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Regulation of oocyte meiotic resumption using cAMP modulators in bovine in vitro maturation

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REGULATION OF OOCYTE MEIOTIC RESUMPTION USING cAMP MODULATORS IN BOVINE *IN VITRO* MATURATION

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for degree of
Master of Science

in

The Interdepartmental Program of
Animal and Dairy Sciences

by

Sarah Emilie Farmer

B.S., University of Louisiana at Lafayette, 2011

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LIST OF ABBREVIATIONS

AC	adenylate cyclase
ART	assisted reproductive technology
ATP	adenosine triphosphate
BCS	bovine calf serum
BMP15	bone morphogenic protein 15
cAMP	3',5'-cyclic adenosine monophosphate
CCs	cumulus cells
cGMP	cyclic guanosine monophosphate
COC	cumulus-oocyte complex
dbcAMP	dibutyl cyclic adenosine monophosphate
D-PBS	Dulbecco's phosphate-buffered saline
EGF	epidermal growth factor
FBS	fetal bovine serum
FSK	Forskolin
GCs	granulosa cells
GDF9	growth differentiation factor 9
GJC	gap junctional communication
GnRH	gonadotropin-releasing hormone
GPR3	g-protein receptor 3
GV	germinal vesicle
GVBD	germinal vesicle breakdown
IBMX	3-isobutyl-1-methylxanthine
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IVC	<i>in vitro</i> culture
IVF	<i>in vitro</i> fertilization
IVM	<i>in vitro</i> maturation
IVP	<i>in vitro</i> embryo production
LH	luteinizing hormone
MI	metaphase I
MII	metaphase II
MAPK	mitogen-activated protein kinase
MOET	multiple ovulation and embryo transfer
MPF	maturation promoting factor
OPU	ovum pick-up
OSFs	oocyte secreted factors
PB	polar body
PDE	Phosphodiesterase
PGCs	primordial germ cells
PKA	protein kinase A
TE	Trophectoderm
TGF- β	transforming growth factor beta
TUGA	transvaginal ultrasound-guided aspiration

ABSTRACT

In vitro maturation (IVM) is a reproductive technique critical to *in vitro* embryo production (IVP) in commercial livestock industries, research, and human infertility treatment. Currently, IVP has low efficiency due to an inadequate IVM system in which premature meiotic resumption results in low oocyte viability. Meiotic arrest is regulated primarily by 3',5'-cyclic adenosine monophosphate (cAMP), and the most successful methods of improving IVM utilize cAMP modulators to maintain high intra-oocyte cAMP, delaying the onset of maturation. This thesis includes experiments comparing standard bovine IVM to a novel extended IVM method similar to the procedure described by Albuz and colleagues (Albuz et al., 2010). Bovine oocytes were obtained from mixed breed cattle by transvaginal ultrasound-guided aspiration. Oocytes from each cow were divided into two groups: standard IVM and extended IVM. Standard IVM consists of a 23-hour maturation composed of TCM-199 based media supplemented with 10% fetal bovine serum, sodium pyruvate, pen/strep, glutamine, and FSH, and cultured in 5% CO₂ at 39°C. Extended IVM is composed of two steps: a pre-IVM of HEPES-TALP supplemented with 100 µM forskolin (FSK) and 500 µM 3-isobutyl-1-methylxanthine (IBMX) for 2 hours at 39°C, and then an extended IVM consisting of standard maturation medium supplemented with 20 µM cilostamide for 31 hours (5% CO₂, 39°C). Oocytes were sampled at various times throughout maturation depending on the experiment. Data was collected either by staining with aceto-orcein to determine nuclear status or by a cAMP ELISA after freezing in groups of ten. Data from the initial experiments showed that cAMP modulators significantly delayed maturation, but overall maturation rates were significantly less than standard IVM (44.5% vs. 81%). Results of the cAMP assay indicated a significant increase in cAMP within the first three hours of oocyte collection after using FSK and IBMX in collection media, but cAMP was not maintained in the cilostamide-only extended IVM medium. Additionally, cilostamide may have had a negative effect on the oocytes since there was a higher percentage arrested at MI in extended IVM.

CHAPTER I

INTRODUCTION

In vitro maturation (IVM) is a reproductive technology whereby oocytes are harvested from follicles in the ovary and matured in a laboratory setting. IVM is an integral part of clinical embryo production. *In vitro* embryo production (IVP) involves three steps: IVM, *in vitro* fertilization (IVF), and *in vitro* culture (IVC). It is widely known that human fertility clinics practice these assisted reproductive technologies (ARTs) to provide infertile women with children. However, IVP is also used extensively in commercial livestock production industries, especially cattle.

IVP is a valuable tool for commercial livestock production because it increases the number of offspring that can be produced from genetically valuable animals. For example, a cow can produce only one calf per year. However, multiple oocytes can be collected from the ovary of this cow, matured *in vitro*, and fertilized to produce many embryos. These embryos can then be transferred into recipient cattle. Therefore, IVP has the advantage that it can produce numerous offspring per year from one valuable animal.

Additionally, IVM can also be utilized in a number of other research and commercial technologies including nuclear transfer (cloning) research, preservation of endangered species, transgenic animal production, and human infertility treatment. IVM is important for human IVF because it would avoid the use of potentially harmful drugs to stimulate ovarian function. IVF for women usually requires treatment for up to a month with high doses of hormones to acquire the optimum number of mature oocytes. This can cause ovarian hyperstimulation syndrome (OHSS), which in severe cases can result in life-threatening complications such as blood clots and kidney failure. Additionally, these drugs are very expensive, and are painful and difficult to administer. Superovulatory drugs are also used in livestock to increase ovarian production. Avoidance of these stimulatory hormones is beneficial here as well, allowing decreased risk of

harmful effects, decreased handling of the animals, and decreased costs from the drugs and the labor to administer them.

IVM has the potential to alleviate these problems alleviating the need for ovarian stimulation, as oocytes could be properly matured *in vitro*. Unfortunately, the current IVM system in cattle is inadequate, and the use of IVM in human infertility treatment is almost non-existent due to poor results. Current literature suggests that *in vitro* matured oocytes produce about half as many embryos as *in vivo* matured oocytes after IVF.

Initiation of maturation in mammalian oocytes is primarily controlled by 3',5'-cyclic adenosine monophosphate (cAMP). Within the ovary, the oocytes are held in an arrested state, by high levels of cAMP. However, once an oocyte is removed from the follicular environment, cAMP levels decrease and the oocyte spontaneously begins to mature. This spontaneous nuclear maturation occurs too quickly and is completed before cytoplasmic maturation, resulting in less viable oocytes for embryo production.

The overall goal of this research is to determine whether modifications to the current *in vitro* maturation system can improve success rates of oocyte maturation using a bovine model. The idea is to delay spontaneous nuclear maturation in order for cytoplasmic maturation to occur concurrently, resulting in a more viable oocyte. Therefore, we propose that the addition of cAMP modulators to the maturation media may increase cAMP levels in the oocyte, delaying maturation and extending the time required for IVM. This would ideally increase oocyte viability, producing more embryos per oocyte collected. Additionally, the use of a bovine model for oocyte maturation may eventually be applied to human embryo IVP in order to avoid ovarian hyperstimulation syndrome and lessen the financial burden of infertility treatments.

CHAPTER II LITERATURE REVIEW

Oocyte Development and Folliculogenesis

The development of an oocyte begins in the fetus and is called “oogenesis”. Oocytes originate from primordial germ cells (PGCs) that are derived from the inner cell mass (ICM) of a developing blastocyst. Once formed, these PGCs migrate from the epithelium of the yolk sac to the gonadal ridges of the mesonephros in the early embryo to occupy the primitive ovary (Byskov, 1986). The fetus’s PGCs will differentiate and undergo mitosis to replicate. Now called oogonia, they will multiply from only a few thousand cells to millions. Once these cells enter meiosis, they develop into primary oocytes. In cattle, this occurs around day eighty-two of fetal development (Billodeau-Goeseels, 2011). These primary oocytes do not progress completely through meiosis but instead are arrested at prophase I, called the dictyate stage. This arrested state is maintained by the follicular environment surrounding the oocyte and continues until the animal begins puberty, at which point a group of oocytes are recruited during each estrous cycle to resume meiosis and complete development.

Meiotic arrest of the oocyte is the ovary’s method of storage, and the oocytes will remain in this paused state until ovulation. For example, by five months of gestation a human female fetus has made all of the oocytes she will ever have (about seven million). At birth this number has been reduced to about two million, and by puberty she will have only three hundred to four hundred thousand oocytes remaining. Because ovulation usually includes one oocyte each month during her reproductive life, there is an apparent mechanism to prevent the remaining oocytes from maturing at the same time. Therefore the ovary stores these oocytes in the arrested dictyate stage to keep them from maturing until needed for ovulation.

The initiation of meiosis in the primary oocyte occurs at the same time as the onset of follicle development, or folliculogenesis. Before the follicle is formed, there is a colonization of

mesonephric cells in the ovary of the fetus. These mesonephric cells surround the primary oocyte and may be a precursor to follicular cells, called pre-granulosa cells. Each oocyte is enclosed in a single layer of these pre-granulosa cells, forming primordial follicles. Any primary oocyte that lacks a primordial follicle will degenerate. The follicle now serves to maintain a controlled environment for and to protect the oocyte (Picton, 2001), as well as to provide an inhibitory environment for the maintenance of meiotic arrest. During the course of folliculogenesis, the oocyte will gradually and sequentially obtain meiotic and developmental competence (Eppig, 1992; Longergan et al., 1994; Schramm and Bavister, 1995; Gilchrist et al., 1997). It is during this phase of oogenesis that the oocyte acquires the cytoplasmic and molecular machinery necessary to support embryo development (Brevini-Gandolfi and Gandolfi, 2001; Sirard et al., 2006).

***In Vivo* Oocyte Maturation**

In vivo oocyte maturation describes the signals, pathways, and processes involved in the production of a viable oocyte within the body. It also involves the expansion of the surrounding cumulus cells (CCs) and maturation of the cytoplasm to support fertilization and embryo development. Oocytes must grow and differentiate inside the ovary before they become developmentally competent, which is defined as the ability of the oocyte to resume and complete meiosis, divide, develop into a healthy embryo, induce a pregnancy, and produce healthy offspring. Maturation is the process whereby the oocyte gains this developmental competence, and once it is completed these oocytes are ovulated and can be fertilized to produce a viable embryo.

Oocyte maturation involves the resumption of meiosis from Prophase I, a germinal vesicle (GV) stage oocyte, to the extrusion of the first polar body and metaphase II (MII). Once meiosis resumes, the arrested GV oocyte undergoes Germinal Vesicle Breakdown (GVBD),

then progresses through Metaphase I (MI), Anaphase I (AI), Telophase I (TI), and MII sequentially. The final stage, MII, is a fully matured oocyte ready for fertilization.

As previously stated, the follicular environment keeps oocytes arrested in the dictyate stage within the ovary, and this arrest is necessary for oocyte storage. But what is inside the follicle that keeps the oocyte arrested, and how is this inhibitory affect reversed in follicles recruited for development? Before an oocyte can be fertilized, it must resume meiosis, complete maturation, and be ovulated. In the past decade, there have been many advances into the understanding of the interactions between the oocyte, the surrounding CCs, and the follicular cells. These advances have resulted in a greater knowledge of the processes of maturation *in vivo*.

It was first observed in rats that meiosis resumes after a drop in cyclic adenosine 3', 5'-monophosphate (cAMP) within the oocyte, and that maturation will subsequently take place. cAMP appears to be a regulator of gap junctional communication (Dekel and Beers, 1978; Dekel and Beers, 1980). Poor bovine oocyte development has been linked to low cAMP levels, which cause a premature interruption in gap junctional communication (GJC) (Modina et al., 2001). These gap junctions connect the oocyte to its surrounding follicular cells, allowing for direct communication (Gilula et al., 1978). Gap junctions are channels inside the cell membrane which consist of a hexamer of proteins called connexin. These channels permit the transfer of small molecular weight molecules (less than 1,000 Da) such as ATP, metabolites, amino acids, sodium, chloride, calcium, and of course cAMP, all of which assist in the growth and differentiation of the oocyte and CCs (Ozawa et al., 2008).

It is apparent that cAMP is an important player in oocyte maturation, so intracellular regulation of cAMP is of interest. cAMP levels are regulated by two types of enzymes: adenylate cyclases (AC) and phosphodiesterases (PDE). Adenylate cyclases generate cAMP from adenosine triphosphate (ATP), and PDEs degrade cAMP via hydrolysis. cAMP itself is a vital step in a signaling cascade that maintains oocyte meiotic arrest, as it phosphorylates the

cAMP-dependent protein kinase A (PKA) (Figure 2.1). PKA in turn inhibits maturation promoting factor (MPF) (Han and Conti, 2006; Downs, 2010; Luciano et al., 2011), which controls the mitotic and meiotic cell cycles. MPF is composed of a catalytic p34Cdc2 kinase subunit and the Cyclin B regulatory subunit. PKA inhibits MPF by directly regulating a kinase and phosphatase for the catalytic Cdc2 kinase subunit. Another important player in oocyte maturation is mitogen-activated protein kinases (MAPKs). The inhibition of MAPK will arrest bovine oocytes in the germinal vesicle (GV) stage (Tian et al., 2002; Fan and Sun, 2004; Marei et al., 2009). MAPKs are also involved in regulating microtubule dynamics, especially in the maintenance of metaphase organization (Alberts et al., 1994, Tian et al., 2002), and have a role in initiating translation in bovine oocytes. MAPK interacts with other protein kinases such as MPF, PKA, protein kinase C, and calmodulin-dependent protein kinase II, as well as with protein phosphatases in meiotic cell cycle regulation, and these extensive interactions are essential for proper oocyte maturation to ensue (Fan and Sun, 2004; Marei et al., 2009).

There are two locations where cAMP is generated endogenously: within the oocyte and within the surrounding somatic cells of the follicle. Intra-oocyte cAMP is generated by active G protein-coupled receptor type 3 (GPR3) (Mehlmann, 2002; Yang et al., 2012) which activates adenylyl cyclases. The accumulation of cAMP is stimulated in early antral follicles by FSH, which binds to receptors located on the surface of the CCs and granulosa cells. The binding of FSH to its receptor activates heterotrimeric G proteins in these cells, which in turn stimulates adenylyl cyclase to produce cAMP (Ozawa, 2008).

Following cAMP production in the surrounding CCs, it is transported into the oocyte via gap junctions (Anderson and Albertini, 1976). These CCs also supply cyclic guanosine monophosphate (cGMP) to the oocyte (Tornell et al., 1991), which inhibits PDE activity and thereby decreases the amount of cAMP degraded in the oocyte.

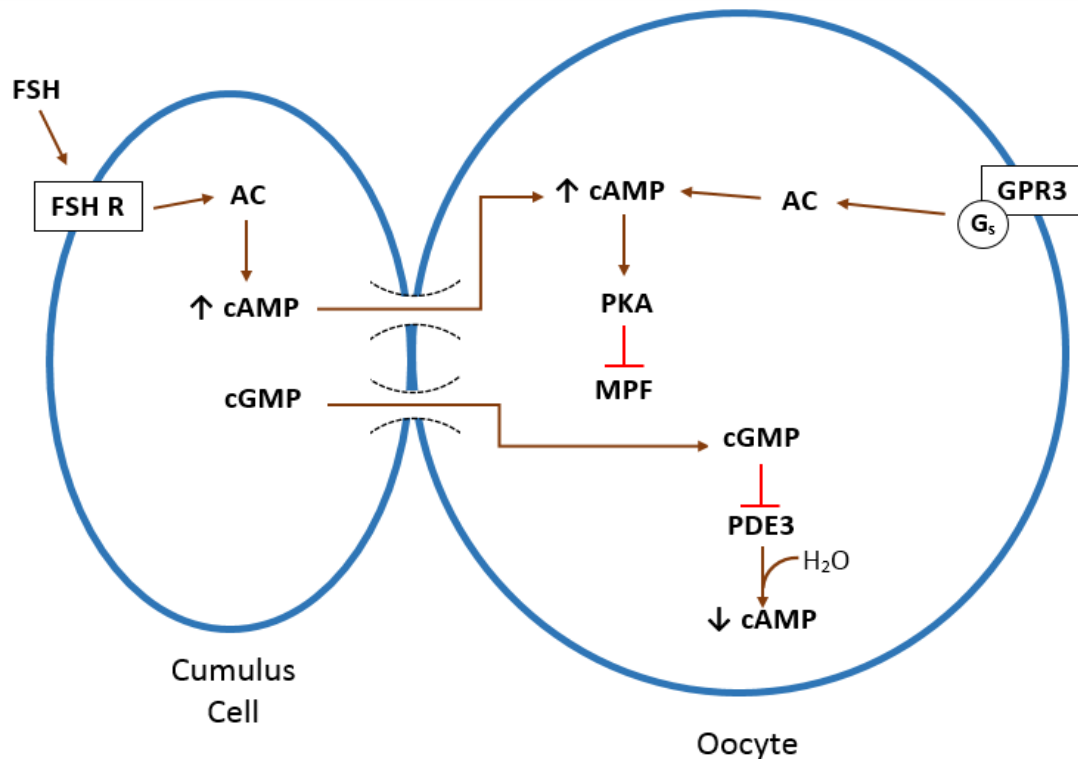


Figure 2.1. Maintenance of meiotic arrest via a signaling cascade beginning with high cAMP. Accumulation of cAMP in cumulus cells is caused by FSH binding to receptors on early antral follicles and activating adenylate cyclase (AC). The cAMP is transported through gap junctions into the oocyte. cAMP is also produced within the oocyte by AC through activation of G-protein coupled receptor type 3 (GPR3). cAMP phosphorylates Protein Kinase A (PKA), and activated PKA inhibits Maturation Promoting Factor (MPF) by phosphorylating the catalytic Cdc2 kinase subunit. In addition, CCs also transport cGMP into the oocyte via gap junctions. cGMP inhibits phosphodiesterase 3 (PDE3), which degrades cAMP via hydrolysis. This inhibition of PDE3 also keeps cAMP levels high.

