSS	1.4	Downregulated in Serum compared to in vivo
ubiquinol cytochrome c reductase	1.3	Downregulated in Serum compared to in vivo
cytochrome c oxidase	4.1	Downregulated in IVM compared to in vivo
atp synthase h+ transporting FO complex	1.7	Downregulated in Serum compared to in vivo

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Table 4. Differential expression of genes involved in the MAPK pathway between in vitro and in vivo matured bovine oocytes.

MAPK kinase	**Mean Expression Ratio**	
RGS3	1.7	Upregulated in Serum compared to in vivo
Rheb	2.1	Upregulated in IVM compared to in vivo
PP1	1.6	Upregulated in IVM compared to in vivo
erk 1/2	3.2	Downregulated in IVM compared to in vivo
tau	1.3	Downregulated in Serum compared to in vivo
hsp70	3.3	Downregulated in IVM compared to in vivo

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Table 5. Metabolism of COCs recovered from 3-6 mm follicles following in vitro maturation under 20% or 5% oxygen tension.

	Glucose Uptake	Lactate Production
20% O_2	759±32	1633±168
5% O_2	815±29	1949±167

Values are represented as means±SEM.

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FIGURE LEGENDS

Figure 1. Gene transcripts altered by more than 1.3 times in the oxidative phosphorylation pathway in IVM oocytes compared to in vivo matured oocytes. Transcripts denoted in red were upregulated. Transcripts denoted in green were down regulated. *Transcripts with differential gene expression in serum group only.

Figure 2. Gene transcripts altered by more than 1.3 times in the MAPK pathway in IVM oocytes compared to in vivo matured oocytes. Transcripts denoted in red were upregulated. Transcripts denoted in green were down regulated. *Transcripts with differential gene expression in serum group only.

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Figure 1.

