

2016

## Vitrification of Immature and Mature Bovine Oocytes

Paige T. Hardin

*Louisiana State University and Agricultural and Mechanical College*

Follow this and additional works at: [https://digitalcommons.lsu.edu/gradschool\\_theses](https://digitalcommons.lsu.edu/gradschool_theses)



Part of the [Animal Sciences Commons](#)

---

### Recommended Citation

Hardin, Paige T., "Vitrification of Immature and Mature Bovine Oocytes" (2016). *LSU Master's Theses*. 4293.

[https://digitalcommons.lsu.edu/gradschool\\_theses/4293](https://digitalcommons.lsu.edu/gradschool_theses/4293)

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact [gradetd@lsu.edu](mailto:gradetd@lsu.edu).

# VITRIFICATION OF IMMATURE AND MATURE BOVINE OOCYTES

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 in  
Animal and Dairy Sciences

by

Paige Taylor Hardin  
B.S., Louisiana State University, 2013  
August 2016

## **ACKNOWLEDGEMENTS**

I would first like to express my very great appreciation to Dr. Bondioli, who gave me this opportunity to expand my education in the field of Reproduction here at Louisiana State University and for his valuable and constructive suggestions during the planning and development of this research. His willingness to give his time has been greatly appreciated. I would also like to extend a special thanks to the members of my graduate committee, Dr. Carlos Pinto and Dr. Cathy Williams for their support and advice.

This research was supported by the School of Animal Science, at Louisiana State University and by the Audubon Center for Research of Endangered Species to whom I thank sincerely.

I would also like to express my deep gratitude to my fellow graduate students, for their patient guidance, enthusiastic encouragement and useful critiques of this work. I would not have been able to perform this research without them. An exceptional thanks to Brittany Foster, who always provided assistance with class work, and lab work, as well as moral support throughout my research. Her help in every aspect of my experiment, from OPU to fertilization, is very much appreciated. Special thanks to Fabian Diaz for being my go to OPU technician and statistical analysis wiz and to Emilio Gutierrez who helped with OPU those early mornings. My thanks also to Whitney Coley Gaspard and Sam Lanjewar for providing aid and friendship throughout this process.

Without the persistence and motivation of Dr. Jairo Sarmiento-Guzmán to encourage me to work harder and strive for greatness during my undergraduate degree I most likely

would not have had the willingness to pursue a master's degree in this field. He is a great mentor and a better friend and I thank him for everything he has done for me.

I would also like to acknowledge Ms. Sonyja Thomas and the crew at the Reproductive Biology Center for all their help with handling the cattle for this project.

The greatest gratitude goes to Jeffery O'Brien for his continual help and support in my mental health. His advice and encouragement during these years of my thesis research were much appreciated and without his support this endeavor would have been much more difficult and I probably would have lost my mind by now.

Last but certainly not least, none of this would have been possible without the support of my siblings and my parents, John Hardin and Jorja Renwick. They have always encouraged me to be my best, and I am eternally grateful for everything they have done for me. I would not be where I am today without their help, guidance, and motivation. There are absolutely no words to express how much they are valued, and I am blessed to have such a magnificent family.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	II
LIST OF TABLES.....	VI
LIST OF FIGURES.....	VII
LIST OF ABBREVIATIONS .....	VIII
ABSTRACT .....	IX
CHAPTER	
I. INTRODUCTION .....	1
II. LITERATURE REVIEW.....	3
HISTORY .....	3
OOGENESIS .....	4
COLLECTION.....	6
CRYOPRESERVATION .....	7
CRYOPROTECTANTS.....	9
SLOW RATE CRYOPRESERVATION .....	14
VITRIFICATION .....	16
CRYOPRESERVATION TOOLS.....	20
WARMING.....	22
EMBRYOS .....	23
MATURE OOCYTES.....	24
IMMATURE OOCYTES .....	26
IN VITRO FERTILIZATION PRODUCTION.....	27
CURRENT ISSUES FOR FUTURE RESEARCH .....	31
III. VITRIFICATION OF BOVINE IMMATURE AND MATURE CUMULUS CELL OOCYTE COMPLEXES.....	33
INTRODUCTION .....	33
MATERIALS AND METHODS .....	35
EXPERIMENTAL DESIGN .....	35
EXPERIMENT ONE DESIGN.....	35
EXPERIMENT TWO DESIGN.....	36
IVF VALIDATION.....	37
PREPARATION .....	38
OOCYTE COLLECTION .....	39
TRANSVAGINAL ULTRASOUND-GUIDED FOLLICULAR ASPIRATION .....	39
VITRIFICATION AND WARMING PROCEDURE .....	40