High levels of cAMP within the oocyte maintains meiotic arrest at the dictyate stage, and maturation is paused. For *in vivo* maturation to occur, the oocyte must resume meiosis in preparation for ovulation and fertilization, and this meiotic resumption begins with the pre-ovulatory gonadotropin surge. Gonadotropin-Releasing Hormone (GnRH) is released from the hypothalamus and stimulates the release of Luteinizing Hormone (LH) from the pituitary, which is the key hormone regulating oocyte maturation. FSH also has an important role, as it is thought to stimulate the expression of LH receptors (Chen et al., 1994; Webb et al., 2003). The surge of LH from the pituitary will bind to these receptors and cause the closure of the oocyte-

CC gap junctions by targeting the granulosa cells (GCs) and causing the build-up of hyaluronic acid, which acts as a spacer between the cells, pushing them apart and breaking the gap junctions. Thus, communication between the oocyte and cumulus cells ceases (Granot and Dekel, 1994; Sela-Abramovich et al., 2005). In bovine oocytes, gap junctional breakdown occurs nine to twelve hours after the LH peak (Hyttel, 1987). cAMP in the oocyte declines because the gap junctions no longer transport cAMP and cGMP into the oocyte from the surrounding cells. Some studies also indicate that the LH surge may turn off GPR3, which produces cAMP inside the oocyte, resulting in a decrease of cAMP (Mehlmann et al., 2004; Mehlmann et al., 2005). The LH surge also leads to PDE activation, which degrades cAMP via hydrolysis. This decrease in cAMP releases the oocyte from meiotic arrest by a decrease in the activity of PKA, which is cAMP dependent, and therefore it can no longer inhibit Maturation Promoting Factor (MPF).

Recall that MPF (also called M-phase promoting factor) controls the mitotic and meiotic cell cycles and consists of a cyclin B and p34cdc2 kinase subunit. MPF is a protein kinase that is present in all dividing cells and regulates the G2/M transition (Nurse, 1990). It is activated during oocyte maturation after there is a dephosphorylation of the p34cdc2 kinase and synthesis of cyclin B regulatory protein (Nurse, 1990; Levesque and Sirard, 1996). Once an increase in MPF occurs, meiosis resumes and the oocytes enter metaphase I, then progresses through anaphase and telophase to arrest at metaphase II until fertilization.

***In Vitro* Oocyte Maturation**

In vitro maturation (IVM) is the process whereby oocytes are removed from a follicle of the ovary and matured in culture medium in a laboratory setting. IVM is an integral part of clinical embryo production, and it is the first step of *in vitro* embryo production (IVP). R.G. Edwards was one of the first to describe *in vitro* maturation of oocytes in 1965, having a need for obtaining mature oocytes for research without having to give exogenous hormones to

stimulate the ovaries. His research included mouse, sheep, cow, pig, dog, rhesus monkey, and human oocytes, and was based on previous work with rabbits and rodents (Pincus and Enzmann, 1935; Chang, 1955; Edwards, 1962), in which oocytes were removed from the follicle and placed in a culture medium where they resumed meiosis within 3 hours. However, canine, primate, and human oocytes did not resume meiosis even after 20 hours of culture (Edwards, 1962). Oocytes from mice, pigs, cows, sheep, monkeys, and humans were placed in various culture media supplemented with serum, antibiotics, and a bicarbonate buffer. Edwards successfully matured mouse oocytes in Difco Medium 199 supplemented with 15% serum, finding that 80% of the oocytes resumed meiosis (Edwards, 1965).

Currently, several commercially available media are used as a base medium for IVM, including TCM-199, SOF (synthetic oviductal fluid), KSOM (K⁺ simplex optimized medium, Ham's F-12, MEM (minimal essential medium), DMEM (Dulbecco's modified Eagle's medium), etc. Maturation culture media generally consists of a balanced salt solution, a bicarbonate buffer, amino acids, an energy source, and water. A major function of the balanced inorganic salts is to regulate the osmolarity of the culture medium (Wright Jr. and Bondioli, 1981). The reported osmotic pressure for bovine embryo culture media is between 265 to 300 mOsmol (Rosenkrans, Jr. et al., 1993; Thompson, 1996). These inorganic ions also play roles in metabolic and signaling pathways., and always include sodium (Na⁺), potassium (K⁺), and calcium (Ca⁺²) (Thompson, 1996). The function of the bicarbonate buffer is to regulate pH in a 5% CO₂ environment which the oocytes are cultured in. The bicarbonate is at a concentration of 25 mM to keep the pH between 7.2-7.4, which is physiological pH. The energy source in maturation media is typically pyruvate. In addition to these media components, temperature is also an important factor of the maturation system. The ideal temperature for culturing bovine oocytes is 39°C.

For bovine oocytes, the most common type of maturation medium currently used is a TCM-199 medium supplemented with 10% fetal bovine serum (FBS), pen/strep, sodium

pyruvate, glutamine, and FSH (Sirard et al., 1988). Variations of this also include supplementing LH, epidermal growth factor (EGF), and estradiol.

Why is IVM important for commercial *In Vitro* embryo production?

IVM is imperative for any large scale embryo production, including commercial cattle production industries, human fertility clinics, research of reproduction or biotechnology, etc. IVM substantially increases the number of oocytes available for use in IVF. In the cow, primary oocytes begin the process of meiosis around day eighty-two of fetal development, but before it can be completed, meiosis is paused and the oocytes are arrested at prophase I (the dictyate stage). This arrest is maintained by the follicular environment, and maturation is inhibited until after puberty when the animal begins. During each estrous cycle, a group of follicles are recruited in a follicular wave to develop in preparation for ovulation and fertilization. However, only one follicle will fully develop and be ovulated, called the dominant follicle. The remaining recruited follicles will undergo atresia and degenerate before they become viable.

If embryo production industries were only able to utilize one *in vivo*-matured oocyte per animal for each cycle, embryo production would be limited and IVP would be impractical. However, if all oocytes in the follicular wave are collected before degeneration and made to mature *in vitro*, the number of potential embryos increases substantially, and this is the value of *in vitro* oocyte maturation.

There is another method of generating higher numbers of oocytes and embryos, but it has drawbacks. It is possible to increase ovarian production by using hormonal stimulation, known as superovulation. In commercial cattle production, superovulation coupled with transvaginal ultrasound-guided aspiration is a common practice, followed by maturation *in vitro*, IVF, and embryo culture. Subsequent embryos are transferred to recipient cattle. Superovulation can also be used in conjunction with artificial insemination and non-surgical embryo collection, which is termed Multiple Ovulation and Embryo Transfer (MOET). The cow

is administered hormones to mature and ovulate multiple oocytes, the oocytes are fertilized *in vivo* by AI. The resultant embryos are collected non-surgically by uterine lavage and are subsequently transferred to recipient cattle.

MOET can increase embryo production, but it is expensive and can have negative effects on the animals. Costs arise from the hormones as well as from the labor of cattle handling and drug administration. These drawbacks apply to humans as well. A single round of IVF requires a month of hormone shots, which are expensive and require diligent monitoring via blood tests and ovarian ultrasounds. Additionally, this approach can result in severe Ovarian Hyperstimulation Syndrome (OHSS), especially in females at high risk such as those with Polycystic Ovary Syndrome (PCOS). OHSS results in enlarged ovaries accompanied by pain, and complications can be life-threatening. About 20-30% of cases can be classified as mild, 5% as moderate, and 1-2% as severe (Navot et al., 1992). Women with moderate to severe cases regularly require hospitalization because they are at risk of thromboembolic disease, respiratory compromise, and renal failure (Baumgarten et al., 2013).

Although stimulating ovarian production does result in increased oocyte and embryo production, it is restrictive in commercial livestock industries due to its high costs. Ovarian hyperstimulation has been successful in obtaining fully mature oocytes in women undergoing IVF, and is almost always used in fertility clinics. However, it is expensive and time-consuming for patients, and can cause life-threatening complications if OHSS results. Therefore, a method to avoid the need for hormonal stimulation would be ideal, and an adequate *in vitro* maturation system resulting in high oocyte developmental competence and good maturation rates would accomplish this goal.

Problems with the Current *In Vitro* Maturation System

IVM is ideal because it allows oocytes from a considerable range of follicle sizes and developmental stages to be used for *in vitro* embryo production. However, when compared to

using *in vivo* matured oocytes, the success rates of IVM are about half. For example, a study in mice found that the live birth rate using *in vivo* matured oocytes with IVF was 52% versus 21% using IVM oocytes (Eppig et al., 2009). In a study of bovine oocytes, it was found that blastocyst yield was about 80% compared to about 50% with IVM (Albuz et al., 2010).

The current system of IVM is referred to as “spontaneous” oocyte maturation because there is no outside stimulus or signal required for maturation to begin. As previously stated, an oocyte is held in meiotic arrest by the follicular environment via high cAMP levels. Through this mechanism, the oocyte is arrested and will not undergo maturation until selected for ovulation, at which point the LH peak occurs and causes a decrease in cAMP, thus releasing MPF from inhibition.

When bovine oocytes are collected for IVP, the oocyte is physically removed from the follicle inside ovary. There are a few methods by which oocytes can be collected including 1) follicular aspiration of abattoir ovaries, 2) “slicing” of abattoir ovaries, and 3) transvaginal ultrasound-guided aspiration (TUGA) in live animals. TUGA, also known as ovum pick-up or OPU, was developed for use in cattle by Pieterse and colleagues (1988). The technique has since been further refined (Looney et al., 1994; Hasler et al., 1995; Meintjes et al., 1995), and involves a technician inserting an ultrasound probe fitted with a needle into the vaginal canal until adjacent to the ovary. The follicles are viewed on an ultrasound monitor, the needle of the probe is inserted, and the oocyte is aspirated. An advantage of this method of collection is that it can be used to collect large numbers of oocytes from genetically valuable live donor animals. These genetically superior oocytes can then be used for IVF, and the resulting embryos transferred into recipient cattle. This is beneficial for commercial cattle industries because a greater number of offspring can be obtained from valuable cows compared with cows that were naturally or artificially inseminated. TUGA can recover an average of 10 oocytes per non-stimulated donor female twice a week for up to three months with no harmful effects (Broadbent

et al., 1997; Godke et al, 2002). In addition, TUGA can be used to obtain oocytes from pre-pubertal, old, pregnant, and infertile cattle.

Collecting oocytes through aspiration or slicing of abattoir ovaries is beneficial because it results in the largest number of oocytes in the least amount of time and is relatively inexpensive. Hamano and Kuwayama (1993) found that aspiration recovered an average of 22.1 oocytes per ovary, and slicing recovered 63.3 oocytes per ovary. Unfortunately the genetic merit of these oocytes is typically unknown because ovaries are obtained from slaughterhouses, so these methods are most often reserved for research rather than commercial cattle production.

Regardless of collection method, the removal of the oocyte from the inhibitory environment of the follicle causes the oocyte to spontaneously begin maturation. This situation is the same for removal of oocytes from human women as well as pigs, mice, and other mammals. This spontaneous maturation results in nuclear maturation in response to low cAMP and the removal of the block on MPF, while cytoplasmic maturation is not spontaneous and requires time. This lack of cytoplasmic maturation results in a less viable oocytes. Oocytes that do not complete both nuclear and cytoplasmic maturation will not acquire full developmental competence. It appears that the low efficiency of IVM oocytes compared with *in vivo* matured oocytes used for IVF is the result of inadequate IVM. Therefore, a more successful IVM system would be beneficial for large scale embryo production, whether for livestock or humans, as it would allow the greatest number of oocytes to be utilized with the fewest drawbacks.

Previous Methods for Improving *In Vitro* Maturation

There have been a few different methods established by which meiotic resumption might be controlled or delayed *in vitro*. Follicular fluid, monolayers of follicular cells, portions of follicle walls, and culture of whole follicles have been studied (Leibfried and First, 1980; Sirard and Bilodeau, 1990; Sirard and Coenen, 1993; Richard and Sirard, 1996a,b; Fouladi Nashta et al., 1998). While these methods showed some success in inhibiting maturation, the dissection and

culture of follicles with oocytes is highly impractical and time-consuming so more convenient methods were sought.

Inhibition of protein synthesis or phosphorylation has been successful at inhibiting oocyte maturation, but these nonspecific inhibitors tend to result in lower developmental competence overall. For example, culture of bovine oocytes with the protein synthesis inhibitor cycloheximide resulted in inhibition of meiosis but also decreased developmental potential (Kastrop et al., 1991; Saeki et al., 1997). Inhibition of MPF has also been examined in several studies using purine derivatives that bind to the ATP site of the Cdc2 kinase of MPF. However, most resulted in blastocyst rates similar to standard IVM oocytes (Mermillod et al., 2000; Longergan et al., 2000; Kulbelka et al., 2000; Ponderato et al., 2001; Hashimoto et al., 2002; Ponderato et al., 2002; Adona and Lima Verde Leal, 2004). Roscovitine is a commonly used MPF inhibitor that has been tested in a number of animal models, including cattle, pigs, mice, goats, and even cats (Sananmuang et al., 2010) and horses (Lange Consiglio et al., 2010). Additionally, MPF inhibitors have been shown to cause a range of deleterious effects, including ultra-structural changes in the oocyte. For example, roscovitine was found to disrupt CC/oocyte connections, degenerate cortical granules, and cause convolution of the nuclear membrane (Fair et al., 2002; Lonergan et al., 2003).

Although most studies indicate that FSH and LH play a vital role in resumption of meiosis and the maintenance of gap junctional communication, a few studies have shown that these can take place without gonadotropin stimulation. In 2004, it was demonstrated that gap junctional communication (GJC) could be extended in bovine oocytes without FSH by inhibiting PDE3, resulting in delayed timing of germinal vesicle breakdown (GVBD) (Thomas et al., 2004). Tsafiriri et al. (1996) found that by increasing cAMP levels in CCs of rat oocytes, PDE4 (which is CC-specific) can mimic the stimulation of LH that induces oocyte maturation. Also, the addition of invasive adenylate cyclase to a gonadotropin-free medium resulted in a higher percentage of MII bovine oocytes, accompanied by prolongation of GJC between the CCs and the oocyte

(Luciano et al., 2004). Regardless of whether gonadotropin stimulation is required, these studies indicate that manipulating intracellular adenylate cyclase and/or PDEs may be crucial for prolonging GJC and the resumption of meiosis in oocytes (Ozawa et al., 2008).

There have also been studies evaluating components of maturation media, such as glutathione (GSH), which is thought to have a role in protecting oocytes from oxidative stress (Luberda, 2005). CCs are believed to maintain GSH levels through gap junctional communication (de Matos et al., 1997; Mori et al., 2000), and a study on bovine oocytes found that supplementing IVM medium with thiol precursors to GSH (such as cysteamine, β -mercaptoethanol, cysteine, and cystine) result in an increase in GSH synthesis in these bovine oocytes. Researchers evaluated the effects of supplementing these in IVM on embryo development, and found that the percentage of embryos that developed to morula and blastocyst stages were significantly higher for treated oocytes than for oocytes matured in control medium. They concluded that high intracellular GSH levels produced by the increased GSH synthesis improved developmental rates (de Matos and Furnus, 1998). A later study on porcine oocytes examined the relationship between GSH content and oocyte nuclear maturation, fertilization, and embryo development. Results indicated that GSH content correlated with the rate of male pronucleus formation, blastocyst formation, and number of cells per blastocyst. These researchers concluded that “GSH synthesized by intact cumulus cells during maturation culture improved oocyte maturation and played an important role in fertilization and embryonic development” (Maedomari et al., 2007). Many additional studies on supplementing porcine, bovine, macaque, and goat IVM medium with GSH or a GSH precursor exhibited positive effects on *in vitro* production of embryos (Zhou et al., 2008; Choe et al., 2010; Curnow et al., 2010; Lott et al., 2011; Nabenishi et al., 2011; Whitaker et al., 2012; Merton et al., 2013). However, one study in goats found that adding 1 mM GSH caused an increase in intracellular GSH in prepubertal goat oocytes but did not improve fertilization rates (Mayor et al., 2001). Another study utilizing goats supplemented IVM media with either 100 mM of

cysteamine, 100 mM of b-mercaptoethanol, 0.57 mM of cysteine, or 0.57 mM cystine. It was reported that only the cysteamine improved embryo development although all thiol compounds increased intracellular GSH content (Rodríguez-González et al., 2003). It has also been shown in pig oocytes that FSH stimulates the synthesis of GSH in cumulus cells (Ozawa et al., 2009). A review by Deleuze and Goudet (2010) stated that supplementing IVM media with cysteamine does not affect nuclear maturation rates, but it can improve pronucleus formation, cleavage rates, and embryo development. This suggests a positive effect on cytoplasmic maturation and oocyte competence. However, due to the variety of results after thiol supplementation to IVM media, these researchers hypothesized that it appears to be highly species-specific and dose-dependent, therefore making it difficult to use for a standardized IVM system.