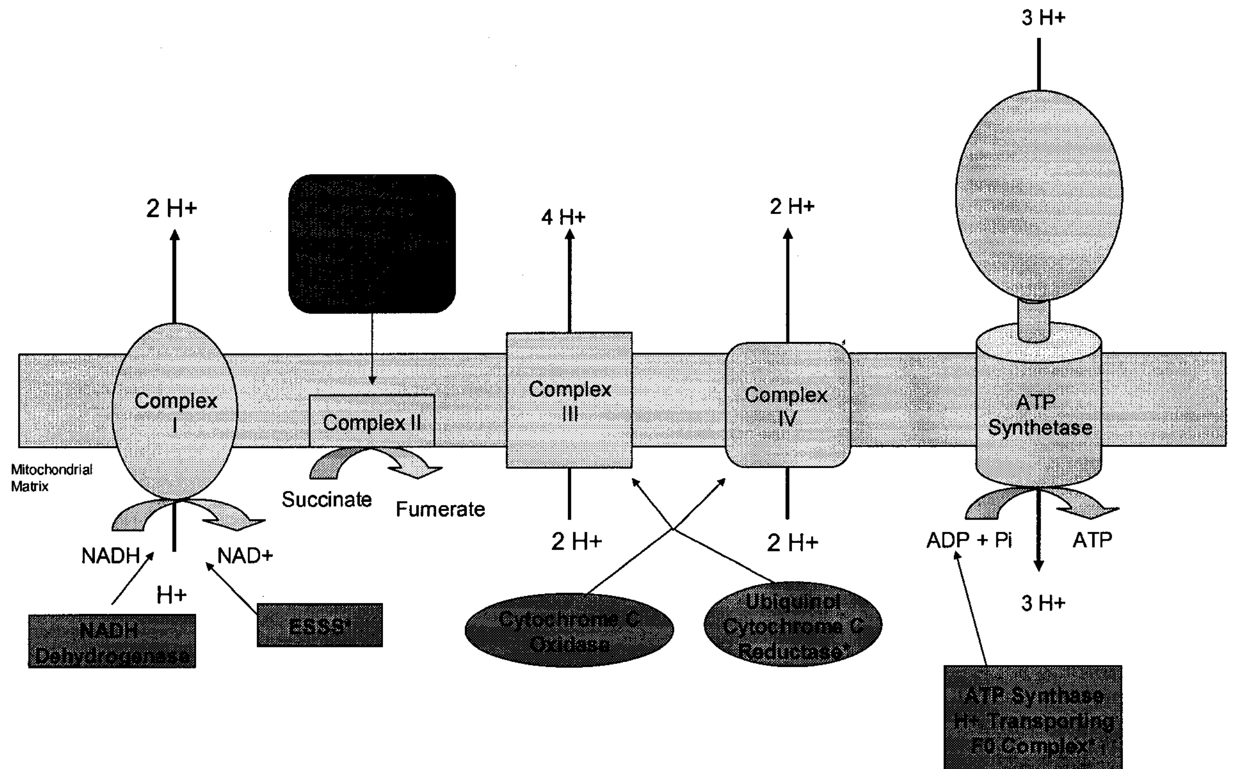

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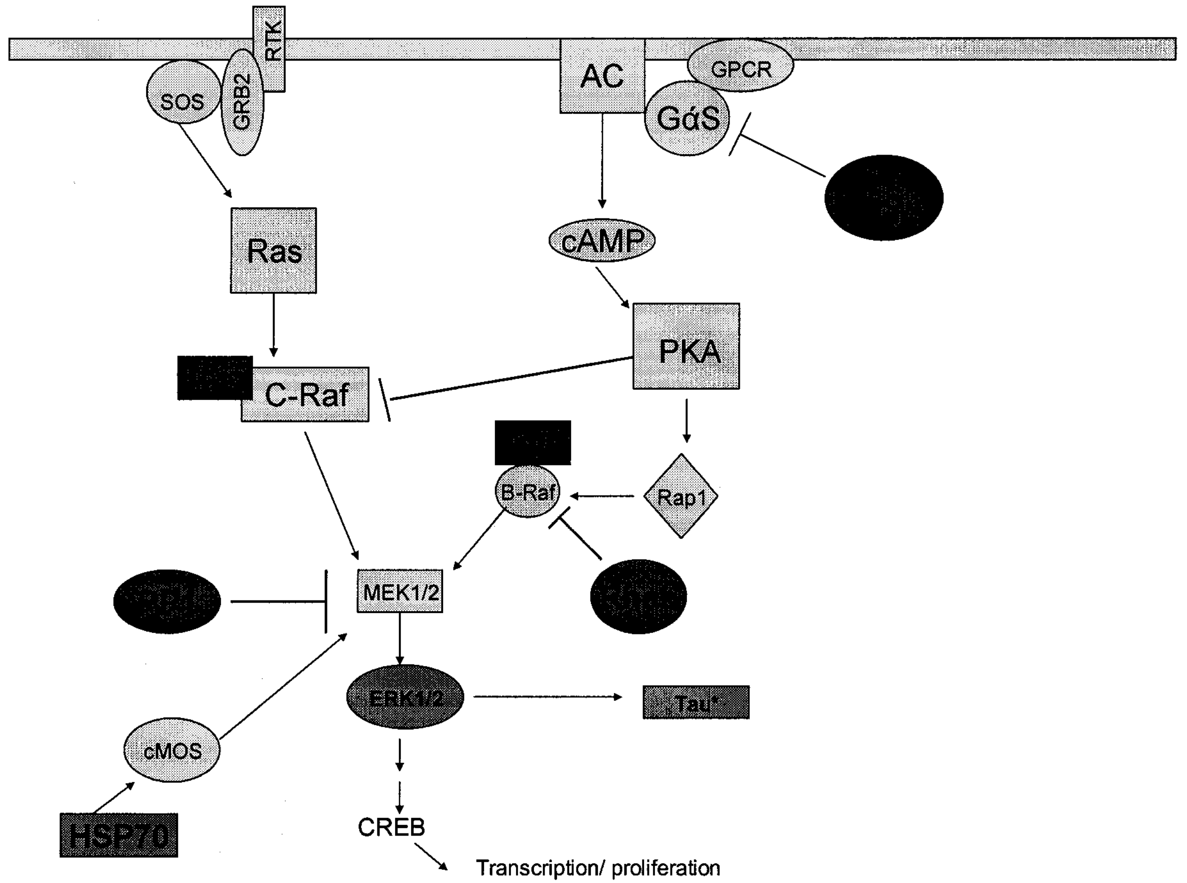
Figure 2.