IN VITRO FERTILIZATION .....	42
STAINING AND DATA COLLECTION .....	43
STATISTICAL ANALYSIS.....	43
RESULTS .....	44
EXPERIMENT ONE.....	44
EXPERIMENT TWO.....	46
IVF VALIDATION RESULTS.....	48
MATURATION CHECK .....	48
DISCUSSION.....	49
IV. SUMMARY AND CONCLUSION .....	59
SUMMARY .....	59
CONCLUSION .....	61
LITERATURE CITED .....	64
APPENDIX A: PROTOCOLS.....	78
APPENDIX B: MEDIA FORMULATIONS AND STOCK SOLUTIONS .....	85
APPENDIX C: DETAILED OOCYTE ASSESSMENT BREAKDOWN .....	88
VITA.....	91

## LIST OF TABLES

2.1	Factors Associated with Cooling and Cryopreservation that Contributes to Cellular Injury and Death in Biological Systems .....	12
2.2	Survival and development rate of human pronuclear embryos cryopreserved by either slow cooling or vitrification using the Cryotop method.....	19
3.1	Composition of Cryoprotectant Solutions for both Treatments.....	37
3.2	Dilution Solution for both Experiments for both Treatments.....	37
3.3	Nuclear status of Immature Oocytes after IVF with DMSO (N=106) and Glycerol (n=81)).....	44
3.4	Nuclear status of Mature Oocytes Group after IVF with DMSO (N=103) and Glycerol (n=88).....	47

## LIST OF FIGURES

3.1	Diagram of transvaginal ultrasound-guided follicular aspiration.....	40
3.2	Cryolock vitrification and warming procedure.....	42
3.3	Denuded bovine oocyte loaded onto Cryolock.....	42
3.4	Different stages of nuclear maturation of Immature DMSO (A) and Glycerol (B) cryoprotectant treatments.....	45
3.5	Different stages of nuclear maturation of Mature DMSO (A) and Glycerol (B) cryoprotectant treatments.....	48

## LIST OF ABBREVIATIONS

ART -	assisted reproductive technology
BCS -	bovine calf serum
CCs -	cumulus cells
COC -	cumulus-oocyte-complex
D-PBS -	Dulbecco's phosphate- buffered serum
FBS -	fetal bovine serum
GCs-	granulosa cells
GnRH-	gonadotropin- releasing hormone
GV -	germinal vesicle
GVBD -	germinal vesicle breakdown
ICM -	inner cell mass
IVC -	<i>in vitro</i> culture
IVF -	<i>in vitro</i> fertilization
IVM -	<i>in vitro</i> maturation
IVP -	<i>in vitro</i> production
LH -	luteinizing hormone
MI -	metaphase I
MII -	metaphase II
OPU -	ovum-pick up
PB -	polar body
PGCs -	primordial germ cells
TUGA -	transvaginal ultrasound-guided aspiration

## **ABSTRACT**

Vitrification is the latest technique used in cryopreservation, the ability to utilize this method with oocytes and embryos has become a valuable system. Vitrification has been successful with bovine embryos and oocytes but is far from optimal. Following cryopreservation storage discarding embryos can cause ethical issues, and mature oocytes have fragile organelles that can be detrimentally affected by ice crystal formation during freezing. Immature oocytes have not formed some of these temperature sensitive microfilaments and can circumvent these detrimental effects. The common intracellular cryoprotective agents are dimethyl sulfoxide, glycerol and ethylene glycol. Different combination of these agents have been reported for vitrification of oocytes.

The overall objective of this experiment was to determine if immature and mature cumulus cell complexes vitrified in solutions of dimethyl sulfoxide or glycerol in combination with ethylene glycol would be competent to produce pronuclei following thawing and in vitro fertilization. Two experiments evaluated two cryoprotectant solutions and their ability to fertilize bovine cumulus cell complexed oocytes (n=400). The first study used DMSO and glycerol containing vitrification solution with immature bovine oocytes (n=200) followed by IVM and IVF with an end point of pronuclei formation to indicate fertilization. The second used DMSO and glycerol containing vitrification solutions with mature bovine oocytes (n=200) followed by IVF. We found that vitrifying immature oocytes with DMSO or Glycerol containing solutions prior to IVF resulted in higher fertilization for DMSO  $P<.01$ . Vitrifying mature oocytes with DMSO or glycerol containing also resulted in higher fertilization for DMSO solution  $P<.05$ .

These results suggest that DMSO may be the more appropriate choice when used in combination with ethylene glycol for vitrification of immature and mature oocytes.