Researchers have also investigated oocyte-secreted factors (OSFs) and their role in the mechanisms required for oocyte competence. As discussed, the follicular environment is important for oocyte development and is primarily mediated through granulosa cells (GCs) and cumulus cells (CCs). Communication from these cells to the oocyte via gap junctions provide the oocyte with molecules (such as ATP, amino acids, metabolites, etc.) that are necessary for growth and differentiation of the oocyte. However, there appears to be cross-communication, from the oocyte to the surrounding CCs. This communication involves oocyte-secreted factors, and two key OSFs are growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) (Gilchrist et al., 2008). Both belong to the transforming growth factor- β (TGF- β) family and activate signaling pathways in the CCs which regulate genes and cellular processes necessary for CC differentiation. These pathways are the BMP pathway utilizing SMAD 1/5/8 messengers and the TGF β /activin pathway utilizing SMAD 2 and 3 (Shimasaki et al., 2004). Because of this bidirectional communication, there is an oocyte-CC regulatory loop in which the oocyte directs the surrounding cells to perform the functions needed for its own development. Therefore, it has been proposed that supplementing IVM medium with exogenous OSFs may improve the developmental potential of oocytes. A study utilizing bovine oocytes and IVM

medium supplemented with BMP15 alone, GDF9 alone, or both combined all resulted in an increase in blastocyst formation (41% compared with 58%, 50%, and 55% respectively) (Hussein et al, 2006). This suggests that OSFs may enhance oocyte competence and regulate the COC environment. Another study on mice found that supplementing IVM medium with only exogenous GDF9 improved embryo development and fetal viability, as indicated by higher rates of development, percentages of hatching blastocysts, and blastocyst total cell number and ICM cell number (Yeo et al., 2008). A third study in humans reported the culture of ovarian follicles in the presence of GDF9 for 7 days resulted in 53% of follicles reaching the secondary stage of development vs. 32% in the control. Follicle viability was also improved, resulting in fewer follicles undergoing atresia (Hreinsson et al., 2002).

cAMP Modulators Regulate Oocyte Meiotic Resumption in Multiple Species

To date, the most promising methods of delaying oocyte maturation have been those that regulate cAMP levels in the oocyte. The substances that regulate cAMP are referred to as cAMP modulators, which are agents that either induce cAMP production or prevent its breakdown. cAMP modulators have been studied extensively in numerous animal models (cattle, pigs, mice, sheep, dogs, etc). This particular approach to oocyte maturation endeavors to prevent the drop in intra-oocyte cAMP which causes the release from meiotic arrest and the initiation of maturation. The cAMP modulators will cause an increase in, and prevent the drop of, cAMP levels in the oocyte after removal from the follicle. The goal is to delay meiotic resumption to allow the oocyte to obtain developmental competence through delaying nuclear maturation so that cytoplasmic maturation can occur.

There are three types of cAMP modulators: cAMP analogues, adenylate cyclase activators, and phosphodiesterase (PDE) inhibitors. In bovine oocytes, cAMP analogues have previously been demonstrated to transiently delay meiotic resumption and nuclear maturation (Homa, 1988; Sirard and First, 1988; Bilodeau-Goeseels 2003a,b). The cAMP analogue that is

most frequently studied is dibutyryl cAMP (dbcAMP). Adenylate cyclase activators, which are the second type of cAMP modulator, include FSH, forskolin (FSK), and invasive adenylate cyclase. FSH activates adenylate cyclase via G-protein activation. Forskolin (FSK) has been shown to swiftly and reversibly stimulate the catalytic subunit of adenylate cyclase (Seamen and Daly, 1981), and therefore increase intracellular cAMP production (Moriwaki et al., 1982; Litosoch et al., 1982). FSK has been found to increase cAMP in the oocytes of mice (Bornslaeger and Schultz, 1985; Salustri et al., 1985), rats (Sherizly et al., 1988), rabbits (Yoshimura et al., 1992), and cows (Thomas et al., 2002). The third type of cAMP modulator are PDE inhibitors, which can be specific or nonspecific for different types of PDE families. IBMX (3-isobutyl-1-methylxanthine) is nonspecific, and inhibits all PDE families except PDE8 and PDE9. Rolipram inhibits PDE4, dupyridamale inhibits PDE8, and PDE3 inhibitors include cilostamide, milrinone, and Org9935 (Gilchrist and Thompson, 2007).

Some of the initial studies on regulating oocyte maturation with cAMP modulators utilized mouse oocytes. One of the first cAMP modulators to be utilized in an experiment was dbcAMP, where it was reported to reversibly inhibit meiotic maturation of mouse oocytes (Stern and Wassarman, 1973; Cho et al., 1974). Nekola and Smith (1975) found that dbcAMP does prevent spontaneous maturation of mouse oocytes, but the inhibitory effects were not reversible even after treatment with gonadotropins LH and FSH. Other studies have confirmed the hypothesis that a decrease in intra-oocyte cAMP initiates meiotic maturation in murine oocytes (Bornslaeger and Schultz, 1985; Bornslaeger et al., 1986). There have been multiple studies on murine oocyte meiotic regulation with cAMP modulators since the 1970s, confirming the antagonist effect of cAMP on meiotic resumption (Schultz et al., 1983; Urner et al., 1983; Vivarelli et al., 1983; Sato and Koide, 1984; Sato et al., 1985; Salustri et al., 1985; Eppig and Downs, 1988; Sun et al., 1999; Nogueira et al., 2003)

In bovine oocytes, one study utilizing cAMP modulators for *in vitro* maturation included: 8-bromo-cAMP, dbcAMP, forskolin, or IBMX. Culture for 12 hours in 8-bromo-cAMP and FSK

significantly inhibited GVBD, and IBMX also caused inhibition of GVBD in a dose-dependent manner following culture for 24 hours. These results suggested that higher levels of cAMP caused a transitory inhibition of GVBD in bovine oocytes. dbcAMP also inhibited GVBD, although less effectively, and the researchers suggested this may be due to the oocyte having active PDEs. They hypothesized that cAMP degradation by endogenous PDEs would inhibit dbcAMP and FSK from raising intracellular levels sufficiently to maintain meiotic arrest, whereas 8-bromo-cAMP is only partially hydrolyzed by PDE and IBMX is an inhibitor of PDE (Homa, 1988). In another early study, bovine oocytes were treated with 5 mM dbcAMP plus 1 mM IBMX which inhibited the transition of the oocyte to metaphase, and these oocytes were reputed to be sensitive to cAMP levels (Milovanov and Sirard, 1994). Numerous other studies have been conducted on bovine oocytes using various cAMP modulators to regulate oocyte maturation, resulting in increased cAMP levels, reversibility of inhibitory effects, and/or delaying meiotic resumption in oocytes (Sato et al., 1990; Sanbuissho et al., 1992; Atkas et al., 1995a; Atkas et al., 1995b; Guixue et al., 2000; Mayes and Sirard, 2002; Bilodeau-Goeseels, 2003b; Luciano et al., 2005; Barretto et al., 2007). In 2009, a study in dairy cattle determined the effects of supplementing maturation media with the adenylate cyclase activator FSK in addition to linolenic acid, which resulted in an improvement of both blastocyst quality and developmental potential of oocytes to achieve the blastocyst stage. Researchers determined intracellular cAMP levels at 0, 3, 6, and 24 hours maturation after supplementing IVM media with linolenic acid alone, FSK alone, or both combined. Supplementation of linolenic acid resulted in a higher cAMP level at 3 hours only, while supplementation of FSK resulted in higher cAMP at all time points. It was reported that the addition of linolenic acid and FSK did not result in any further increase in cAMP compared with FSK alone. This supports the hypothesis that FSK causes an increase in cAMP levels within the oocyte (Marei et al., 2009).

The effect of cAMP modulators has also been examined in sheep oocyte maturation. In 2013, Gharibi and colleagues experimented with a two-step maturation system in which a

“prematuration culture” included the PDE3 inhibitor cilostamide. Prematuration consisted of 1, 10, or 20 μ M cilostamide for 22 hours followed by 22 hours in standard ovine maturation medium. Researchers analyzed the effect on gap junctional communication (GJC), nuclear status, chromosome organization, polar body (PB) extrusion, and embryo development. Cilostamide delayed maturation and inhibited CC expansion, which maintained gap junctional communication. Additionally, the inhibitory effects of 1 μ M cilostamide were reversible, but not at higher concentrations, resulting in deleterious consequences on chromosome organization and spindle patterns. Blastocyst rates after prematuration with 1 μ M cilostamide were not different from standard IVM, but were less in oocytes pre-matured in the higher concentrations of cilostamide (Gharibi et al., 2013). It was concluded that although this method maintained GJC and delayed maturation, the reduction of cAMP by PDE3 is not the only mechanism that controls nuclear maturation in ovine oocytes. Another study on bovine oocytes supports this conclusion that increased cAMP maintained gap junctional function in COCs, finding that in gap junctions which were exogenously interrupted, chromatin quickly condensed and RNA synthesis ceased. The addition of cilostamide prevented these effects, suggesting that GJC facilitates chromatin structure and function via cAMP exchange from the CCs to the oocyte. Extending GJC during maturation and preventing the degradation of cAMP enhanced the ability of oocytes to undergo meiosis and embryo development, suggesting this may be important in achieving meiotic competence (Luciano et al., 2011).

In addition to mice, cattle, and sheep, there has been some success in porcine oocyte maturation using cAMP modulators. The nonspecific PDE inhibitor IBMX has been shown to increase cAMP in porcine cumulus-oocyte complexes (COCs) and to prolong gap junctional communication between the oocyte and surrounding CCs. In addition, PDE inhibition via IBMX resulted in increased expression of LH receptor mRNA in CCs and enhanced the binding abilities of this receptor on CC membranes. In the same study, FSH was also shown to increase cAMP content in porcine oocytes by about seven-fold from 0 to 20 hours of culture

(Ozawa et al., 2008). A study combining FSH and dbcAMP in porcine oocyte maturation medium for five days was found to prolong meiotic arrest and promote the growth and acquisition of meiotic competence in oocytes from early porcine antral follicles. Results showed that FSH alone resulted in 11% MII oocytes after maturation, while dbcAMP alone resulted in 19% MII oocytes, and a combination of FSH and dbcAMP resulted in 68% MII oocytes (Cayo-Colca et al., 2011). It was reported that 1 mM dbcAMP for the first 22 hours of IVM led to an increase in porcine blastocyst rates for prepubertal but not adult porcine oocytes derived from 3 mm follicles, but exhibited no effect on larger 4-8 mm follicles (Bagg et al., 2006; Bagg et al., 2007). Kim et al. (2008) demonstrated that dbcAMP treatment for the first 22 hours of IVM of porcine oocytes promoted maturation to the MII stage, and also indicated an increase in MPF and mitogen-activated protein kinase (MAPK) activity after 44 hours of maturation, both of which are essential M-phase regulating enzymes. They also reported an increase in PKA activity in dbcAMP-treated oocytes compared with control oocytes, and treatment with cAMP reduced polyspermy during fertilization but may have negatively affected structural integrity, mitochondrial membrane potential, and apoptosis in IVF and NT porcine embryos. It was also suggested that dbcAMP-treated oocytes resulted in blastocysts that had a higher number of ICM cells, trophectoderm (TE) cells, and total cells compared with the controls (Kim et al., 2008).

Finally, there has been some recent work with human oocyte maturation using cAMP modulators, and the aforementioned animal studies can be considered as models for human ART. A study by Zeng et al (2013) was designed to determine the effects of heparin and cAMP modulators during pre-IVM using both mouse and human oocytes. Results on murine oocytes were consistent with past studies comparing maturation using cAMP modulators (in this case, IBMX and FSK in a 1 hour pre-IVM phase) to standard IVM, finding that cAMP was elevated and that there were higher rates of maturation, embryo development, and improved embryo quality. Treatment with heparin alone in mouse pre-IVM significantly delayed GVBD at 0 and 1

hours IVM, but not by 2 or 3 hours. Supplementation of IBMX and FSK produced a large delay in GVBD, and addition of heparin in the pre-IVM further delayed GVBD in a dose-dependent manner. However, supplementation of heparin eliminated the IBMX+FSK-stimulated increase in mitochondrial membrane potential and ATP production ($p<0.05$), and had a negative effect on embryonic cleavage, development, and quality. In human oocytes, in the absence of heparin in pre-IVM culture, cAMP modulators did not affect oocyte MII competence after 40 hours. In standard IVM, supplementation of heparin in pre-IVM without cAMP modulators did not affect oocyte competence either, and when heparin was combined with cAMP modulators, MII competence was significantly reduced from 65% to 15% ($p<0.05$). Researchers concluded that heparin ablated the advantageous effects of cAMP modulators during pre-IVM (Zeng et al., 2013). Another study on human oocyte maturation investigated effects of a 24 hour and 48 hour “prematurization culture” with Org9935 (a PDE3 inhibitor) on oocytes from small diameter follicles. After prematurization culture for 24 and 48 hours, 99% and 97% of the oocytes were still in GV stage respectively, suggesting that meiotic resumption was effectively blocked. Following removal from prematurization culture, oocytes were placed in IVM and 67% in both treatments extruded a PB, which did not differ from the control (63% PB extrusion). After ICSI of each treatment group, embryos were cultured to day 3. *In vivo* matured oocytes resulted in about 84% good quality embryos, whereas control IVM resulted in about 65%. Oocytes prematured for 24 hours resulted in about 85% good embryos, which was significantly higher than control IVM and not different from *in vivo* matured oocytes. Oocytes prematured for 48 hours resulted in the lowest rate, about 45%, which was significantly different from *in vivo* matured oocytes but not from control IVM (Nogueira et al., 2006).

Utilizing cAMP Modulators within a Novel Extended IVM System

Recently a promising new system of IVM utilizing the concept of cAMP modulators was developed for bovine and murine oocytes, and was subsequently repeated in ovine oocytes

(Albuz et al., 2010; Rose et al., 2013). This IVM system involves two phases: Pre-IVM and IVM. This method was termed “Simulated Physiological Oocyte Maturation” or SPOM (Figure 2.2). Because high cAMP levels maintain meiotic arrest, the pre-IVM phase included cAMP modulators FSK and IBMX, which were found to cause a large spike (from about 3 fmol/oocyte to almost 200 fmol/oocyte) in cAMP within the oocyte, resulting in delayed meiotic resumption. FSK increases the activity of adenylate cyclase, which causes increased cAMP production, and IBMX is a nonspecific PDE inhibitor, which inhibits most PDEs in the COC, effectively preventing cAMP degradation. This pre-IVM phase followed by an extended IVM phase containing the cAMP modulator cilostamide and FSH allowed the oocytes to resume and complete maturation slowly. Cilostamide inhibits only PDE3 (located only in the oocyte) and does not affect PDEs in the CCs, which allows for a gradual degradation of cAMP in the COCs and a slower, delayed reversal of meiotic inhibition.

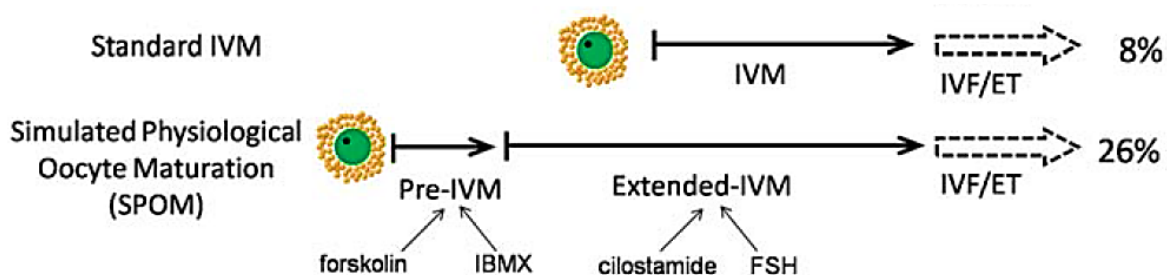


Figure 2.2. The standard IVM system compared to the SPOM system developed by Albuz and colleagues (Albuz et al., 2010).