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CHAPTER VIII

SUMMARY

The studies described within this thesis were designed to enhance knowledge of oocyte physiology during in vitro maturation. The main goal was to optimize a defined maturation system capable of supporting meiotic and developmental competence of immature oocytes. This was achieved in murine and bovine models, with maturation and developmental rates equivalent to systems including blood serum. This defined maturation system permitted evaluation of various maturation conditions. Assessment of carbohydrate and oxygen metabolism, mitochondrial membrane potential, and gene expression indicated that conditions of in vitro maturation impact oocytes on a physiological and molecular level relative to in vivo matured oocytes.

The use of quantitative markers increased our understanding of oocyte physiology during in vitro maturation. Metabolic profiles of developmentally competent mouse oocytes changed throughout maturation; glucose uptake and lactate production increased over the final 8 h of maturation. The increased metabolic activity by competent COCs during this time may reflect the need to support various processes associated with completion of nuclear and cytoplasmic maturation. These results not only provided valuable time points of metabolic assessment for subsequent studies, but also revealed that glucose and lactate metabolism were potential markers of oocyte developmental competence.

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A defined maturation system and established metabolic criteria allowed assessment of the specific effects of medium supplements, as well as culture conditions in terms of oocyte maturation and subsequent development of embryos after fertilization. Evaluation of in vivo matured oocytes provided a baseline comparison with respect to in vitro matured oocytes. In the cow, the inclusion of serum increased lactate production of in vitro matured COCs compared to in vivo matured COCs and COCs matured with recombinant human serum albumin. In addition, maturation of COCs in the presence of serum resulted in altered metabolic activity of subsequent embryos, indicating that conditions of maturation impact quality of resulting embryos. In the mouse, EGF significantly increased metabolic activity of COCs recovered from both eCG-stimulated and unstimulated donors. As metabolism of in vivo matured COCs was higher than that of in vitro matured COCs, supplementation of IVM medium with EGF altered metabolism in a manner more consistent with in vivo matured COCs. Supplements to base medium clearly impact oocyte physiology and subsequent embryo quality, and results of these studies indicate that supplementation with recombinant human serum albumin and EGF enhances developmental competence of in vitro matured oocytes.

In addition to medium supplementation, effects of oxygen tension (20% or 5% O_2) during maturation were assessed. Interestingly, oxygen tension did not alter carbohydrate metabolism in bovine COCs recovered from ovaries of abattoir cattle; however, reduced oxygen tension significantly increased metabolic activity of murine COCs recovered from unstimulated mice. In addition, mouse oocytes matured under low O_2 produced

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embryos of higher quality than oocytes matured under high O_2, again confirming that conditions of maturation clearly affect embryo quality.

Oxygen utilization also differed between mouse and cow oocyte, as maturation under 20% O_2 resulted in reduced oxygen uptake by denuded mouse oocytes; however, no difference in oxygen uptake was observed between denuded bovine oocytes matured under 20% or 5% O_2. As oxygen uptake is associated with ATP production, it appears that atmospheric concentrations of oxygen during maturation may reduce ATP production in mouse oocytes, but not bovine oocytes. This was further evidenced by decreased mitochondrial membrane potential in denuded mouse oocytes following IVM under 20% O_2 compared to 5% O_2. Clearly, oocyte requirements during IVM differ between species; therefore, to optimize IVM conditions, baseline measurements of in vivo matured oocytes from the species of interest will be required. However once baseline measurements are established, results of studies in this thesis indicate that non-invasive metabolic assessment can potentially serve as a marker to distinguish developmentally competent from less viable oocytes.