## **CHAPTER I INTRODUCTION**

In vitro embryo production (IVP) including in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) is an invaluable tool for human fertility clinics as these assisted reproductive technologies help to provide infertile men and women with children of their own (Hansen, 2013). IVP is also a valuable tool for commercial livestock production due to the fact that it increases the number of offspring that can be produced from each animal per year (Hansen, 2013). Through the use of transvaginal ultrasound guided oocyte pickup, it is possible to retrieve many oocytes per cow on a weekly basis. These oocytes can then be matured and fertilized in vitro before transfer into recipient cattle to produce many viable embryos and offspring (Hiroyoshi, 2002). This is especially useful for maximizing the offspring potential of genetically valuable animals.

In vitro production can be further maximized through use of cryopreservation for long term oocyte storage (Shaw and Troson, 2000). However, cryopreservation of embryos can be costly, hard to manage, and at times may pose moral and ethical issues. Cryopreserving mature oocytes can result in damage to the metaphase spindle due to the temperature sensitivity of microfilaments and microtubules as well as other morphological issues (Baka et al, 1995). Cryopreservation of immature oocytes may circumvent this problem because these structures have not yet formed and the genetic material is enclosed within a nuclear envelope (Benson, 2008). Cryoprotectants, such as dimethyl sulfoxide and glycerol, help to prevent ice crystal damage during cryopreservation (Fuller, 2004). There has been discussion on which cryoprotectant and concentration works better for certain cell types and sizes. Recent studies

have also shown that a newer technique, termed vitrification, results in a significant improvement over the traditional slow-rate freezing procedure (Lawson, 2011). These results vary with cell type, cell size and species used in vitrification, a fast freezing technique that transforms a liquid into a glass like structure. It has also been shown that intact cumulus-oocyte complexes (COC) are essential for normal maturation and should be intact during the vivo or vitro maturation and fertilization periods (Senger, 2005).

The overall goal of this research is to determine if immature and mature COC's cryopreserved via vitrification with dimethyl sulfoxide (DMSO) or glycerol will form pronuclei following thawing and in vitro fertilization, indicating viability. This study will compare DMSO and glycerol cryoprotectants within each oocyte group. Survival rates will be compared to determine if it is possible to vitrify immature oocytes as an alternative to mature oocytes for future cryopreservation procedures. This will help to determine which cryoprotectant has a higher survival rate with bovine oocytes, immature or mature and determine whether one cryoprotectant treatment may be optimal for a particular oocyte stage and identify whether or not these oocytes can become viable embryos.

## **CHAPTER II**

### **LITERATURE REVIEW**

#### **HISTORY**

Gamete cryopreservation was initially established primarily for use in male infertility treatments, but has since expanded to become an important tool in many aspects of assisted reproduction (Bunge, 1954). Fowl spermatozoa were first cryopreserved in 1949 using glycerol, thus making them the first mammalian reproductive cells to be successfully frozen, (Polge and Parks, 1949) and remain the easiest gamete to freeze today. The development of techniques for cryopreservation of human spermatozoa for use in human reproduction is credited to Sherman and Bunge's work in the mid 1950's (Bunge and Sherman, 1954). The use of glycerol was further studied in the 1950's to attempt cryopreservation of unfertilized mouse oocytes (Sherman, 1959) and rabbit zygotes (Smith, 1953) but with minimal success. Cryopreservation of oocytes and their ability to become viable embryos proved more challenging than cryopreservation of the spermatozoa cells, mainly because many procedures caused damage to the zona pellucida (Shaw, 2000, Baka, 1995, Magistrini and Szollosi, 1980). Another problem was thought to be due to the meiotic spindles sensitivity to cold temperatures, causing irregularities in meiosis (Shaw, 2000 and Baka, 1995). This was demonstrated in mouse oocytes where upon return to 37°C, the spindle would reorganize in an abnormal shape with the chromatids scattered throughout the ooplasm (Pickering, 1987). This was believed to be a possible cause for increased aneuploidy in cryopreserved oocytes (Pickering, 1987). In the 1980's cryopreservation protocols for embryos and spermatozoa success was established resulting in live mice births (Pickering, 1987). Other studies at the time raised concerns about

fertilization of cryopreserved mature oocytes after observations of physical damage (Todorow, 1989) and structural modifications to the zona pellucida (Todorow, 1989). These studies established the necessity for a slow cooling cryopreservation protocol for oocytes and cleavage-stage embryos. Successful cryopreservation of spermatozoa and embryos has made assisted reproductive technologies (ART) more practical and available for commercial use (Baldassarre and Karatzas, 2004). The advantages of cryopreservation being the ability to transport gametes long distances, to remove the requirement of estrous synchronization and therefore reduce the number of recipients required (Do and Taylor-Robinson, 2014). There are now a range of cryopreservation protocols that can be used to preserve both mature and immature oocytes, embryos and spermatozoa.