Researchers hypothesized that this system more closely resembles the maturation process *in vivo*, and determined that SPOM doubled implantation rates (53% vs. 28%) and substantially improved bovine embryo yield (26% vs. 8%) compared to spontaneous IVM. This SPOM system allowed for maturation to be extended by six hours in bovine oocytes and four

hours in murine oocytes. This delay seemingly allowed oocytes enough time for both nuclear and cytoplasmic maturation to occur. Therefore, more oocytes obtained full developmental competence, increasing viability and embryo production after IVF (Albuz et al., 2010).

As mentioned, a similar study was conducted using this SPOM system in sheep oocyte maturation. Sheep COCs were first cultured for 2 hours in a pre-IVM medium with 100 μ M FSK and 500 μ M IBMX, which increased cAMP concentrations 10-fold compared with controls ($p<0.05$). When followed by an IVM step with FSH and cilostamide, the cAMP concentrations increased and the SPOM system had the highest cAMP levels of all experimental groups by the end of maturation. In addition, 77.9% of COCs reached the MII stage by 21 hours of standard IVM compared with 54.1% in the SPOM system ($P=0.001$). By 27 hours in SPOM, the oocytes reached approximately the same percentage of MII as standard IVM at 24 hours (which is the standard insemination time). Therefore, SPOM caused a delay in nuclear maturation by three to four hours but did not inhibit maturation completely. However, unlike the study by Albuz et al. (2010) using bovine and murine oocytes, the sheep blastocyst and cleavage rates did not increase compared with the controls. Here, 79% of oocytes successfully cleaved in both treatments and 54% of all cleaved zygotes developed into blastocysts in both standard IVM and SPOM. However, there was a 1.6-fold increase in total cell number of sheep blastocysts when compared with the control ($p<0.001$). These results suggest that regulating ovine oocyte cAMP concentrations with cAMP modulators during IVM improved the quality of embryos when compared with standard ovine IVM methods (Rose et al., 2013).

CHAPTER III

EFFECT OF cAMP MODULATORS ON MEIOTIC RESUMPTION OF BOVINE OOCYTES

Introduction

The spontaneous resumption of meiosis that occurs after removing oocytes from the follicular environment is thought to cause a lack of oocyte developmental competence, resulting in lower success rates of *in vitro* embryo production. There is no doubt that the current spontaneous IVM system is the major rate-limiting step of commercial IVP, and it is now widely accepted that the second messenger cAMP has a key role in maintaining oocyte meiotic arrest within the follicle. It is also known that there is a drop in cAMP within the COCs after removal from the follicle, causing a signal cascade which results in meiotic resumption. This experiment focuses on maintaining high cAMP in the oocyte in order to keep it from spontaneously resuming meiosis, resulting in a slower maturation process compared to standard IVM.

These experiments compare the standard IVM system to a two-step extended IVM similar to the Simulated Physiological Oocyte Maturation (SPOM) system previously described by Albuz et al. (2010), although these experiments utilize a different base media for maturation. Here, standard IVM consists of a 23-hour maturation period in a typical TCM-199 based maturation medium (Sirard et al., 1988). The extended IVM system consists of a 2-hour pre-IVM phase of HEPES-TALP supplemented with FSK and IBMX, followed by a 31-hour IVM phase of standard maturation medium supplemented with cilostamide. FSK is an adenylate cyclase activator, and IBMX is a nonspecific PDE inhibitor. IBMX is termed “nonspecific” because it inhibits multiple types of PDEs, both within the oocyte and the CCs. In contrast to IBMX, cilostamide only inhibits PDE3, which is located exclusively in the oocyte (Tsafiriri et al., 1996). The use of only cilostamide in the IVM phase should allow a gradual reversal of inhibition, causing the oocytes to resume and complete meiosis slowly. This novel extended IVM system should provide the oocytes more time for both nuclear and cytoplasmic maturation

to occur, and therefore a greater number of oocytes should reach full developmental competence compared to spontaneous standard IVM.

The aim of these experiments is to determine whether or not the cAMP modulators in this two-step extended IVM system causes a delay in meiotic resumption compared to standard IVM, and also how the overall maturation rates compare in extended IVM to standard IVM. Data will be collected on the percentage of oocytes reaching each nuclear stage every five hours during standard IVM and extended IVM. The stages examined are germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII), where oocytes reaching MII are fully matured and ready for fertilization.

In addition, experiment 1 will also examine the effects of the IVM treatments on two different breeds of cattle, Brahman and mixed breed/Angus cattle. This will determine whether one treatment may be optimal for a particular breed of cattle, and will identify whether or not these breeds react differently to treatment with cAMP modulators.

Materials and Methods

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Experimental Design

Experiment 1

Oocytes from both Brahman and Angus cattle were collected on separate collection days, divided into two groups (standard IVM and extended IVM), cultured in 5% CO₂ at 39°C, and sampled every five hours. Sample times for standard IVM are 8, 13, 18, and 23 hours. Sample times for extended IVM are 8, 13, 18, 23, 28, and 33 hours. Each subset of oocytes was removed from their respective maturation media at these times, then CCs were removed and the oocytes were fixed on slides in a methanol/acetic acid solution for three to five days

before staining with aceto-orcein. Nuclear status of each oocyte was assessed to determine how far the oocyte developed at each time sample. A chi-square test was performed to determine if any significant differences exist among the results. In addition, a comparison between Brahman and Angus oocytes was examined to determine if these breeds reacted differently to either treatment.

Experiment 2

Experiment 2 was conducted similarly to experiment 1, except that cAMP modulators FSK and IBMX were added to the oocyte holding medium (HEPES-TALP) during oocyte collection for the extended IVM treatment. Oocytes from Angus cattle were divided by cow into two groups: control standard IVM and extended IVM. The control treatment consisted of standard HEPES-TALP holding medium during oocyte collection, then oocytes were moved into standard IVM medium. Extended IVM for this experiment consisted of a HEPES-TALP holding media supplemented with FSK and IBMX during oocyte collection. Oocytes were moved into pre-IVM for 2 hours, then extended IVM for 31 hours. The remainder of the experiment was conducted exactly as experiment 1. All oocytes in IVM were cultured in 5% CO₂ at 39°C and sampled every 5 hours. CCs were removed and oocytes stained with aceto-orcein. Nuclear status was assessed to determine oocyte development at each time sample, and a chi-square test was performed to determine any significant differences among the results.

Preparation

Media required for this experiment includes Dulbecco's Phosphate Buffered Saline (D-PBS), HEPES-TALP, standard maturation medium, Pre-IVM medium with IBMX and FSK, and extended IVM medium with cilostamide. D-PBS was prepared and stored until needed, then placed in the incubator to warm overnight. 10 mL of bovine calf serum (BCS) and 1 mL of

Heparin (Sagent Pharmaceuticals) were added to D-PBS the morning of collection. HEPES-TALP and all maturation media were prepared the afternoon before oocyte collection and stored in the refrigerator overnight. HEPES-TALP consists of 20 mL HEPES-TL (Caisson Labs, North Logan, UT, USA) with 60 mg BSA, 200 μ L sodium pyruvate, and 200 μ L pen/strep (Gibco, No.15140). Standard maturation medium is composed of TCM-199 with 10% fetal bovine serum (FBS), 0.2 mM sodium pyruvate, 1% pen/strep, 2 mM glutamine, and FSH (5.0 μ g/mL). The pre-IVM medium is HEPES-TALP supplemented with 100 μ M FSK and 500 μ M IBMX. Extended IVM medium consists of standard TCM-199 maturation medium supplemented with 20 μ M cilostamide. (See Appendix B for media formulations and stock solutions.)

On collection day, all necessary dishes of media were prepared and equilibrated for a minimum of 2 hours in the incubator before oocytes were added. Ten 35 mm petri dishes (BD Falcon) of HEPES-TALP were prepared for oocyte collection, washing, and sorting into treatment groups and placed in a non-CO₂ incubator at 39°C to warm. Dishes of maturation medium included four 35 mm wash dishes and four 4-well plates (Nunc, Thermo Fisher Scientific Inc., Waltham, MA, USA) each of which had two 35 μ L drops of maturation medium covered with 570 μ L of pre-warmed, pre-equilibrated mineral oil (embryo tested). The dishes were labeled “8”, “13”, “18”, or “23” for each time sample and placed in a 5% CO₂ incubator at 39°C. The pre-IVM HEPES-TALP medium with IBMX and FSK was prepared in one 35 mm wash dish and one 4-well plate with 500 μ L of pre-IVM media in each well, and these were placed in a non-CO₂ incubator at 39°C. The extended IVM medium with cilostamide was prepared similarly to standard IVM media, with four 35 mm wash dishes and six 4-well plates each with two 35 μ L drops of media covered with mineral oil. These were labelled “8”, “13”, “18”, “23”, “28”, or “33” for each time sample, and placed in the CO₂ incubator at 39°C and 5% CO₂.

Oocyte Collection

For experiment 1, oocytes (n=589) were collected by transvaginal ultrasound-guided follicular aspiration over 6 collection days. All follicles between 3 to 8 mm were aspirated. Collection medium for TUGA was pre-warmed D-PBS with 0.1% heparin and 1% BCS. Oocytes with less than two layers of CCs were discarded, and the remaining oocytes were transferred to a 35 mm petri dish containing pre-warmed HEPES-TALP holding medium and placed in a non-CO₂ incubator at 39°C. Collection time ranged from 2 to 2.5 hours. Oocytes were separated by cow into two groups, where forty percent were placed into standard IVM which had four sample times, and the remaining sixty percent were placed into extended IVM having six sample times.

For experiment 2, oocytes (n=327) were collected with TUGA in the same manner as the first experiment. There were three collection days consisting of all mixed breed/Angus cattle. Oocytes were divided into treatment groups during collection, rather than after collection was completed. The oocytes selected for the extended IVM treatment were immediately moved into HEPES-TALP with FSK and IBMX, where they were held for the duration of oocyte collection. Oocytes in standard IVM were held in the standard HEPES-TALP media without the modulators. Total collection time ranged from 2 to 2.5 hours.

Transvaginal Ultrasound-Guided Follicular Aspiration

Oocytes were obtained via transvaginal ultrasound-guided aspiration (TUGA), a method of oocyte collection from live cattle (Figure 3.1). Each donor cow is retained in a holding chute and given an epidural injection of 6 mL of 2% lidocaine (Vet One, Product No. 510212). An ultrasound probe with a concave 7.5 MHz transducer is inserted into the vaginal canal. The ovary is grasped through the rectum and positioned against the vaginal wall next to the transducer at the distal end of the ultrasound probe. The ovary is viewed on the ultrasound monitor and follicles are located. An 18-gauge disposable collection needle, located inside the

ultrasound probe, is connected to a suction pump via polyethylene tubing. The collection needle is inserted through the vaginal wall into each follicle, and the follicular fluid along with the oocyte is aspirated out of the ovary. The needle is changed after each cow, tubing and needles are rinsed with collection medium (D-PBS with 0.1% heparin and 1% BCS), and the follicular fluid and oocytes are deposited into an embryo collection filter (Agtech Inc., Manhattan, KS, USA). All media used during collection is maintained at 39°C.

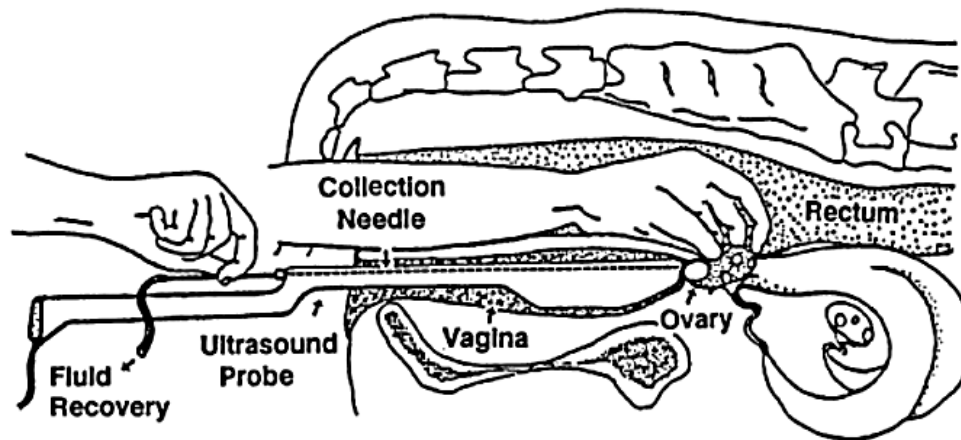


Figure 3.1. Diagram of transvaginal ultrasound-guided follicular aspiration. (Drawing by E. Meintjes, obtained from Godke et al., 2002.)

Oocyte Culture

Oocytes in treatment 1 were washed four times through standard IVM medium, and were divided evenly into four groups for the 8, 13, 18, and 23-hour time samples. Individual four-well plates were previously prepared and labelled for each of these time samples, and contained nine to twelve oocytes in each 35 μ L drop. Oocytes were cultured in 5% CO₂ at 39°C for the required times, ranging from 8 to 23 hours.

Oocytes in treatment 2 (extended IVM) were washed four times through the pre-IVM medium with FSK and IBMX, and then placed in this medium for two hours in a non-CO₂ incubator at 39°C. Oocytes were then taken out and washed through HEPES-TALP, then

washed four times through extended IVM medium with cilostamide. Oocytes were then divided into six groups for the six time samples, and each group of nine to twelve oocytes was placed in individual four-well plates and cultured in 5% CO₂ at 39°C for the required time.

Sampling

For standard IVM, time was measured beginning when the oocytes were placed in maturation medium, and for extended IVM time was measured beginning when the oocytes were placed into pre-IVM media. Samples were taken every five hours beginning at 8 hours and ending at 23 hours for standard IVM and 33 hours for extended IVM. For each time sample, the groups of oocytes were removed from maturation medium and placed in warmed hyaluronidase solution (Appendix B) in a 15 mL sterile centrifuge tube. Oocytes in the hyaluronidase solution were placed in a warm water bath at 39°C for about 5 minutes, then vortexed at max speed for 4 to 8 minutes as needed to remove all CCs. Oocytes were then fixed on a slide and placed in coplin jars containing a previously prepared 3:1 methanol/acetic acid solution.

Staining and Data Collection

Slides of oocytes were stored in the methanol/acetic acid solution for three to five days. Oocytes were then stained with 1% aceto-orein (refer to Appendix A for staining protocol and Appendix B for stain preparation). Slides were viewed under a microscope at 20X and nuclear status was ascertained for each oocyte. Data was recorded for each stage at each time sample for experiment 1 (Tables 3.1-3.5 and Figure 3.2) and experiment 2 (Table 3.6 and Figure 3.3). Oocytes could be at any of the following stages: germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), anaphase I (AI), telophase I (TI), or metaphase II (MII) (Figures 3.2 and 3.3).

Statistical Analysis

All data was analyzed using Sigma Stat Statistical Software Version 3.5 (Systat Software, Inc., Chicago, IL, USA). For both experiments, a chi-square test was performed to detect significant differences between the numbers of oocytes in each stage during standard IVM compared to extended IVM at each time sample. For experiment 1, statistics was completed on Brahman oocytes individually (Table 3.1), Angus oocytes individually (Table 3.2) and a comparison of Brahman and Angus oocytes to each other (Tables 3.3 and 3.4) to determine whether these breeds reacted differently to either treatment. Lastly, Brahman and Angus oocytes were pooled together (Table 3.5) after significant differences were analyzed. Differences of $p < 0.05$ were considered significantly different.

Results

Experiment 1 Results

Table 3.1. Nuclear status of Brahman cattle oocytes after standard IVM (n=104) and after extended IVM with cAMP modulators (n=166).

	Standard IVM			Extended IVM with cAMP modulators		
	GV, no. (%)	MI, no. (%)	MII, no. (%)	GV, no. (%)	MI, no. (%)	MII, no. (%)
8 hour	6 (21.4) ^a	13 (46.4) ^A	0	24 (82.8) ^b	1 (3.4) ^B	0
13 hour	1 (4.0) ^a	20 (80.0)	0	10 (34.5) ^b	15 (51.7)	0
18 hour	1 (4.2)	6 (25.0) ^a	10 (41.7) ^A	4 (14.8)	16 (59.3) ^b	1 (3.7) ^B
23 hour	0	3 (11.1) ^a	23 (85.2) ^A	1 (3.8)	8 (30.8) ^b	9 (34.6) ^B
28 hour				3 (11.1)	7 (25.9)	15 (55.6)
33 hour				2 (7.1)	13 (46.4)	9 (32.1)

^{abAB} Different superscripts within rows indicate significant differences ($p < 0.05$).

Note: The number of MII oocytes at 33 hour Extended IVM is significantly different from the number of MII oocytes at 23 hours Standard IVM for Brahman cattle ($p \leq 0.001$).

Table 3.2. Nuclear status of Angus cattle oocytes after standard IVM (n=130) and after extended IVM with cAMP modulators (n=189).