The development of reliable oocyte viability markers will augment existing approaches to determine the effectiveness of oocyte maturation protocols. Although fertilization and embryo culture and embryo transfer will still be required to assess the quality of a maturation system, markers of oocyte developmental competence can be implemented in preliminary screening of systems, thereby reducing cost and time necessary for development of an optimal maturation system. Viability markers could also be utilized

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by pharmaceutical companies developing protocols for exogenous ovarian stimulation, in order to assess the impact of their product on oocyte physiology. In addition, for clinical laboratories and breeding programs, non-invasive techniques will provide the opportunity to assess oocyte viability without compromising fertilization and development. Non-invasive techniques for evaluating oocytes, followed by established techniques to evaluate embryo viability, will greatly enhance selection of the most viable embryos for transfer. In animal programs, the selection of healthy embryos will reduce the number of recipients required and increase the percentage of successful transfers. In human infertility clinics, selection of viable embryos for transfer will lead to increased numbers of successful single embryo transfers, and ultimately eliminate the risk of multiple pregnancies.

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APPENDICES

Appendix A

Plastic-ware for murine research

Murine Procedures	Plastic-ware
COC Recovery	Center Well Organ Culture Dish (Falcon, 3037)
	35x10 mm Tissue Culture Dish (Falcon, 3001)
COC Maturation, Fertilization and Embryo Culture	60x15mm In Vitro Fertilization Dish (Falcon, 3652)
Metabolic Incubation	60x15mm In Vitro Fertilization Dish (Falcon, 3652)

Plastic-ware for bovine research

Bovine Procedures	Plastic-ware
COC Recovery	35x10 mm Tissue Culture Dish (Falcon, 3001)
	60x15mm Tissue Culture Dish (Falcon 3004)
COC Maturation, Fertilization and Embryo Culture (Exp 6,7,8)	60x15mm Tissue Culture Dish (Falcon 3004)
COC Maturation, Fertilization and Embryo Culture (Exp 9)	4 Well Nunc Dish
Metabolic Incubation	60x15mm In Vitro Fertilization Dish (Falcon, 3652)

Falcon, Becton Dickenson, Franklin Lakes, NJ, USA
Nunc, Kamstrupvej, Denmark

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APPENDIX B

G2 Metabolism Medium

Stock	Component		Volume for 10 ml Medium
H2O			7.1
A	105.08 mM	NaCL	1
	5.5 mM	KCl	
	0.25 mM	NaH2PO4H2O	
	1.8 mM	MgSO4-7H2O	
	0.5 mM	Glucose	
	0.06 g/l	Penicillin-G	
	100 ml	H20	
B	25 mM	NaHCO3	1
	0.0005 g/l	Phenol Red	
	100 ml	H20	
C	0.23 mM	Na Pyruvate	0.1
	10 ml	H2O	
D	1.0 mM	CaCl2-2H2O	0.1
	10 ml	H2O	
G	1.0 mM	Alanyl-Glutamine	0.1
	10 ml	H2O	
N	Non-Essential MEM Solution		0.1
ESS	Essential AA MEM Solution		0.1
V	0.0042 mM	D-Ca Pantothenate	0.1
	0.0049 mM	Pyridoxal-HCL	
	0.00296 mM	Thiamine-GCl	
	0.000265 mM	Riboflavin	
	10 ml	H2O	

Add stocks in the order in which they appear.
Osmol Spec: 255±6
pH Spec: 8.00±0.05

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APPENDIX C

Preparation of pyruvate buffer, reagent and standard for pyruvate assay.