## **OÖGENESIS**

In mammals, oögenesis (the development of an oocyte) begins early in fetal development and ends months to years later in the sexually mature female, puberty. Oögenesis begins with primordial germ cell formation (Senger, 2005). Once established they are then referred to as oogonia and will eventually undergo a final round of DNA replication before entering meiosis and becoming oocytes. After puberty oocytes will enter meiotic prophase one before arresting at the diplotene phase. This arrest is maintained until the dominant follicle is stimulated by a gonadotropin surge (Senger, 2005). Oocytes must grow in follicles to become competent to resume nuclear maturation and undergo fertilization and cleavage divisions. When oocyte growth is complete they are capable of progressing through germinal vesicle breakdown and resume meiosis. During the oocytes growth phase, the oocytes undergo a complex reorganization of organelles within the cytoplasm. Replication of

the cytoplasmic organelles during oogenesis, specifically the mitochondria and mitochondrial DNA, is crucial as the cytoplasmic inheritance of the zygote originates in the oocyte (Alberts, 2002). During oocyte growth phase, the mitochondria rapidly multiply, and organelles become less active. Within the maturing oocytes ribosome numbers increase and organelles move towards the cell periphery (Senger, 2005). As the oocyte continues to grow an increasing number of pores appear in the nuclear membrane around the nucleolus, indicating the beginning of maturity (Alberts, 2002). At the end of the growth period and just before meiotic maturation, the oocyte nucleus is large, pale, and immature, referred to as a 'germinal vesicle' (GV) (Senger, 2005). Once the dominant follicle is stimulated the primary oocyte starts to develop into an ootid, through resumption of meiosis. This stage occurs after puberty and development continues to a secondary oocyte with extrusion of the first polar body, these oocytes are now considered mature and are ready for ovulation and fertilization (Senger, 2005).

Surrounding both immature and mature oocytes are layers of cumulus cells. These cells develop from specialized granulosa cells that differentiate into mural granulosa cells and cumulus cells during follicular antrum formation. Cumulus cells are biologically different from other follicular cells and execute specific roles including, transmitting signals within the ovary and supporting oocyte growth and maturation during the later stages of follicular development (Senger, 2005). They surround and nourish the oocyte. The communication between cumulus cells and oocytes is bidirectional and imperative for regulating both oocyte and cumulus cell development. Oocytes have fewer energy sources than necessary for proper oocyte growth so the cumulus cells are needed to transfer energy to the oocyte through gap

junctions (Franciosi et al., 2014). After the luteinizing hormone surge, granulosa cells produce epidermal growth factors that act on cumulus cells to induce cumulus expansion and oocyte maturation (Hung et. al, 2015). Cumulus cells regulate oocyte growth, oocyte maturation and the fertilization process (Da Costa et. al, 2015). A high proportion of fertilized oocytes express an abnormal fertilization rate following maturation when there is an absence of cumulus cells around the oocyte. Matured oocytes with intact cumulus cells before fertilization were able to develop to viable fetuses at similar rates to ovulated oocytes after in vitro fertilization (Vanderhyden and Armstrong, 1989). This demonstrates the essential role for cumulus cells in assisting normal cytoplasmic maturation of oocytes necessary for pronuclear formation and complete developmental capacity (Vanderhyden and Armstrong, 1989). In another study completed by Jin and associates, it was concluded that the maintenance of cumulus cells can improve the developmental ability of vitrified thawed mature human oocytes resulting in successful pregnancy. In comparison, oocytes with the cumulus cells removed prior to cryopreservation had a significantly lower fertilization rate (Jin et. al, 2012). An additional study by Purohit found that immature cumulus enclosed compact goat oocytes better tolerate cryopreservation stress, as assessed by fertilization rates when compared to immature, denuded and in vitro matured oocytes (Purohit et. al, 2012). In conclusion cumulus cells are needed on oocytes regardless of maturity, especially with in vitro maturation of the oocytes.

## **COLLECTION**

While there are different methods for the recovery of oocytes, aspirating follicles from ovaries of slaughtered animals is currently the most common procedure. However, due to its terminal nature, this technique cannot be repeated on any single animal (Hashimoto et. al,

1999). To continually repeat collection of bovine oocytes from naturally cycling cows, the transvaginal ultrasound guided follicular aspiration technique (TUGA) was developed (Pieterse et. al, 1988). With TUGA, an epidural anesthetic is normally given to prevent abdominal straining and to ease palpation. The standing cow is restrained so that little movement is possible during oocyte collection (Manik and Palta, 2003). After retraction of the uterus the right or left ovary is placed against the head of the transducer which had been inserted into the vagina adjacent to the cervix. The antral follicles appear as black round spots on the monitor of the scanner and the puncture needle is depicted by a hyperechoic line going into the ovary (Manik and Palta, 2003). The transducer is positioned so that the puncture line on the monitor transects the follicle to be punctured. When a follicle is positioned steadily on the puncture line, the needle will be pushed through the vaginal wall until its tip becomes visible within the follicle (Pieterse et. al, 1991). This is directly followed by aspiration of the follicular contents. After the needle is withdrawn from the ovary and the transducer from the vagina, the needle is flushed with phosphate-buffered saline solution (PBS). The fluid of each follicle is collected in a filter and inspected for the presence of an oocyte (Pieterse et. al, 1991).