	Standard IVM			Extended IVM with cAMP modulators		
	GV, no. (%)	MI, no. (%)	MII, no. (%)	GV, no. (%)	MI, no. (%)	MII, no. (%)
8 hour	4 (10.8) ^a	27 (73.0) ^A	1 (2.7)	24 (68.6) ^b	4 (11.4) ^B	0
13 hour	0 ^a	19 (57.6) ^A	2 (6.1)	12 (35.3) ^b	10 (29.4) ^B	0
18 hour	0	7 (25.0) ^a	17 (60.7) ^A	3 (10.7)	16 (57.1) ^b	6 (21.4) ^B
23 hour	0	7 (21.9)	25 (78.1) ^a	3 (9.1)	14 (42.4)	8 (24.2) ^b
28 hour				3 (12.0)	6 (24.0)	13 (52.0)
33 hour				6 (17.6)	6 (17.6)	19 (55.9)

^{abAB} Different superscripts within rows indicate significant differences ($p < 0.05$).

Note: The number of MII oocytes at 33 hour Extended IVM is *not* significantly different from the number of MII oocytes at 23 hour Standard IVM for the Angus cattle ($p=0.098$).

Table 3.3. Comparison of Angus oocytes after standard IVM (n=130) with Brahman oocytes after standard IVM (n=104).

	Angus Standard IVM			Brahman Standard IVM		
	GV, no. (%)	MI, no. (%)	MII, no. (%)	GV, no. (%)	MI, no. (%)	MII, no. (%)
8 hour	4 (10.8)	27 (73.0)	1 (2.7)	6 (21.4)	13 (46.4)	0
13 hour	0	19 (57.6)	2 (6.1)	1 (4.0)	20 (80.0)	0
18 hour	0	7 (25.0)	17 (60.7)	1 (4.2)	6 (25.0)	10 (41.7)
23 hour	0	7 (21.9)	25 (78.1)	0	3 (11.1)	23 (85.2)

There are no significant differences between Angus and Brahman oocytes after standard IVM.

Table 3.4. Comparison of Angus oocytes after extended IVM (n=189) with Brahman oocytes after extended IVM (n=166).

	Angus Extended IVM			Brahman Extended IVM		
	GV, no. (%)	MI, no. (%)	MII, no. (%)	GV, no. (%)	MI, no. (%)	MII, no. (%)
8 hour	24 (68.6)	4 (11.4)	0	24 (82.8)	1 (3.4)	0
13 hour	12 (35.3)	10 (29.4)	0	10 (34.5)	15 (51.7)	0
18 hour	3 (10.7)	16 (57.1)	6 (21.4)	4 (14.8)	16 (59.3)	1 (3.7)
23 hour	3 (9.1)	14 (42.4)	8 (24.2)	1 (3.8)	8 (30.8)	9 (34.6)
28 hour	3 (12.0)	6 (24.0)	13 (52.0)	3 (11.1)	7 (25.9)	15 (55.6)
33 hour	6 (17.6)	6 (17.6) ^a	19 (55.9)	2 (7.1)	13 (46.4) ^b	9 (32.1)

^{ab} Different superscripts within rows indicate significant differences (p=0.030).

Table 3.5. Nuclear status of Brahman and Angus oocytes after standard IVM (n=234) and after extended IVM with cAMP modulators (n=355).

	Standard IVM			Extended IVM with cAMP modulators		
	GV, no. (%)	MI, no. (%)	MII, no. (%)	GV, no. (%)	MI, no. (%)	MII, no. (%)
8 hour	10 (15.4) ^a	40 (61.5) ^A	1 (1.5)	48 (75.0) ^b	5 (7.8) ^B	0
13 hour	1 (1.7) ^a	39 (67.2) ^A	2 (3.4)	22 (34.9) ^b	25 (39.7) ^B	0
18 hour	1 (1.9)	13 (25.0) ^a	27 (51.9) ^A	7 (12.7)	32 (58.2) ^b	7 (12.7) ^B
23 hour	0	10 (16.9) ^a	48 (81.4) ^A	4 (6.8)	22 (37.3) ^b	17 (28.8) ^B
28 hour				6 (11.5)	13 (25.0)	28 (53.8)
33 hour				8 (12.9)	19 (30.6)	28 (45.2)

^{abAB} Different superscripts within rows indicate significant differences (p < 0.05).

Note: The number of MII oocytes at 33 hours Extended IVM is significantly different from the number of MII oocytes at 23 hours Standard IVM for all cattle (p ≤ 0.001).

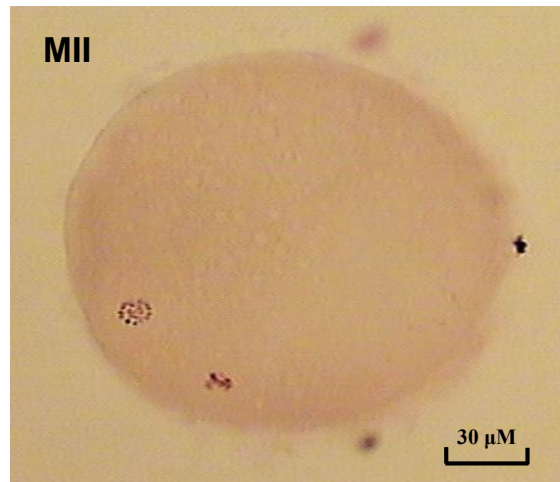
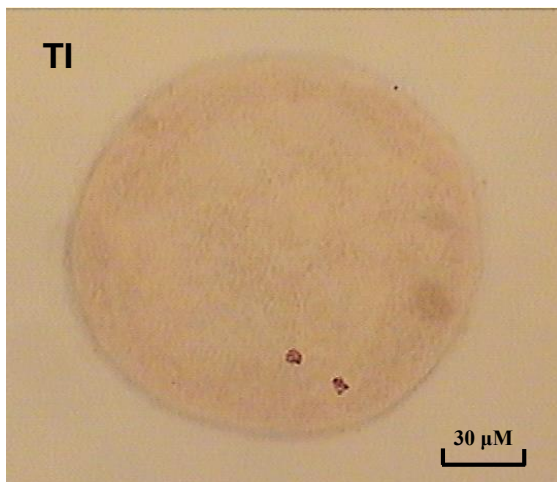
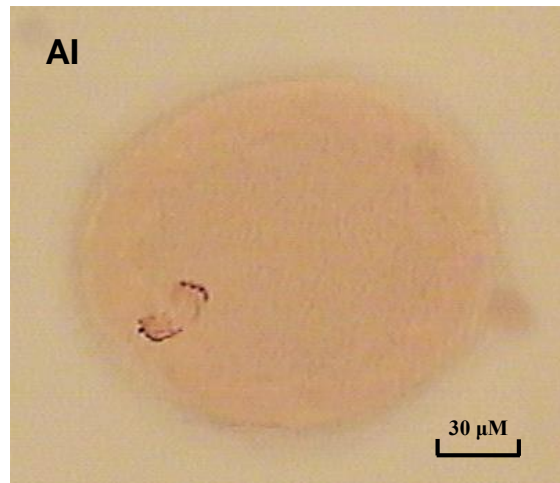
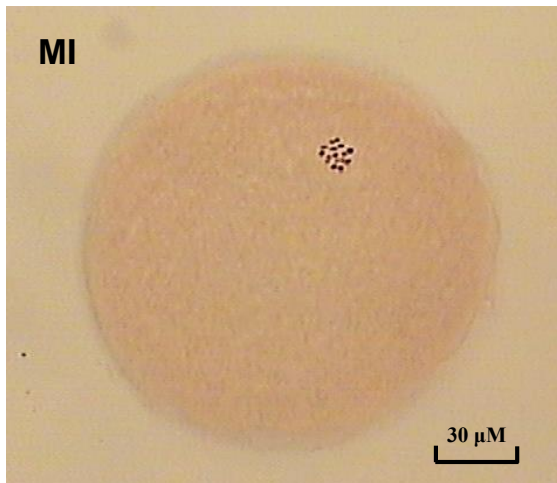
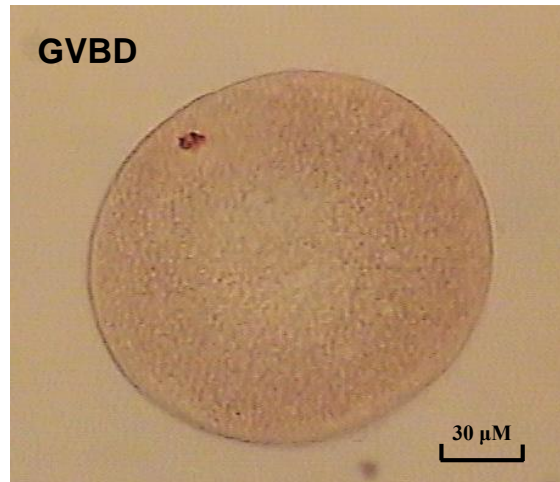


Figure 3.2. Stages of nuclear maturation after staining with aceto-orcein (20X). GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphase of the first meiotic division; AI: anaphase of the first meiotic division; TI: telophase of the first meiotic division; MII: metaphase of the second meiotic division.

Experiment 2 Results

Table 3.6. Nuclear status of bovine oocytes after standard IVM (n=133) and after use of cAMP modulators in oocyte holding media followed by extended IVM (n=194).

	Standard IVM			Extended IVM with cAMP modulators		
	GV, no. (%)	MI, no. (%)	MII, no. (%)	GV, no. (%)	MI, no. (%)	MII, no. (%)
8 hour	16 (44.4) ^a	9 (25.0) ^A	0	29 (90.6) ^b	0 ^B	0
13 hour	2 (5.1) ^a	31 (79.5) ^A	0	22 (62.9) ^b	7 (20.0) ^B	0
18 hour	0 ^a	8 (29.6)	12 (44.4) ^A	11 (31.4) ^b	14 (40.0)	0 ^B
23 hour	0 ^{aa}	5 (16.1) ^a	25 (80.6) ^A	5 (16.7) ^{bb}	13 (43.3) ^b	5 (16.7) ^B
28 hour				7 (23.3)	10 (33.3)	8 (26.7)
33 hour				8 (25.0)	6 (18.8)	14 (43.8)

^{abAB} Different superscripts within rows indicate significant differences ($p < 0.05$).

Note: The number of MII oocytes at 33 hour Extended IVM is significantly different from the number of MII oocytes at 23 hours Standard IVM ($p=0.006$).

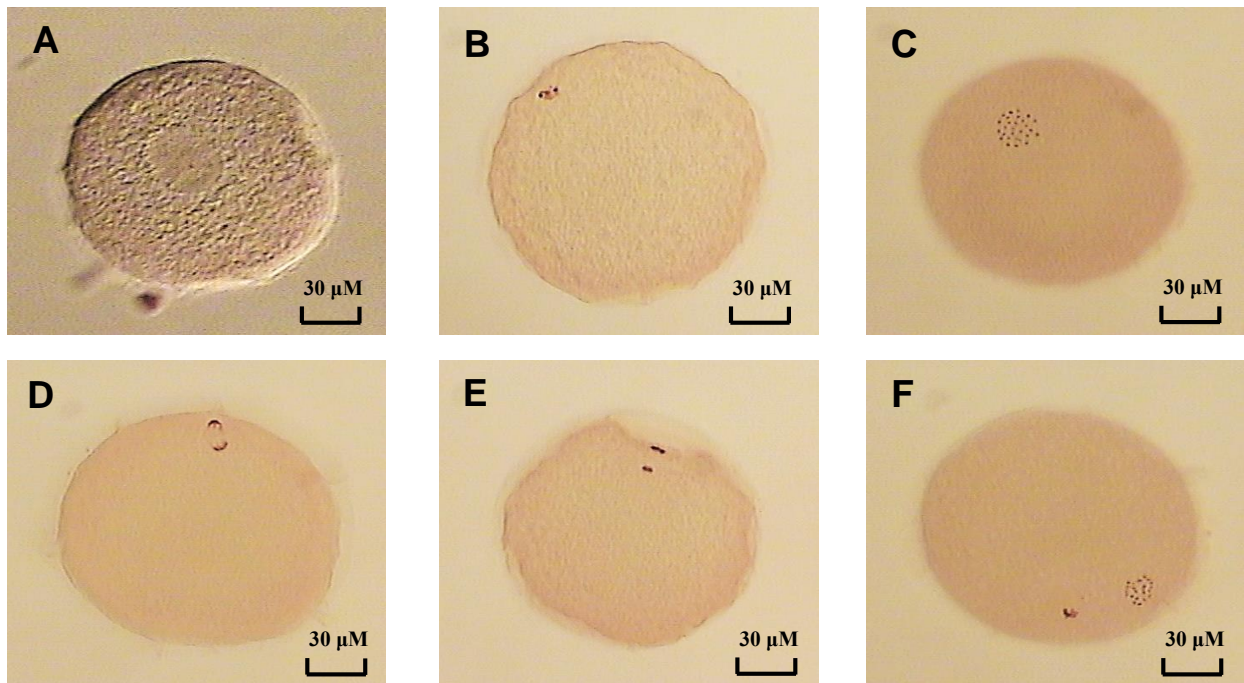


Figure 3.3. Stages of nuclear maturation after staining with aceto-orcein (20X). A) GV stage oocyte at 13 hours IVM, B) GVBD at 13 hours IVM, C) MI oocyte at 18 hours IVM, D) AI oocyte at 13 hours IVM, E) TI oocyte at 23 hours extended IVM, F) Mature MII oocyte at 23 hours IVM.

Discussion

The aim of these experiments was to determine whether or not the cAMP modulators utilized in this study (IBMX, FSK, and cilostamide) cause a delay in meiotic resumption of bovine oocytes. A delay in resumption of meiosis could be beneficial in allowing oocytes enough time to acquire improved developmental competence during oocyte *in vitro* maturation, which is a key step of *in vitro* embryo production. Additionally in the first experiment, both Brahman and Angus oocytes were collected to determine whether there was any differences between these breeds after either treatment.

For experiment 1, Table 3.5 presents the data for both Brahman and Angus oocytes pooled together (n=589). A significant difference ($p<0.05$) exists between standard IVM and extended IVM at all meiotic stages during all time samples that could be statistically analyzed with a chi-square test (see Table 3.5), indicating that the cAMP modulators do significantly delay oocyte maturation. For example, the comparison between standard IVM and extended IVM at 8 hours for the GV stage is 15.4% vs. 75.0% respectively ($p\leq 0.001$). This suggests that during standard IVM, almost 85% of the oocytes have already resumed meiosis, but only 25% have resumed meiosis in extended IVM with the cAMP modulators present. This trend is observed at each time sample throughout all meiotic stages. At the completion of standard IVM, which is 23 hours, 81.4% of oocytes have reached the MII stage and are ready to be fertilized. However, only 28.8% of the oocytes have achieved the MII stage after 23 hours extended IVM, again indicating that meiosis was significantly delayed during extended IVM ($p\leq 0.001$). Tables 3.1 and 3.2, which contain data for Brahman oocytes and Angus oocytes individually, both present this same trend.

It is also of note that the standard IVM oocytes in experiment 1 followed an expected pattern of maturation through the GV, GVBD, MI, AI, TI, and MII stages progressively. There is a decreasing number of early stage oocytes and an increasing number of later stage oocytes as maturation time increases. However, in extended IVM there is one irregularity in this pattern.

Notice in Table 3.5 that there is a higher percentage (53.8%) of MII at the 28 hours compared with 33 hours (45.2%), which is abnormal since there has consistently been a higher percentage of MII as time progresses before this point, indicating that maturation is gradually taking place as it should. This irregularity results mainly from the Brahman cattle oocytes (Table 3.1), in which there is a large difference between 28 hour MII (55.6%) and 33 hour MII (32.1%). This does not occur in Angus cattle (Table 3.2), where the number of MII oocytes is slightly higher at 33 hours compared to 28 hours (55.9% vs. 52.0%, respectively). Although this could be a result of some difference between breeds, it is more likely due to random variation in oocyte quality. This irregularity probably occurred as a result of lower quality oocytes being grouped into the 33 hour extended IVM sample due to random chance, and may be resolved by increasing the sample size to account for random variation within oocytes.

The results of experiment 2 are shown in Table 3.6. In this experiment, cAMP modulators were added to the holding media during oocyte collection in order to avoid the drop in cAMP that occurs within 2 hours of removal from the follicle in bovine oocytes (Luciano et al., 2004; Albuz et al., 2010). In this experiment, the overall maturation rate for standard IVM compared with extended IVM was 80.6% vs. 43.8% ($p=0.006$). For standard IVM, maturation once again progressed as expected. As with the first experiment, there were significant differences shown between standard IVM and extended IVM at all time points for all nuclear statuses, except for 18 hour MI (29.6% standard vs. 40.0% extended, $p=0.563$). This irregularity was probably due to a variation in oocyte quality and a low sample size, where a larger number of oocytes may have shown a significant difference, as the power of this particular chi-square test was low (0.083). Overall, results were similar to the first experiment and once again indicated that the cAMP modulators were delaying maturation. For example, a comparison between GV stage oocytes at 8 hours standard IVM to 8 hours extended IVM resulted in a significant difference (44.4% vs. 90.6%, $p\leq 0.001$). At 13 hours, the majority of oocytes in standard IVM were MI stage, but only 20% reached MI in extended IVM. By 23 hours, 80.6% of

oocytes were fully matured in standard IVM compared to only 16.7% in extended IVM ($p \leq 0.001$).