EPPS Buffer: 2.52g *N*-(2-Hydroxyethyl)piperazine- *N'*- (3-propanesulfonic acid; Epps) in 150 ml H_2O
pH to 8.0 with 1M NaOH
Dilute to 200ml
10 mg penicillin-G
10 mg streptomycin sulfate

5 mM NADH 17.73 mg in 5 ml H_2O

Preparation of Pyruvate Cocktail:
14 ml EPPS buffer
0.3 ml 5mM NADH solution
0.4 ml Lactate Dehydrogenase (LDH; 5mg/ml)

500 µl aliquots of Pyruvate Cocktail were stored in Eppendorf tubes for up to 3 m at -80°C.

Preparation of 1 mM standard:
0.0110g pyruvate to 100 ml H_2O

** NADH and LDH were purchased from Roche Diagnostic, Indianapolis, IN, USA

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Preparation of glucose buffers, reagent, and standard for glucose assay.

EPPS Buffer:	2.52g *N*-(2-Hydroxyethyl)piperazine- *N'*- (3-propanesulfonic acid; Epps) in 150 ml H_2O pH to 8.0 with 1M NaOH Dilute to 200ml 10 mg penicillin-G 10 mg streptomycin sulfate
5 mM Dithiothreitol (dtt)	7.72 mg in 10 ml H_2O
37 mM $MgSO_4$	91.2 mg in 10 ml H_2O
10 mM ATP	30.3 mg in 5 ml H_2O
10 mM NADP+	39.4 mg in 5 ml H_2O
Glucose Cocktail	15 ml EPPS buffer 2 ml 5 mM dtt 2 ml 37 mM $MgSO_4$ $7H_20$ 1 ml 10 mM ATP 3 ml 10 mM NADP+ 1 ml Hexokinase/G6PDH (3mg/ml)

500 μl of Prepared glucose cocktail was stored in Eppendorf tubes for up to 3 m at -80°C.

Glucose Standard:

0.0180 g glucose in 100 ml H_2O

** ATP, NADP+, Hexokinase/G6PDH were purchased from Roche Diagnostic, Indianapolis, IN, USA.

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Preparation of lactate buffers, reagent and standard for lactate assay.

Glycine/ Hydrazine Buffer	7.5 g Glycine 5.2 g Hydrazine 0.2 g EDTA
	Suspend in 10 ml H_2O, add 51 ml 2M NaOH
	Dilute to 100 ml
NAD+	40 mg NAD+ 1 ml H_2O

75 µl aliquots of NAD were frozen in Eppendorf tubes for up to 3 m at -80°C.

Preparation of Lactate Cocktail (day of assay)

75 µl NAD+
0.45ml glycine/hydrazine buffer
0.4 ml H_2O
25 µl Lactate Dehydrogenase (LDH; 5mg/ml)

**NAD+ and LDH were purchased from Roche Diagnostic, Indianapolis, IN, USA.

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APPENDIX D

Protocol for RNA extraction (Acturus Pico Pure kit with modifications)

Components needed for procedure: Frozen oocyte samples in extraction buffer
Conditioning buffer
70% Ethanol
Wash Buffer I
Wash Buffer II
Elution Buffer
RNA purification column with collection tubes (one per sample)
Microcentrifuge tubes

1. Incubate samples at 42°C for 30 min
2. Pipette 250 µl conditioning buffer onto the filter of the purification column
    a. Incubate for 5 min
    b. Centrifuge at 16,000g for 1 min
3. Add 10 ul of 70% EtOH to each sample and pipette to mix.
4. Pipette sample/EtOH mixture directly onto the filter of the purification column
    a. Centrifuge at 100g for 2 min
    b. Centrifuge at 16,000 for 30 sec
5. Add 100 µl wash buffer I onto the filter.
    a. Centrifuge at 8,000g for 1 min
6. DNase treat each sample.
    a. Add 5 µl DNase stock to 35 µl buffer RDD
    b. Add 40 µl DNase mixture onto each filter
    c. Incubate for 15min
    d. Add 40 µl wash buffer I
    e. Centrifuge at 8,000g for 15 sec
7. Add 100 µl wash buffer II onto the filter
    a. Centrifuge at 8,000g for 1 min
8. Add another 100 µl wash buffer II to the filter.
    a. Centrifuge at 16,000g for 2 min
9. Transfer column to a new 0:5 ml tube (tubes were labeled with sample number and treatment group)
10. Add 20 µl elution buffer onto each filter.
    a. Incubate for 1 min
    b. Centrifuge at 1,000g for 1min
    c. Centrifuge at 16,000g for 1 min
11. Freeze RNA samples at -80°C.