## **CRYOPRESERVATION**

Cryopreservation of oocytes enables scientists to store oocytes long term from patients that are in danger of losing ovarian function, while relieving many of the ethical concerns associated with embryo cryopreservation (Shaw and Troson, 2000, Baka et. al, 1995). The first challenge in cryopreserving cells from warm-blooded animals is in cooling the cells below body temperature. Cells may incur damage, referred to as chilling injury, from the low temperatures (Baka et.al, 1995). Cryopreservation is the use of very low temperatures to preserve intact

living cells and tissues at approximately  $-196^{\circ}\text{C}$ . At these low temperatures, all biological activity stops, including the biochemical reactions that lead to cell death and DNA degradation (Shaw and Troson, 2000). Water is the major component of all living cells and must be available for the processes of life to occur, cellular metabolism stops when water in the structure is converted to ice crystals. The behavior and function of membrane lipids and proteins may be affected during the freezing process as well (Shaw and Troson, 2000).

Cryopreservation relies on the ability of small molecules to enter the cell's cytoplasm and prevent both dehydration and formation of ice crystals within the cell. Ice crystals form at different times during the freezing process. At temperatures at or below  $0^{\circ}\text{C}$ , water resists the breaking of hydrogen bonds and the molecules lock together, this creates pockets of open space between tight parts of the structure where ice crystals may form (Pickering, 1987). Ice crystals have the capacity to grow into complex clusters forming networks that can grow exponentially as more and more water molecules interact to form increasingly complex crystals. Ice nucleation affects the structural, osmotic, and colligative integrity of cells causing physical ruptures and mechanical injury (Benson, 2008). With the help of cryoprotectants ice does not begin to form until about  $-5^{\circ}\text{C}$ . Extracellular water begins to freeze between  $-5$  and  $-15^{\circ}\text{C}$  but the cell contents remain unfrozen and super cooled. The plasma membrane blocks the growth of any ice crystals into the cytoplasm (Albrecht, 1982) and the water within the cell has a higher chemical potential than the water that is partially frozen outside of the cell, therefore the water flows out of the cells to freeze extracellularly (Albrecht, 1982). The actual physical events within the cell depend on the cooling speed (Alink and Offerigns, 1977). When cooling the cell slowly, the cell is able to lose water by exosmosis. The cell will then dehydrate

and intracellular freezing will be avoided. During rapid cooling, the cell is not able to lose water fast enough to maintain the equilibrium between the external and intercellular membranes, it becomes increasingly super cooled and will eventually reach equilibrium by the intracellular freezing as a glass like substance (Alink and Offerigns, 1977).

Scientists are challenged with stabilizing replicable genetic material to preserve it unchanged. The aim of cryopreservation is to enable cells to be stored and to prevent the need to have all cell lines in culture at all times. This is invaluable when dealing with cells of limited life span. Other main advantages of cryopreservation are the reduced risk of microbial contamination, the reduced risk of cross contamination with other cell lines, the reduced risk of genetic drift and morphological changes, and the reduced costs (Sigma,2010).

Cryopreservation also plays an important role in assisted reproduction, it enables the reproductive cells collected and/or fertilized in one treatment cycle, to be used for fertilization and/or implantation into the uterus in a future treatment cycle.

## **CRYOPROTECTANTS**

Cryoprotectants (CPA) is the functionally derived term created to describe any additive which can be provided to cells before freezing to yield a higher post thaw survival than can be obtained in its absence. The first recorded example of deliberately adding a cryoprotectant, was the works of Polge and colleagues (Polge and Parks, 1949). They established factors for success of cryoprotectant activity of a particular solute, the addition of glycerol (Polge and Parks, 1949). In order for the cells to survive the cryopreservation process, they must maintain intact organelle membranes and have no intracellular ice crystal formation (Agca et. al, 1998). Cryoprotectants guard the cell during freezing, are nontoxic in low concentrations and are