Within experiment 2, the addition of cAMP modulators to the holding media during oocyte collection did not seem to positively affect the outcome of extended IVM compared to the first experiment. The rates at each sample time and each nuclear stage were very similar between these two experiments, especially when comparing the overall maturation rates. For experiment 2, 80.6% of oocytes reached MII after standard IVM and 43.8% reached MII in extended IVM, which is very similar to the 81.4% vs. 45.2% attained in experiment 1. Therefore, maturation rates for extended IVM did not improve after the addition of cAMP modulators to the holding media, and these rates were still significantly less than those achieved in standard IVM.

In the first experiment, a comparison between cattle breeds was analyzed in addition to determining whether maturation was effectively delayed. Data comparing Brahman and Angus oocytes for standard IVM is located in Table 3.3. There are no significant differences between Angus and Brahman oocytes after standard IVM, so any differences between these breeds after extended IVM would probably be due to some effect of the cAMP modulators on the oocytes of these breeds. However, as indicated in Table 3.4 comparing both breeds' oocytes after extended IVM, all cattle reacted similarly to the extended IVM treatment. There is one significant difference found between Angus MI oocytes (17.6%) and Brahman MI oocytes (46.4%) at 33 hours extended IVM, but this further supports the proposition that the Brahman cattle had a lower number of good quality oocytes at the 33 hour time sample due to random chance. A higher percentage of oocytes stopping at the MI phase during maturation is an indicator of poor quality oocytes, and is not due to a difference in breed. Therefore, there were no differences found between Angus cattle and Brahman cattle as a result of either maturation treatment.

To summarize, both of these experiments indicate that the cAMP modulators delay oocyte maturation, which is consistent with previous research utilizing these specific

modulators. One initial study in 1996 supplemented IBMX and cilostamide in a dose-dependent manner on rat oocyte maturation, finding that GVBD was significantly inhibited using cilostamide at 10 and 100 μM and IBMX at concentrations $>200 \mu\text{M}$ (Tsafiri et al., 1996). A study on bovine oocytes in 2002 supplemented 50 μM cilostamide for 16 hours after exposure to 2 mM IBMX during collection, finding that 35% of oocytes reached MII (Thomas et al., 2002), which is about 10% less than experiments 1 and 2. This suggests that the much higher IBMX concentration (2 mM vs. 500 μM) and higher cilostamide concentration (50 μM vs. 20 μM) may have had a greater detrimental effect on the oocytes than the extended IVM used for these experiments. The previously discussed research by Albuz, which used the same types and concentrations of cAMP modulators, found that after 20 hours of culture in the SPOM system with modulators only about 30% of oocytes reached the MII stage, compared to almost 100% MII in the control IVM after 24 hours culture (Albuz et al., 2010).

Also of concern is whether or not the effects of these cAMP modulators are reversible. The goal is to delay maturation, not to halt it, and in order for this to occur the inhibitory effects of the cAMP modulators must be reversible. In addition, the percentage of oocytes that reach the MII stage after extended IVM must be comparable to the percentage of MII after standard IVM for this system to be useful for IVP. MII oocytes are ready for fertilization, thus this is the stage that needs to be reached in order for IVF to be successful. To determine whether the inhibitory effects are being reversed, the number of MII oocytes at 23 hour standard IVM should not be significantly different from the number of MII oocytes at 33 hour extended IVM. However, this is not the case in either experiment. The number of MII oocytes at 23 hour standard IVM is significantly different from the number of MII oocytes at 33 hour extended IVM: 81.4% vs. 45.2% ($p \leq 0.001$) in the first experiment, and 80.6% vs. 43.8% ($p = 0.006$) in experiment 2. These results indicate that the inhibitory effects of cAMP on meiotic resumption is not completely reversible, and that extended IVM produces about half as many matured oocytes as standard IVM. For Brahman cattle oocytes in experiment 1, there is also a difference between MII at 23 hours

standard IVM and 33 hours extended IVM (85.2% vs. 32.1%, $p \leq 0.001$). However, as previously explained this difference is due in large part to poor quality oocytes randomly selected for the 33 hour extended IVM time samples in Brahman cattle. In Angus oocytes (Table 3.3), there was 78.1% MII at 23 hours standard IVM compared to 55.9% MII at 33 hours extended IVM. This data is not significantly different ($p=0.098$), although this may be due to sample size as the power of this particular chi-square test is low.

Experiments 1 and 2 suggest that the effects of the cAMP modulators used in this extended IVM are not reversible. Contrary to these findings, Tsafiriri and colleagues reported in their 1996 study on rat oocytes that the effects of IBMX and cilostamide were reversible after 3 hours of culture followed by washing and transfer to control medium, obtaining GVBD in 100% of oocytes in 10 μM cilostamide and 96% of oocytes in 200 μM IBMX (Tsafiriri et al., 1996). A major difference here is that these modulators were used individually, on rat oocytes, and in smaller concentrations. A second study on mice oocytes also found the effects of 40 μM IBMX on meiotic resumption to be reversible after culturing oocytes for up to 24 hours and then transferring to standard media for 3 hours (Vivarelli et al., 1983). However, again the concentration of IBMX used was much lower than in the extended IVM treatment. The study by Albuz and colleagues also found that the effects of the modulators were reversible, reporting that their SPOM system resulted in about 95% MII oocytes after 28 hours culture compared to almost 100% in the control (Albuz et al., 2010). Alternatively, Gharibi reported in 2013 that the effects of 22 hour culture of sheep oocytes in cilostamide at 1 μM was reversible, but the effects at higher concentrations of 10 and 20 μM were not reversible, and blastocysts rates were significantly less in these oocytes (Gharibi et al., 2010). Concentrations used in this particular study were more similar to the extended IVM system than both Vivarelli's and Tsafiriri's culture conditions, and Gharibi's results agree that effects are not reversible, although this study used sheep oocytes rather than bovine oocytes.

To summarize, results of these experiments both indicate that the cAMP modulators do delay bovine oocyte maturation in both Brahman and Angus cattle. However, the final maturation rates in the extended IVM system are significantly lower than those achieved in standard IVM, suggesting that extended IVM is less optimal than standard spontaneous IVM for IVP because standard IVM produces a greater number of matured oocytes.

CHAPTER IV

UTILIZING A cAMP ENZYME IMMUNOASSAY TO DETERMINE VARIATION IN OOCYTE cAMP LEVELS DURING IVM TREATMENTS

Introduction

In the previous experiments it was determined that the oocytes in the extended IVM treatments did undergo a significantly delayed, prolonged maturation, shown by a delay in the progression of nuclear stages throughout the extended IVM treatment compared to standard IVM. These results support the idea that cAMP modulators keep cAMP levels high enough to maintain meiotic arrest, therefore extending the time required for maturation. However, the results of the previous experiments also indicated that the effects of the cAMP modulators on the oocytes may not have been completely reversible due to a significantly lower maturation rate in extended IVM compared to standard IVM (45.2% vs. 81.4% for experiment 1; 43.8% vs. 80.6% for experiment 2). This obviously makes the extended IVM system less than optimal for use on a large scale basis, and the standard IVM system remains the most effective in obtaining mature oocytes for use in IVP.

It is hypothesized that the low maturation rates result from an inadequate decrease in cAMP levels, whereby the oocytes are unable to resume and complete maturation properly. If the cAMP modulators are found to keep cAMP levels too high, or if cAMP is not decreasing in a similar manner to standard IVM, this may be the major issue with the extended IVM system. Therefore, the next experiment will examine the levels of cAMP within the oocytes throughout both standard and extended IVM. The aim is to determine whether intra-oocyte cAMP levels in the extended IVM treatment are higher than standard IVM, as is suspected. It will also determine whether the levels of cAMP in the extended IVM are eventually decreasing, and if this decrease is similar to that which is observed in standard IVM. To accomplish this, a cAMP enzyme immunoassay will be performed after collecting oocytes at various times throughout both treatments. The treatments will be similar to those utilized in the second experiment,

where cAMP modulators are added to the holding media in extended IVM to prevent any drop in cAMP that may occur before the oocytes can be placed in IVM.

Determining the cause of the deficiency in the maturation rate of extended IVM would be useful in order identify modifications that can be made to improve this system. If the results of the cAMP assay indicate that oocyte cAMP concentrations in extended IVM are remaining higher than standard IVM throughout maturation, the procedure may be adjusted to compensate for this. For example, the time frame for exposure to cAMP modulators could be modified, the amount of cAMP modulators used in the media could be changed, or different modulators could be examined. However, if the cAMP levels are decreasing in extended IVM similarly to standard IVM, there may be other unknown reasons for the lack of maturation rates in extended IVM, and further research would need to be conducted on this issue.

Materials and Methods

Unless otherwise stated, all chemicals were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Experimental Design

Oocytes were collected with TUGA on multiple collection days and divided into two groups: standard IVM and extended IVM. Oocytes in standard IVM were held in HEPES-TALP medium during collection, and oocytes in extended IVM were held in HEPES-TALP with FSK and IBMX. Once collection was complete, oocytes were moved into their respective maturation treatments, which were standard 23-hour IVM and extended IVM consisting of a 2-hour pre-IVM and 31-hour extended IVM. Sample times for standard IVM were 0, 8, and 23 hours, and times for extended IVM were 0, 8, 18, and 33 hours. Cumulus cells were removed by vortexing in a hyaluronidase solution, then oocytes were pooled in groups of ten and frozen at their respective

sampling times. This was repeated until enough oocytes were obtained to conduct a cAMP assay with at least seven replicates for each of the sample times, and then the cAMP biotrak enzyme immunoassay (GE Healthcare) was performed as per the protocol of the kit.

Preparation

Required media included Dulbecco's Phosphate Buffered Saline (D-PBS), HEPES-TALP, standard maturation medium, HEPES-TALP medium with IBMX and FSK, and extended IVM medium with cilostamide. D-PBS was prepared and stored until needed. On the afternoon before oocyte collection, D-PBS is placed in the incubator to warm overnight. 10 mL of bovine calf serum (BCS) and 1 mL of Heparin (Sagent Pharmaceuticals, Schaumburg, IL, USA) are added to the D-PBS the morning of collection. All media was prepared the afternoon before oocyte collection, and stored in the refrigerator overnight. HEPES-TALP holding media for the control group consists of 20 mL HEPES-TL (Caisson Labs, North Logan, UT, USA) with 60 mg BSA and 200 μ L sodium pyruvate and pen/strep (Gibco, No.15140). The holding medium for the treatment group consisted of the same HEPES-TALP medium supplemented with 500 μ M IBMX and 100 μ M FSK. The standard maturation medium is composed of TCM-199 with 10% fetal bovine serum (FBS), 0.2 mM sodium pyruvate, 1% pen/strep, 2 mM glutamine, and FSH (5.0 μ g/mL). The pre-IVM medium is made up of HEPES-TALP supplemented with 100 μ M FSK and 500 μ M IBMX. Extended IVM medium consists of the standard TCM-199 maturation medium supplemented with 20 μ M cilostamide. (See Appendix B for media formulations and stock solutions.)

On collection day, all necessary dishes of media were prepared and equilibrated for at least two hours in the incubator before being used. Petri dishes (35 mm, BD Falcon) of HEPES-TALP were prepared for oocyte collection, washing, and sorting into treatment groups and placed in a non-CO₂ incubator at 39°C to warm. Dishes of maturation medium included 35 mm wash dishes and 4-well plates (Nunc, Thermo Fisher Scientific Inc., Waltham, MA, USA) each of

which had 35 μ L drops of maturation medium covered with 570 μ L of pre-warmed, pre-equilibrated mineral oil (embryo tested). The four well plates were labeled for each sample hour and placed in a 5% CO₂ incubator at 39°C. The pre-IVM HEPES-TALP medium with IBMX and FSK was prepared in one 35 mm wash dish and one 4-well plate with 500 μ L of pre-IVM media in each well, and these dishes were placed in a non-CO₂ incubator at 39°C. The extended IVM medium with cilostamide was prepared similar to the standard maturation medium, with 35 mm wash dishes and 4-well plates each with 35 μ L drops of media covered with mineral oil. These were also labelled for each sample time, and placed in the CO₂ incubator at 39°C and 5% CO₂.

Oocyte Collection

Oocytes were collected from 3 to 8 mm follicles by transvaginal ultrasound-guided follicular aspiration over four collection days. Collection media was a pre-warmed D-PBS with 0.1% heparin and 1% BCS. Oocytes from each cow were sorted to include only those with two or more layers of CCs and transferred to a 35 mm dish containing pre-warmed HEPES-TALP holding medium. Oocytes were then divided by cow into two treatment groups: standard IVM and extended IVM. Oocytes selected for extended IVM were moved from standard HEPES-TALP into HEPES-TALP with IBMX and FSK. All oocytes were then placed in a non-CO₂ incubator at 39°C. Total oocyte collection time ranged from 2 to 2.5 hours.

Transvaginal Ultrasound-Guided Follicular Aspiration

Oocytes were obtained by TUGA (Figure 3.1). Each cow is retained in a holding chute and given an epidural injection of 6 mL lidocaine (Vet One, Product No. 510212). An ultrasound probe with a 7.5 MHz transducer is inserted into the vaginal canal, and the ovary is grasped through the rectum and positioned against the vaginal wall next to the transducer at the end of the ultrasound probe. The ovary is viewed on an ultrasound monitor and follicles are located.

An 18-gauge disposable collection needle, located inside the ultrasound probe, is connected to a suction pump via polyethylene tubing. The collection needle is inserted through the vaginal wall into the follicle, and follicular fluid along with the oocyte is aspirated out. The needle is changed after each cow, tubing and needles are rinsed with collection media (D-PBS with heparin and BCS), and the follicular fluid and oocytes are deposited into an embryo collection filter (Agtech Inc., Manhattan, KS, USA). All media is maintained at 39°C during collection.

Oocyte Culture

Oocytes in standard IVM were washed four times through IVM media, then divided evenly into three groups for the 0, 8, and 23-hour time samples. Individual four-well plates were previously prepared and labelled for each of these time samples, and contained ten to twelve oocytes in each 35 μ L drop. Oocytes were cultured in 5% CO₂ at 39°C for the required times, ranging from 8 to 23 hours.

Oocytes in extended IVM were washed through pre-IVM medium with FSK and IBMX, and then left in this medium for two hours in a non-CO₂ incubator at 39°C. Oocytes were washed once through HEPES-TALP, then washed four times through the extended IVM media with cilostamide and divided evenly into four groups for 0, 8, 18, and 33 hour time samples. Each group of oocytes was placed in individual 4-well plates and cultured in 5% CO₂ at 39°C for the required times.

Sampling

Samples were taken at 0 hours for both treatments, 8 hours for both treatments, 18 hours extended IVM, 23 hours standard IVM, and 33 hours extended IVM. The first samples (time 0) were completed before the oocytes entered each respective IVM treatment. For both treatments, groups of oocytes were removed from maturation medium at the appropriate time

and then placed in warm hyaluronidase solution in a 15 mL sterile centrifuge tube. These were placed in a water bath at 39°C for 5 minutes, then vortexed at max speed for 8 minutes to remove CCs. Oocytes were then moved in minimal media (2-3 μ L) in groups of ten to small centrifuge tubes, and 100 μ L lysis reagent 1B (prepared from the assay kit) was added to the tube. These were stored at -80°C until the cAMP assay was performed.

cAMP Enzyme Immunoassay

The assay was purchased as a kit from GE Healthcare (Product No. RPN225). It is a competitive ELISA utilizing a rabbit anti-cAMP antibody. The plate is coated with donkey anti-rabbit IgG. This kit is dual range, with an acetylation protocol measuring in the range of 2-128 fmol per well and a non-acetylation protocol measuring 25-6400 fmol per well. Additionally, these protocols can be used with or without a novel lysis reagent. For this experiment, the acetylation protocol with the lysis reagent was used for maximum sensitivity (Appendix A).

Results were analyzed using a microplate reader (Biotek FLx800) to measure the optical density of each well and results were viewed using the Gen5 Microplate Data Collection and Analysis Software program (Biotek). A standard curve was built with standards for 2, 4, 8, 16, 32, 64, and 128 fmol. Results were calculated by obtaining the mean optical density of each sample and subtracting the optical density of the non-specific binding wells. The concentration of cAMP in each sample was calculated using the standard curve (Figure 4.1). See assay protocol for specific calculations.

Statistical Analysis

Assay results were analyzed using Sigma Stat Statistical Software Version 3.5 (Systat Software, Inc., Chicago, IL, USA). A one-way ANOVA was performed followed by a Tukey's

pairwise test to detect significant differences between any time points in either treatment.

Differences of $p < 0.05$ were considered significantly different.