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APPENDIX E

Protocol for Transcriptor First Strand cDNA Synthesis kit

Kit Components required for procedure: Frozen samples of extracted RNA
Water
Transcriptor RT Reaction Buffer
dNTPs
Oligo dT primer
RNase Inhibitor
Reverse Transcriptase
Microcentrifuge tubes

1. Thaw all components and put them on ice. Centrifuge everything before starting.
2. Make mastermix (20 µl mastermix for each sample)
    a. 4 µl H2O
    b. 8 µl Transcriptor RT Reaction Buffer
    c. 4 µl dNTPs
    d. 2 µl Oligo dT primer
    e. 1 µl RNase Inhibitor
    f. 1 µl Reverse Transcriptase
    g. Total volume = 20 µl (multiple volume of each component by number of samples)
3. Aliquot 20 µl mastermix into each tube
4. Add 20 µl RNA to each tube containing mastermix
5. Mix well by pipetting, spin briefly
6. Incubate 30 min at 55°C.
7. Inactivate by heating to 85°C for 5 min

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Appendix F

Protocol for Real Time PCR (Light Cycler FastStart DNA Master SYBR Green I Kit

Step	Action
1	Thaw vial of Reaction Mix and shield from light
2	Pipette 14 ul from enzyme vial into Reaction Mix (Creates Master Mix)
3	Mix master mix by pipetting, shield from light
4	In separate vials, mix 12 ul H2O, 2 ul Primer, 4 ul Master Mix (Multiply volumes by number of samples)
5	In a separate PCR capillary tube for each sample, add 18 ul of mix from step 4
6	Add 2 ul of cDNA to PCR capillary tube and mix by pipetting
7	Seal each capillary and place in light cycler carousel and centrifuge
8	Place carousal in light cycler

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GAPDH

Cycle	Analysis Mode	Cycles	Segment	Target Temp	Hold Time	Acquisition Mode
Pre-incubation	None	1		95	10 min	none
Amplification	Quantification	45	Denaturation	95	10 sec	none
			Annealing	60	5 sec	none
			Extension	72	3 sec	single
Melting Curve	Melting Curves	1	Denaturation	95	0	none
			Annealing	65	15 sec	none
			Extension	95	0	continuous
Cooling	None	1		40	30 sec	none

Succinate Dehydrogenase

Cycle	Analysis Mode	Cycles	Segment	Target Temp	Hold Time	Acquisition Mode
Pre-incubation	None	1		95	10 min	none
Amplification	Quantification	45	Denaturation	95	10 sec	none
			Annealing	59	5 sec	none
			Extension	72	4sec	single
Melting Curve	Melting Curves	1	Denaturation	95	0	none
			Annealing	65	15 sec	none
			Extension	95	0	continuous
Cooling	None	1		40	30 sec	none

NADH Dehydrogenase

Cycle	Analysis Mode	Cycles	Segment	Target Temp	Hold Time	Acquisition Mode
Pre-incubation	None	1		95	10 min	none
Amplification	Quantification	45	Denaturation	95	10 sec	none
			Annealing	58	5 sec	none
			Extension	72	5 sec	single
Melting Curve	Melting Curves	1	Denaturation	95	0	none
			Annealing	65	15 sec	none
			Extension	95	0	continuous
Cooling	None	1		40	30 sec	none

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