inexpensive (Felipe de Lara Janz et. al, 2012). Cryoprotectants also help to ensure high levels of cell viability post thaw, and lower freezing temperatures allowing for greater dehydration of cells before water freezes (Watson, 1995). Cryoprotectants act not only as an osmotic gradient to aid in the water movement across the membrane, but also as antifreeze, to reduce the temperature at which ice crystals form. Cryoprotectants are separated into two main classes intracellular agents, and extracellular agents. Intracellular agents include ethylene glycol, dimethyl sulfoxide, and glycerol, these penetrate inside the cell preventing the formation of intracellular ice crystals that could result in membrane rupture or destruction. Intracellular cryoprotectants have low molecular weight and amphipathic properties, permeate cells and are effective in minimizing cell damage in many slow frozen biological systems (Agca et. al, 1998). These are the most commonly used cryoprotectants, with glycerol being the most widely used. Glycerol is a small trihydroxy sugar alcohol, poly-hydroxylated solute with high solubility in water, and low toxicity to living cells with short exposure periods. It can interact by hydrogen bonding with water, and can permeate across the plasma membrane of many different cell types (Agca et. al, 1998). Cells may tolerate exposure to glycerol in concentrations between 1 to 5 mol/L, depending upon cell type and conditions of exposure (Fuller, 2004). There are many debates about which intracellular cryoprotectant to use when freezing as dimethyl sulfoxide, glycerol and ethylene glycol are comparable to one another in many respects. In recent studies dimethyl sulfoxide has been used more often for oocytes and embryos (Schellander et. al, 1994). In one particular study dimethyl sulfoxide treatment led to significantly better cleavage and development up to 4-cell stage in oocytes cryopreserved at the MII stage. However, development beyond the 8-cell stage was obtained only when

immature oocytes were frozen (Schellander et. al, 1994). It was found that when using glycerol and 1,2- propanediol in vitrification of bovine blastocysts the success following the 16 step equilibration minimized the ultrastructural damage to the plasma membrane during freezing and therefore ice crystal formation (Kuwayama and Nagai, 1994). In another study it was noted that when vitrifying immature and mature equine and bovine oocytes in ethylene glycol that the maturation rates after vitrification were higher in bovine oocytes than equine at 70% and 30%, respectively (Hurt et. al, 2000). Depending on the source consulted, there have been variable results on the relative toxicity of the different cryoprotectants, with disagreement on which is the least toxic (Lawson and Sambanis, 2011, Huang et. al, 2006, Aye et. al, 2010, Comizzoli and Pukazhenth, 2004). Many factors for optimizing cryopreservation are dependent on the cell type and species including cryoprotectants used, experimental protocols and thawing time.

The second class of cryoprotectants are extracellular cryoprotective agents, such as sucrose, trehalose, or dextrose. These do not penetrate the cell membrane and act to improve the osmotic imbalance that occurs during cryopreservation. Extracellular CPA's can also be used entirely on their own, to increase the osmolarity of extracellular solution, to help draw water out of the cells. Osmotic imbalance occurs within the cell when water is removed from the extracellular environment and the water going through the cell membrane is moved out of the cell. When the extracellular solute concentration is too high and water moves out of the cells rapidly, it can be harmful. If too much water is inside the cell during cryopreservation, ice crystals will form and recrystallization during thawing can occur and lyse the cell. Felipe de Lara Janz stated that when cells were preserved in sucrose and trehalose alone they had a low

rate of stem cell viability and therefore had an increase in lag phase after thawing (Felipe de Lara Janz et. al, 2012). These problems occurred due to the fact that extracellular agents alone do not adequately prevent the formation of ice crystals within the cell. The combination of

Table 2.1 Factors Associated with Cooling and Cryopreservation that Contribute to Cellular Injury and Death in Biological Systems (Adapted from Shaw and Troson 2000)

System	Type/cause of damage
All	Intracellular ice formations, extracellular ice formation, apoptosis, toxicity, calcium imbalance, free radical, ATP levels, general metabolism, fertilization failure, cleavage failure, pH <sub>i</sub> , parthenogenetic activation,
Membrane	Rupture, leakage, fusion, microvilli, phase transition
Chromosomes	Loss/gain, polyspermy, polygyny (failure to extrude polar body), tertrapliody
DNA	Apoptosis, fusion, rearrangements
Cytoskeleton	Microtubules dissolve, actin
Proteins/enzymes	Dehydration, loss of function
Ultrastructure	Microvilli mitochondria, vesicles, cortical granules, zona pellucida
Zona Pellucida	Hardening, fracture
Lipids	Free radical?

both cryoprotectant agents can optimally preserve cells but there are many factors that need to be evaluated for each individual experiment before creating a protocol. The concentration

of cryoprotectants, the combination of cryoprotectants, length of exposure time to each solution, and other factors affect the viability of the cells being cryopreserved. Table 2.1 shows the type and cause of damage during cryopreservation within the biological system (Shaw and Troson, 2000).