Results

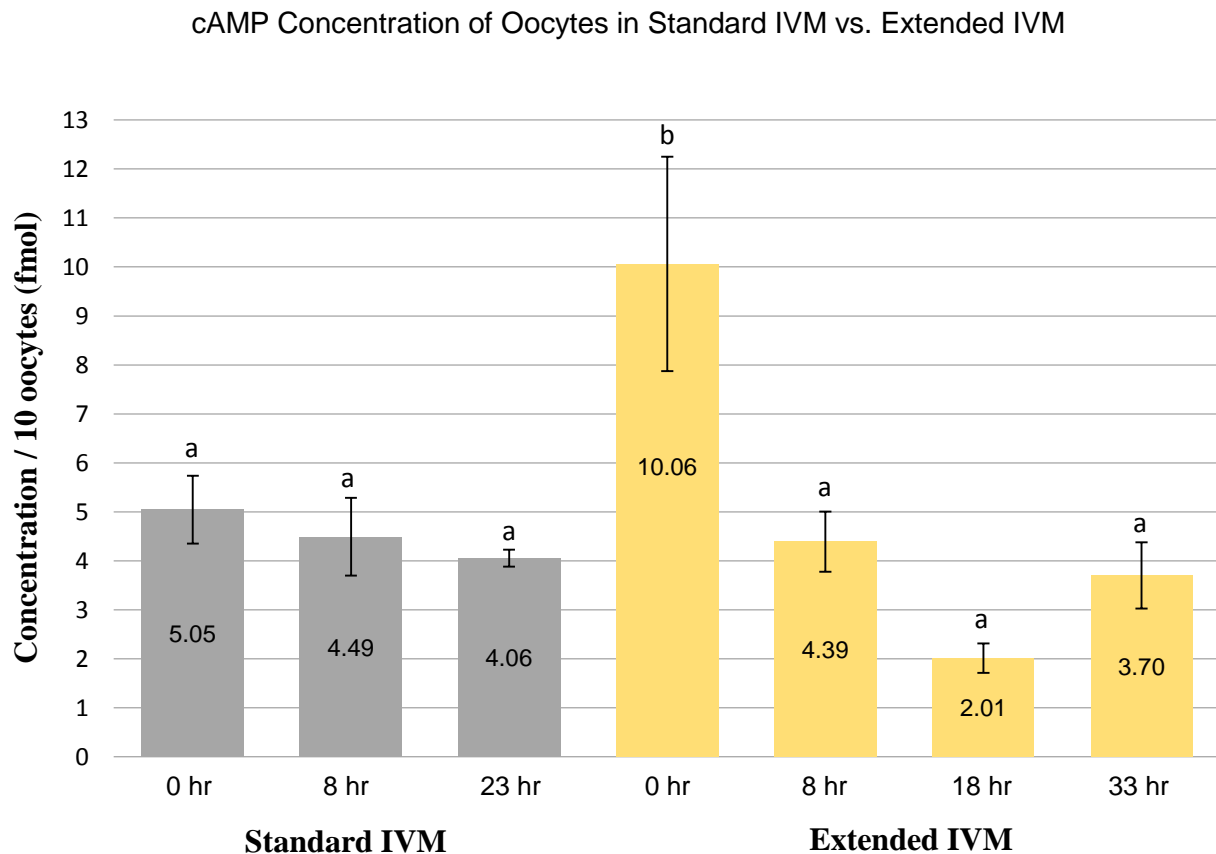


Figure 4.1. cAMP concentrations for each sample time during standard IVM and extended IVM. Concentrations are measured in fmol per 10 oocytes. Change in superscript indicates significant differences ($p < 0.05$). Standard error bars are shown.

Discussion

The aim of experiment 3 was to determine intra-oocyte cAMP levels at different time points during standard and extended IVM using a cAMP enzyme immunoassay. These results may be useful in determining whether the cAMP levels in the oocytes are high as a result of the cAMP modulators, and if the concentration of cAMP decreases in a manner

similar to standard IVM. This may give insight into the low overall maturation rates of extended IVM compared to standard IVM found in the previous experiments.

During standard IVM, there was no detected decrease in cAMP between 0 and 23 hours. At the 0 hour time point, which was about 3 hours after collection, oocytes had a cAMP concentration of 5.05 fmol in ten oocytes. The concentrations at 8 hours and 23 hours (4.49 fmol and 4.06 fmol) were slightly less than but not significantly different from 0 hours. Therefore, no significant decrease in cAMP was detected throughout maturation in standard IVM. These results indicate that the cAMP concentration within the oocytes dropped to base level within 3 hours of being removed from the follicular environment, and meiotic resumption of the oocyte has probably commenced by this time.

There was, however, a significant decrease in cAMP during extended IVM from 0 hours to 8 hours, at 10.06 fmol vs. 4.39 fmol respectively ($p=0.012$). There were also significant differences between 0 hour compared to 18 hour ($p<0.001$) and 33 hour ($p=0.002$). Additionally, after 8 hours extended IVM there were no further decreases in cAMP detected, as there were no significant differences between 8, 18, and 33 hours. Results indicate that by 8 hours, the cAMP concentrations have reached a base level since these time points are also not significantly different from any time point in standard IVM.

Data also shows a significant difference between 0 hour extended IVM and 0 hour standard IVM (10.06 fmol vs. 5.05 fmol, $p=0.035$). This indicates that the cAMP modulators FSK and IBMX in the HEPES-TALP collection medium for extended IVM did keep the cAMP concentration from declining after removal from the follicular environment. By 8 hours extended IVM, the cAMP levels have already dropped significantly to 4.39 fmol/ten oocytes ($p=0.012$) compared to time 0, and this continues through 18 and 33 hours. These final three time points are not significantly different from the concentrations shown in standard IVM, which have probably dropped to the lower threshold level.

In summary, the results of this assay indicate that cAMP modulators IBMX and FSK did cause a significant increase in cAMP levels within the first two hours during oocyte collection, as evidenced by significantly higher concentrations of cAMP at 0 hour extended IVM compared to 0 hour standard IVM. This suggests that FSK and IBMX prevented the substantial drop in cAMP that had already occurred by 0 hours standard IVM. Additionally, there was a significant decrease from 0 hours to 8 hours extended IVM, which may be a result of the cilostamide-only extended IVM medium. The cilostamide added to the extended IVM media did not appear to maintain higher cAMP levels compared to 8 hours standard IVM.

There has not been a large amount data reported on cAMP concentrations using an enzyme immunoassay, although some reports have been shown using radioimmunoassay (Vivarelli et al., 1982; Mattioli et al., 1994; Thomas et al., 2002; Albuz et al., 2010). One study in 2005 which did utilize the enzyme immunoassay (GE Healthcare) examined the effects of supplementing 0.5 mM IBMX for 3 hours, finding a cAMP concentration of 0.497 fmol per oocyte (Luciano et al., 2005). This is about half the concentration observed in this experiment after 3 hour incubation in IBMX and FSK (1.0 fmol/oocyte), which is perhaps due to the supplementation of FSK in addition to IBMX since the concentrations of IBMX were the same. Other studies using the radioimmunassay to determine cAMP concentration found similar ranges, although comparisons are difficult because each uses different modulators in differing concentrations, varying lengths of time, and different animal models. An early study conducted in 1983 on mouse oocytes found the initial cAMP content to be 2.1 fmol, and this drops to 0.9 fmol per oocyte after 2 hours of culture. Addition of 1 mM IBMX for 2 hours increased cAMP content to 3.1 fmol (Vivarelli et al., 1983). Another study supplemented 0.1 mM forskolin with 50, 250, and 500 μ M milrinone (a PDE3 inhibitor similar to cilostamide) for 5 hours, observing a significant increase in

cAMP compared to the control (about 2.5 fmol, 4.5 fmol, 4.9 fmol vs. 0.5 fmol, respectively). Additionally, the greater concentrations at 250 and 500 μ M resulted in significantly higher cAMP compared to the 50 μ M concentration (Thomas et al, 2002). Here, the concentration of FSK was the same but the concentration of milrinone was much higher than cilostamide, although it may not be as effective. After 8 hours culture during experiment 3, oocytes here contained 0.439 fmol per oocyte which is much less than that reported by Thomas and colleagues. Although measurements in Thomas' study were at 5 hours compared to 8 hours in experiment 3, the lower cAMP concentration obtained here suggests that milrinone as a PDE3 inhibitor may be more effective than cilostamide. The aforementioned study by Albuz in 2010 also utilized a radioimmunoassay, and here the types, concentrations, and timing of cAMP modulators were exactly the same as those used in experiment 3, although a different base medium was used. Results after pre-IVM with IBMX and FSK indicated that cAMP was increased almost 10-fold compared to the control (about 200 fmol per COC compared to about 12 fmol, respectively) (Albuz et al., 2010). This measurement was obtained after 2 hours culture, and is much higher than those observed in experiment 3. However, cAMP measurements in the Albuz study were done on full COCs rather than denuded oocytes, which would result in higher levels due to the cAMP in the cumulus cells. Additionally, the pre-IVM treatment in conjunction with 31 hour IVM resulted in 1.2 fmol per oocyte compared to about 0.2 fmol in the control (Albuz et al., 2010). These measurements are more similar to those found in experiment 3, as the radioimmunoassay was done on denuded oocytes rather than COCs.

In summary, the results of experiment 3 indicate that FSK at a concentration of 100 μ M combined with IBMX at a concentration of 500 μ M successfully prevented cAMP decline during the first 3 hours of collection after removal from the follicular environment. However, cilostamide at a concentration of 20 μ M in the extended IVM step did not appear to maintain

higher levels of cAMP by 8 hours. Therefore, adjustments may be required for extended IVM, perhaps by replacing cilostamide with a different modulator, combining it with other modulator, or altering the concentrations that are added to the extended IVM media.

CHAPTER V

SUMMARY AND CONCLUSIONS

Oocyte *in vitro* maturation is a reproductive technique that has applications in a variety of industries, including commercial livestock production, human infertility treatment, nuclear transfer (cloning), stem cell research, transgenic animal production technologies, and reproductive technologies to preserve rare and endangered species. All of these commercial and research industries rely on producing viable, developmentally competent oocytes for optimal success. Currently, IVM is the limiting step of *in vitro* embryo production. It has been found in numerous species that *in vitro* matured oocytes produce about half as many embryos as *in vivo* matured oocytes. Examples of this include sheep blastocyst rates (74% vs. 35%; Thompson et al., 1995), bovine blastocyst rates (58% vs. 39%; Rizos et al., 2002), human implantation rates (17% vs. 9.5%; Child et al., 2002), and mouse live birth rates (52% vs. 21%; Eppig et al., 2009). Additionally, IVM has the advantage that it does not require the use of expensive hormones in order to increase ovarian function, which also avoids the risk of ovarian hyperstimulation syndrome that is especially prevalent in human IVF. With human oocytes, IVM is never used because it is so inefficient. Instead, numerous mature oocytes must be obtained from the ovaries, making ovarian stimulation necessary even though it is harmful to the health of these women.

Improvement of this insufficient *in vitro* maturation system relies on understanding the biological aspects of *in vivo* oocyte maturation in order to successfully mimic this process in the laboratory. Research into this subject over the last decade has made many advances into the overall understanding of the pathways of oocyte maturation. Most notably of these, it was discovered that cAMP levels play a large role in maintaining oocyte meiotic arrest in the ovarian follicle, and that a decrease in cAMP removes the block to maturation that allows the oocyte to prepare for ovulation and fertilization.

This thesis examined the effects of three cAMP modulators, IBMX, FSK, and cilostamide, in an extended two-step IVM system to the standard, spontaneous *in vitro* maturation procedure used currently. To summarize the results, it was determined that these cAMP modulators do significantly delay meiotic maturation. However, it appears that the effects of the modulators are not completely reversible due to the fact that an average of 44.5% of oocytes from both experiments reached the mature MII stage after extended IVM compared to 81% in standard IVM. Results from the second experiment indicated that the addition of cAMP modulators within the holding media did not affect the outcome of extended IVM, as the overall maturation rates were similar to experiment 1. Data also showed that the number of MII oocytes after extended IVM was not comparable to standard IVM in either experiment, which suggests that this system is less favorable than standard IVM to be used for IVP. This is most likely because the effects of cAMP modulators were not completely reversed, although the use of these modulators does have potential for improving IVM. If a similar system could be designed where cAMP levels will eventually decrease in a manner similar to that which occurs *in vivo*, this extended IVM method could result in maturation rates comparable to standard IVM. In addition, these oocytes may be more competent than those from standard IVM, since both cytoplasmic and nuclear maturation will have had increased time to occur properly. This could result in higher success rates of embryos produced with IVP.

In addition to cytoplasmic maturation, another advantage to extending the time of IVM is that it may allow researchers to expose oocytes to a multitude of exogenous substances that may improve oocyte developmental competence. Already research is being conducted on supplementing numerous OSFs, GSH, BMP15, GDF9, etc., with varying success. However, if exposure time to these potentially beneficial substances is increased, perhaps oocyte developmental competence would improve and therefore increase embryo production rates. Thus, the extended length of IVM would allow increased manipulation of the environment *in vitro* in the hope of further improving oocyte developmental competence.

The results of the cAMP ELISA in the third experiment indicated that cAMP modulators IBMX and FSK did cause a significant increase in cAMP concentration within the oocytes. This was evidenced by a much higher concentration at 0 hour extended IVM compared to standard IVM ($p=0.035$). Additionally, cAMP in standard IVM appeared to have already dropped to base level by the 0 hour time point, which was approximately 3 hours post-collection. This is consistent with past literature suggesting an exponential decrease in cAMP within the first two hours of collection (Luciano et al., 2004; Albuz et al., 2010), resulting in the removal of meiotic arrest and spontaneous maturation.

FSK and IBMX appear to have maintained intra-oocyte cAMP levels within the first 3 hours of collection, but it is more difficult to determine the effects of cilostamide on cAMP. Results suggest that cilostamide was not as effective as IBMX and FSK in keeping cAMP high. By 8 hours extended IVM, cAMP had dropped to concentrations similar to those observed in standard IVM, which appear to be the lower threshold levels. This could be due to a number of factors. Perhaps the concentration of cilostamide was too low, or it may be necessary to add an alternate cAMP modulator to extended IVM either in addition to or instead of cilostamide. These findings are somewhat contrary to previous literature, which has maintained that cilostamide in this concentration does delay meiotic maturation. For example, one study supplemented cilostamide at 10 μM and 20 μM for 12 hours, founding a significantly higher number of COCs at the GV stage compared to the control, (30.4% and 45.3%, respectively) (Mayes and Sirard, 2002). However, this study did not measure the concentrations of cAMP after the 12 hour incubation, and these rates are similar to the 34.9% GV stage oocytes at 13 hours extended IVM in these experiments. Therefore, although these oocytes may have been delayed, they may not have matured properly.

The results of the cAMP assay also indicated that the low maturation rates (about 44.5% in the first two experiments) were not likely caused by lack of a decrease in cAMP levels by 33 hours extended IVM. It was previously hypothesized that the effects of these modulators were

not reversible, and that cAMP levels may have remained high throughout extended IVM, keeping the oocytes under meiotic arrest. However, cAMP levels had already dropped to a low concentration by 8 hours extended IVM, so this may not be the cause of the low maturation rates. An alternative explanation may be that cilostamide could be causing low maturation rates via some unknown effect on the oocytes. This may be evidenced by the unusually high percentage of oocytes that arrested at the MI stage in the first two experiments. For example, at the end of standard IVM there were only 16.9% MI oocytes compared to 30.6% MI oocytes at the end of extended IVM in the first experiment. A study in 2013 on sheep oocyte IVM found that prematuration with cilostamide for 22 hours at 10 or 20 μ M followed by 22 hours in standard ovine IVM caused deleterious consequences on chromosome organization and spindle patterns (Gharibi et al., 2013), suggesting that cilostamide could be the cause of low maturation rates since the progression from MI to AI requires proper spindle formation (Fulka et al., 1994; Fulka et al., 1998).

Although recent research has resulted in some improvements to the IVM system, *in vitro* matured oocytes have not yet achieved the viability levels of *in vivo* matured oocytes. The results of this thesis have indicated that the use of IBMX and FSK to maintain high cAMP levels can be successful in delaying nuclear maturation in bovine oocytes. However, it is still unknown whether or not cilostamide played a significant role postponing meiotic resumption. Additionally, although maturation was successfully delayed, the use of these cAMP modulators did not result in improved maturation rates compared to standard IVM. Therefore, the standard IVM system still remains optimal for IVP.

Future research is needed on varying levels of cAMP modulator concentrations, different pairings of modulators, and the use of other types of modulators that may better maintain cAMP concentrations, as cilostamide was not effective. Additionally, the length of culture time with these cAMP modulators may be altered to improve overall maturation rates. For example, since IBMX and FSK successfully maintained high cAMP levels, perhaps exposure to these

modulators for a longer period of time and without the use of cilostamide would have resulted in a successful delay and higher maturation rates than those observed in extended IVM, especially since cilostamide may have had deleterious effects on oocyte development.

There are still many aspects of *in vivo* maturation that are not yet fully understood. cAMP levels are just one small piece in a puzzle that involves many other complicated pathways. Further research into the understanding of the details of *in vivo* maturation is necessary. This may uncover alternate routes of delaying nuclear maturation, or other methods to increase the overall developmental competence of *in vitro* matured oocytes.