High concentrations of cryoprotective agents can be damaging to cells (Meryman, 1971). In general, vitrification requires a two to three step procedure of moving cells through increasing concentrations of cryoprotective agents to minimize osmotic shock (Meryman, 1971). The first solution consists of low concentration to remove the majority of the water from the cell often referred to as an equilibrating solution. The second and third solutions consist of higher concentrations with the cells only exposed for a brief time to minimize the risk of toxicity. A study showed that reducing the amount of time of exposure to vitrification solutions can reduce the risk of toxicity to the cell (Ishimori et. al, 1993). Another study completed by Yamada and colleagues compared the efficiency of vitrifying immature oocytes in different combinations of ethylene glycol, glycerol and dimethyl sulfoxide at various time intervals. In the first experiment, oocytes were exposed to the cryoprotectant for either 30 or 60 seconds in final solutions of 20 % ethylene glycol + 20% dimethyl sulfoxide or 25% ethylene glycol +25% dimethyl sulfoxide. In the second experiment, the oocytes were vitrified in open pulled straws (OPS) using a 30 second exposure to final solutions as above. The post-thaw maturation rates showed that a 30 second exposure did not differ from the control groups but that a 60 second cryoprotectant exposures was toxic to the cells, reducing the maturation rates. The second solution of 25% ethylene glycol + 25% dimethyl sulfoxide enhanced the maturation rates compared to the other solutions used. The concentration of cryoprotectants

used in this study showed their importance on the post thaw development and survival rate of vitrified oocytes (Yamada et. al, 2007). Si and colleagues found that glycerol at concentrations below 5% in comparison to 15% dimethyl sulfoxide led to improved post-thaw survival (Si et. al, 2004). In addition to all these different factors the cooling rate has been proven to have a significant impact on long term cell viability (Fasano, 2014). Not only does cooling rate affect the rate of formation and size of both intracellular and extracellular ice crystals; it can also impact solution effects that occur during the freezing process (Fasano and Vannin, 2014). There are two main freezing procedures that are used today; slow rate cryopreservation and a fast freezing method termed vitrification.

### **SLOW RATE CRYOPRESERVATION**

Most oocyte and embryo cryopreservation systems use a controlled rate freezer for a process known as slow rate cooling or conventional cryopreservation. This freezing system leads to the extracellular solution freezing before intracellular ice crystals form creating an osmotic imbalance across the cell membrane leading to the water leaving the cell. Liquid nitrogen is poured into a closed chamber system that is connected to a computer system, controlling the rate at which the internal chamber, where cells are placed, is cooled. During this process there was an increase in the solute concentration outside and inside the cell due to ice crystal formation, this can be detrimental to cell survival (Mavrides and Morroll, 2002). The freezing rate is set to optimize cell dehydration and so prevent intracellular ice crystal formation. The embryos or gametes are placed into a programmable freezer that can be set and reduced to any temperature. One common protocol starts at 23°C and reduced -2°C per minute to -8°C then seeded and held for 5 minutes. The system then reduces -0.3°C per

minute to  $-30^{\circ}\text{C}$ ,  $-50^{\circ}\text{C}$  per minute to  $-140^{\circ}\text{C}$  and held for 15 minutes. This can take anywhere from an hour to three hours, depending on the set cooling rate, but allows many cells to be frozen at the same time (Mavrides and Morroll, 2002). The frozen suspension is then transferred to a liquid nitrogen tank and maintained at subzero temperatures for indeterminate lengths of time. The consequences of this slow and gradual freezing process have been documented to include cellular injury, resulting in cell death, especially from  $-15^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$  (Djuwantono et. al, 2011). Cells pass through this critical range twice; during freezing and again during thawing. The transition of the cells from fluid to solid state could also cause osmotic injury. In recent literature, the survival of cryopreserved human oocytes following slow rate freezing ranged from 74% to 90%(Chen and Yang, 2009). About 85% of slow rate frozen matured oocytes had normal morphological characteristics in another study (Sinha et.al, 2014). Bianchi questioned if the cryopreservation machine may be obsolete in a study performed in 2012 (Bianchi et.al, 2012) concluded that in infertile human couples undergoing in vitro fertilization treatments he found that slow freezing of oocytes can be a valid tool still in practices with in vitro fertilization when using suitable protocols (Bianchi et. al, 2012). A study aimed to evaluate the ability of slow freezing for cryopreservation of immature, germinal vesicle porcine oocytes found that slow freezing is a feasible approach for cryopreservation of porcine germinal vesicle oocytes when the correct cooling rate is used. The results indicated that the cooling rates of 6, 3 and  $1.5^{\circ}\text{C}$  per minute could be considered as possible cryopreservation protocols when using 2.0M DMSO (Yang et. al, 2012). This study also verified an effective approach for optimal cooling rates by evaluating the intracellular ice crystal formation characteristics of porcine germinal vesicle cumulus cell complexed oocytes (Yang et.

al, 2012). Literature reviews have shown that the majority of pregnancies to date have been accomplished with a slow freezing protocol (Oktay and Bang, 2006). Both slow rate freezing and vitrification methods for oocyte cryopreservation can be used effectively with subsequent successful pregnancies (Oktay and Bang, 2006). However, recent improvements in the vitrification technology have enhanced its effectiveness and because of its simplicity made it the method of choice in oocyte cryopreservation.