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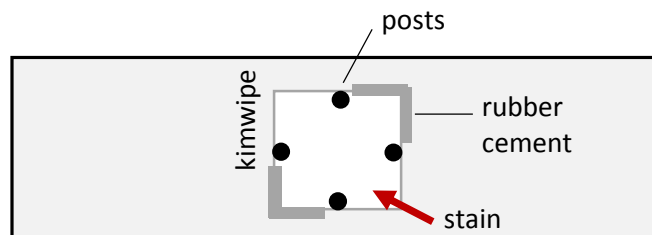
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APPENDIX A PROTOCOLS

Aceto-Orcein Staining Protocol for Oocytes or Embryos

1. Prepare aceto-orcein stain and methanol/acetic acid solution before beginning (see Appendix B).
2. Use a pencil to label the top of a microscope slide with the date, number of oocytes/embryos to be mounted, and any other relevant information.
3. Place small paraffin wax/vaseline posts onto each of the four sides of the coverslip (see diagram below).
4. Place ≈ 10 oocytes/embryos onto the microscope slide, making sure none are on top of each other. Draw off as much excess medium as possible to ensure that they stick to the slide.
5. Quickly place the coverslip onto the slide to prevent dehydration.
6. While viewing the oocytes/embryos under the stereoscope, use a small stick or pencil to apply gentle pressure on each side of the coverslip at the posts. The oocytes/embryos should become slightly flattened to secure them in place but not mashed enough to break the zona. An air bubble will form around the oocyte when adequately secured to the slide.
7. Place rubber cement on two corners of the cover slip.
8. Tilt the coplin jar containing methanol/acetic acid fixative, and gently submerge the slide into the solution. The rubber cement glue will harden on contact with the fixative.
9. Stain and view after at least 48 hours of fixing. Slides can safely be stored in the coplin jars for up to 7 days.
10. Staining:
 - a. View between 48 hours and 7 days after mounting.

- b. Place one drop of aceto-orcein stain on one open side of the coverslip.
- c. Draw stain across the slide by placing a kimwipe on the side of the coverslip opposite the drop of stain. An extra drop of stain can be placed on the slide to prevent the preparation from drying during viewing.
- d. Use microscope to view oocytes/embryos. Record observations and take photographs if necessary.



cAMP Enzymeimmunoassay: Intracellular cAMP Measurement Using the Acetylation Protocol with Lysis Reagents

Reagent Preparation:

Allow all reagents to equilibrate to room temperature. Use distilled or deionized water for reagent preparation. ****Reconstituted reagents should be stored at 2-8°C and re-used within 2 weeks****

1. **Assay Buffer:** transfer the contents of the bottle to a 500 mL cylinder by repeated washing with distilled water. Adjust final volume to 500 mL with distilled water. Mix thoroughly.
2. **Lysis reagent 1:** transfer the contents of the bottle (lysis reagent 1, solid) to a 100 mL graduated cylinder by repeated washing with assay buffer. Dissolve in 60 mL of assay buffer using continuous stirring throughout. Ensure contents of bottle are completely

dissolved. Adjust the final volume to 80 mL with assay buffer and mix thoroughly. The final solution contains 2.5% dodecyltrimethylammonium bromide in assay buffer. This is **lysis buffer 1A**. Stir continuously when used. Take 10 mL of lysis buffer 1A and make up to 100 mL with assay buffer to give a final 0.25% dodecyltrimethylammonium bromide in assay buffer. This is **lysis reagent 1B**. It is used for the intracellular measurement of cAMP and for the preparation of standards.

3. **Lysis reagent 2:** transfer the contents of the bottle (lysis reagent 2, solid) to a 100 mL graduated cylinder. Dissolve in 80 mL of assay buffer using continuous stirring throughout. Ensure the contents of the bottle are completely dissolved.
Adjust the final volume to 100 mL with assay buffer and mix thoroughly. This is **lysis buffer 2A**. Take 10 mL of lysis buffer 2A and make up to a final volume of 40 mL with assay buffer and mix thoroughly. This is **lysis reagent 2B**.
4. **Standard:** Carefully add 4.0 mL Lysis reagent 1B and replace the stopper. Mix until contents are completely dissolved. The final solution should contain cAMP at 2.56 pmol/mL in lysis reagent 1B.
5. **Antiserum:** Carefully add 11 mL lysis reagent 2B and replace the stopper. Gently mix contents of bottle by inversion and swirling until a complete solution is obtained (agitation and foaming should be avoided). The serum in lysis reagent 2B.
6. **cAMP peroxidase conjugate:** Carefully add 11 mL of the diluted assay buffer and replace the stopper. Mix until contents are completely dissolved. *Note: an aliquot of the conjugate should be diluted with an equal volume (1:1) of assay buffer prior to use.* The solution will contain cAMP-horseradish peroxidase in 0.05M acetate buffer pH 5.8, 0.02% (w/v) bovine serum albumin and 0.01% (w/v) preservative.
7. **Wash buffer:** Transfer the contents of the bottle to a 500 mL graduated cylinder by repeated washings with distilled water. Adjust final volume to 500 mL with distilled water

and mix thoroughly. The diluted wash buffer contains 0.01 M phosphate buffer pH 7.5 containing 0.05% (v/v) Tween 20.

Sample Collection:

1. Remove oocytes from maturation media at each time point. Remove cumulus cells by vortexing in hyaluronidase solution. Wash through HEPES-TALP, and freeze oocytes in 100 μ L diluted lysis reagent 1B on dry ice in groups of ten per tube for each time sample. Store in -80°C .

Preparation of Working Standards:

****Use a clean pipette tip for each dilution. Standards should be used within 1 hour of preparation****

1. Label 7 polypropylene tubes (12x75mm): 2 fmol, 4 fmol, 8 fmol, 16 fmol, 32 fmol, 64 fmol, and 128 fmol.
2. Pipette 1 mL lysis reagent 1B (Diluted lysis reagent 1) buffer into all tubes EXCEPT 128 fmol.
3. Pipette 1 mL of stock acetylation standard into the 128 fmol.
4. Pipette 1 mL of stock acetylation standard into the 64 fmol tube and mix thoroughly.
5. Transfer 1 mL from the 64 tube into the 32 tube and mix thoroughly.
6. Repeat this doubling dilution successively with the remaining tubes.
7. Remove 1 mL from the 2 fmol standard and discard. All tubes should now contain 1 mL.
8. 50 μ L aliquots from each serial dilution will give rise to the 7 standard levels of cAMP, from 2-128 fmol.

****These tubes will now be referred to as acetylation tubes containing working standards.**

Working standards should be freshly prepared before each assay and not re-used!**

Assay Method:

Only polypropylene or glass tubes should be used for steps 1-7. Make sure all reagents are equilibrated to room temperature before use. This is particularly important with the enzyme substrate, TMB. It is very important that the refrigerator temperature does not rise above 5°C during the assay. An alternative method for achieving low assay temperatures is to place the microplate on crushed ice during the assay.

1. All of the assay buffer and working standards in the “reagent preparation” section should be prepared as described, including the 1:1 dilution of cAMP peroxidase conjugate. Equilibrate all reagents to room temperature and mix before use.
2. Label polypropylene tubes (12x75mm) for zero standard and unknowns. These will be known as acetylation tubes.
3. Set up the microplate with sufficient wells for running all blanks, standards, and samples as required.
4. Prepare acetylation reagent by mixing 1 volume acetic anhydride with 2 volumes triethylamine in a glass vessel. Mix well. (Sufficient reagent for 50 acetylations may be attained by mixing 0.5 mL acetic anhydride with 1.0 mL triethylamine.)
5. Pipette 1 mL of diluted assay buffer into the zero standard acetylation tube.
6. Pipette 100 µL of lysis reagent 1B into the zero standard acetylation tube.

Steps 6 through 11 should be performed as quickly as possible

7. Pipette 100 µL of each unknown (see sample preparation section) into the appropriately labelled acetylation tubes.
8. Carefully add 2.5 µL of the acetylation reagent to all acetylation tubes containing unknowns. To the 1 mL tubes of standards, add 25 µL of acetylation reagent per mL of standard. Each tube should be *vortexed immediately* following addition of the acetylation reagents, and allow to mix gently on a shaker for 5-10 minutes. Optimum precision is attained by placing the pipette tip in contact with the test tube wall above the

aqueous layer and allowing the acetylation reagent to run down the test tube wall into the liquid.

9. Pipette 100 μL of antiserum (prepared in lysis reagent 2B) into all wells except the blank and non-specific binding (NSB) wells.
10. Pipette 100 μL lysis reagent 2B and 50 μL lysis reagent 1B into the NSB wells.
11. Pipette duplicate 50 μL aliquots from all acetylation tubes including the zero standard into the appropriate wells.
12. Cover the plate with the lid, gently mix, and incubate at 3-5°C for *exactly 60 minutes* (on crushed ice).
13. Pipette 100 μL of cAMP peroxidase conjugate into all wells except the blank. Remember the 1:1 dilution should have been made as recommended – this is critical or curve sensitivity will be compromised.
14. Cover the plate, gently mix, and incubate at 3-5°C for *exactly 60 minutes* (on crushed ice).
15. Aspirate and wash all wells four times with 400 μL wash buffer. Blot the plate on tissue paper, ensuring any residual volume is removed during the blotting procedure.

Thorough washing is essential for good performance!

16. Immediately dispense 150 μL of the enzyme substrate (supplied ready for use) into all wells. Cover the plate and mix on a microplate shaker for *exactly 60 minutes* at room temperature (15-30°C). A blue color will develop.
17. Halt the reaction by pipetting 100 μL of 1 M sulphuric acid into each well, mixing the contents of the plate. Determine the optical density in a plate reader at 450 nm. The optical density determination should be carried out within 30 minutes of the addition of sulphuric acid.

Data Processing/Calculation of Results:

The assay data should be similar to the example supplied in the product booklet protocol.

1. Calculate the average optical density (OD) for each set of replicate wells.
2. Calculate the percent bound for each standard and sample using the following relationship:

$$\%B/B_0 = \frac{(\text{standard or sample OD} - \text{NSB OD})}{(\text{zero standard OD} - \text{NSB OD})} \times 100$$

A standard curve may be generated by plotting the percent B/B₀ as a function of the log cAMP concentration. Plot %B/B₀ (on y-axis) against fmol cAMP standard per well (on x-axis). The curve shape should be similar to the graph provided in the product booklet supplied with the kit. The fmol/well value of samples can be read directly from this standard curve graph.

APPENDIX B MEDIA FORMULATIONS AND STOCK SOLUTIONS

Media Formulations:

HEPES-TALP

Component	Source	Product Number	Amount
BSA, Fraction V	Sigma	A-4503	60 mg
HEPES-TL	Caisson (or stock solution)	IVL01	20 mL
Na Pyruvate	stock solution	P-4562	200 µL
Pen/Strep	Gibco	15140	200 µL

pH should be 7.4. Sterile filter. Date, label, and store at 4°C for no more than one week.

HEPES-TL Stock Solution

Component	Source	Concentration (mM)	Amount
NaCl	Sigma S-5886	114.0	3330 mg
KCl	Sigma P-5405	3.1	120 mg
NaHCO ₃	Sigma S-8875	2.0	84 mg
NaH ₂ PO ₄	Sigma S-5011	0.34	20.4 mg
HEPES	Sigma H-3375	10.0	1200 mg
Lactic Acid	Sigma L-7900	10.0	424.6 µL
CaCl ₂ ·2H ₂ O**	Sigma C-7902	2.0	150 mg
MgCl ₂ ·6H ₂ O**	Sigma M-2393	0.5	50 mg
Phenol Red	Sigma P-0290	1 µL/mL	500 µL

Add NaCl, KCl, NaHCO₃, NaH₂PO₄, HEPES, lactic acid, and phenol red into a beaker. Bring volume to 480 mL with sterile DI water and dissolve ingredients completely.

** Dissolve CaCl₂·2H₂O and MgCl₂·6H₂O in a small amount of sterile DI water before adding to other ingredients. Adjust volume to 500 mL with sterile DI water. Sterile filter into a plastic bottle. Date, label, and store at 4°C for up to one month.

Standard IVM Medium

Component	Source	Product Number	Amount
Medium-199	Sigma	M-4530	8.68 mL
Fetal Bovine Serum	cellgro	35-010-CV	1 mL
Pen/Strep	Gibco	15140	100 µL
Na Pyruvate	stock solution	P-4562	100 µL
Glutamine	100x stock solution	G-8540	100 µL
FSH (Folltropin)	1000x stock solution	Bioniche	10 µL

Sterile filter. Date, label, and store at 4°C for up to one week.

Pre-IVM Medium with FSK and IBMX

Component	Source	Product Number	Amount
HEPES-TALP	stock		5 mL
FSK	100 µM stock	Sigma F-6886	5 µL
IBMX	500 µM stock	Sigma I-7018	5 µL

Date, label, and store at 4°C for up to one week.

Extended IVM Medium with Cilostamide

Component	Source	Product Number	Amount
Standard IVM Medium	stock		5 mL
Cilostamide	20 µM stock	Sigma C-7971	5 µL

Date, label, and store at 4°C for up to one week.

Stock Solutions:

1. **Dulbecco's Phosphate-Buffered Saline:** Add 100 mL of 10X D-PBS solution (Sigma D-1283) to 900 mL of autoclaved DI water to make 1 L of D-PBS. Store at room temperature. Add 10 mL of Bovine Calf Serum (HyClone) and 1 mL of Heparin (Sagent Pharmaceuticals, Schaumburg, IL, USA) to make the D-PBS oocyte collection medium.
2. **Pen/Strep:** Gibco 15140. Aliquot ≈ 500 μ L of new pen/strep solution into sterile centrifuge tubes. Store at -20°C until use.
3. **Na Pyruvate:** Sigma P-4562. Dissolve 22 mg of sodium pyruvate in 10 mL of sterile Millipore-Q water. Sterile filter into an aluminum foil-wrapped 15 mL conical tube and store at 4°C for up to a month.
4. **L-Glutamine:** Sigma G-8540. Make a 100X stock solution with a concentration of 200 mM by dissolving 2.92 g of glutamine in 100 mL of DI water. Aliquot 1.0 mL into sterile centrifuge tubes and store at -20°C .
5. **FSH:** Folltropin-V (Bioniche). Make a 1000X stock solution (5 mg/mL) by diluting a 400 mg vial of folltropin in 80 mL of DI water. Store at -20°C in 100 μ L aliquots.
6. **FSK:** Forskolin, Sigma F-6886. Make a 10 mM stock solution by adding 244 μ L of DMSO (Sigma D-2650) to 10 mg of FSK. Aliquot 5 μ L into small centrifuge tube and store at -20°C until use.
7. **IBMX:** 3-Isobutyl-1-methylxanthine, Sigma I-7018. Prepare a 500 mM stock solution by adding 899 μ L of DMSO (Sigma D-2650) to 100 mg of IBMX. Aliquot 5 μ L into small centrifuge tubes and store at -20°C until use.
8. **Cilostamide:** Sigma C-7971. Prepare a 20 mM stock solution by adding 730 μ L of DMSO (Sigma D-2650) to 5 mg of cilostamide. Aliquot 5 μ L into centrifuge tubes and store at -20°C until use.

9. **Hyaluronidase:** Sigma H-3506. Prepare a 1 mg/mL solution by dissolving 10 mg hyaluronidase into 10 mL of HEPES-TALP (see media formulations above). Aliquot 1 mL into 1.5 mL sterile centrifuge tubes. Store at -80°C indefinitely.
10. **Methanol/Acetic Acid Fixative:** For use with aceto-orcein staining protocol, make a 3:1 methanol/acetic acid solution by adding 300 mL of methanol (Sigma M-1775) and 100 mL of acetic acid (Sigma A-6283). Store at room temperature.
11. **Aceto-Orcein stain:** Prepare a 1% orcein stain by adding 100 mg of orcein (Sigma O-7380) to 10 mL of solvent consisting of 60% DI water, 40% acetic acid (Sigma A-6283).

VITA

Sarah Emilie Farmer was born in New Iberia, Louisiana to James and Madeleine Farmer. She grew up in Jeanerette, Louisiana. She has a twin brother, Alexander Farmer, who holds a degree in Business Management from the University of Louisiana at Lafayette and is currently studying Film Arts at the University of New Orleans. Sarah attended Catholic High School in New Iberia. She participated in many activities, including the Louisiana Science and Engineering Fair for which she obtained a US patent in 2006 for an underground tree anchoring system. She graduated high school in 2006.

Sarah attended the University of Louisiana at Lafayette from 2006 to 2011. While at ULL, she was an officer in the ULL Biological Society, a member of the Student Affiliates for the American Chemical Society, Phi Beta Delta honors society, and Phi Kappa Phi honors society. Sarah also worked in the lab of Dr. Glen Watson. Sarah enjoyed a summer studying abroad in Paris in 2008. She graduated Magna Cum Laude in May 2011 from the University of Louisiana at Lafayette with a B.S. in Biology and a minor in Chemistry.

Sarah began graduate school at Louisiana State University in the fall of 2011. She studied Reproductive Physiology and Biotechnology under the direction of Dr. Kenneth Bondioli. She is now a candidate for the degree of Master of Science in Reproductive Physiology in the School of Animal Sciences at Louisiana State University, Baton Rouge, Louisiana.