## **VITRIFICATION**

Vitrification is a fast freezing technique, that transforms liquid into a glass like state without ice crystal formation. It was developed in 1985 and requires no expensive programmable freezing equipment (Rall, 1985) unlike the slow-freezing process. It relies on an extremely high concentration of CPAs to create highly viscous solutions in which ice crystals cannot form, rather than relying directly on cell dehydration (Rall, 1985). Rall first showed that a murine embryo could be successfully cryopreserved, but the vitrification solution they used was highly toxic (Rall, 1985). Later studies by Rall were successful with live mice born following embryo vitrification (Rall, et al. 1987). The combination of ethylene glycol and dimethyl sulfoxide with sucrose was reported for mouse (Ishimori, et al. 1993) and bovine embryos (Ishimori, et al. 1993), and form the basis of the vitrification protocol in assisted reproduction treatment. By the end of the 1990's, vitrification was being applied to human embryos with high survival leading to blastocyst development and pregnancy establishment (Hsieh, et al. 1999). Lately there has been increased interest in cryopreservation of oocytes. The desire to preserve fertility in young women undergoing gonadotoxic treatments or restrictions by embryo cryopreservation in some countries including Bangladesh, El Salvador, Germany,

Switzerland and Italy, has led to the necessity of oocyte donation or oocyte preservation for fertility maintenance (Gook, et al. 2012). Many studies have shown that vitrification of matured bovine oocytes have very high survival rates, and (Dalvit, et al. 2012) reported a 99% survival rate after thawing. In the same study oocytes were matured and fertilized in vitro and cultured to the blastocysts stage prior to vitrification. After thawing the morphology was evaluated and showed 100% survival. The rest of the vitrified blastocysts were transferred to recipient heifers, with fifteen heifers becoming pregnant which resulted in the birth of eleven calves. Pregnancy rates with in vitro produced embryos showed no difference compared to fresh blastocysts. Dalvit and others concluded that successful vitrification is possible when using bovine oocytes and embryos. They further noted that the survival and developmental rate are very similar to one another when comparing vitrified thawed oocytes and fresh oocytes (Dalvit, et al. 2012). Vitrification normally consists of two to three solutions that are prepared in TCM-199 with 10% fetal bovine serum (FBS). The equilibration solution consists of a relatively low concentration of cryoprotectants which is then increased in the second or third solution. These solutions also contain sucrose or trehalose as an extracellular cryoprotective agent (Kuleshova et. al, 1999). A study done by Dutta and colleagues evaluated the post-thaw developmental competence of vitrified bovine cumulus-oocyte complexes in vitro. Oocytes were aspirated from three to eight millimeter ovarian follicles and cryopreserved via vitrification (Dutta, et al. 2013). They used two vitrification solutions in TCM 199 and 10% FBS, 7.5% ethylene glycol and 7.5% dimethyl sulfoxide followed by 15% ethylene glycol, 15% dimethyl sulfoxide and 0.6M sucrose. The immature oocytes were placed into the first solution for three minutes followed by 30 seconds in the second solution at room temperature. The

oocytes were then immediately loaded into 0.25mL French straws that were preloaded with 0.6M sucrose in the holding medium with air gaps in between and plunged directly into liquid nitrogen. During the thawing stage straws were put into a 37°C water bath for 30 seconds and were gradually rehydrated in 0.6M sucrose solution for one minute. After washing, the morphology of the cells was assessed and put into vitro maturation. After 24 hours in the maturation medium they were then subjected to in vitro fertilization. In the vitrified group in vitro maturation on the basis of cumulus cells expansion was more than 80% and the unfrozen control had 93% maturation showing that vitrification had minimal effect on the survival rates and the ability of the oocytes to mature in vitro. High proportions of the oocytes retained their post thaw morphology after short exposure to high concentrations of permeating cryoprotectants with sucrose. Overall this protocol for vitrification had minimal effects on survival rates and the ability for the oocytes to mature in vitro (Dutta, et al. 2013).

Different procedures and protocols work as well and solution concentrations can be variable, and the amount of time in each solution may differ, as a single optimum protocol has not been established. In another study, Luvoni evaluated the effect of vitrification of feline ovarian cortex on follicular morphology and oocyte integrity, as well as meiotic competence. Oocytes that were isolated from vitrified tissues were able to resume meiosis (39.8%), showing that vitrification preserves the integrity of ovarian follicles and that oocytes that are taken from cryopreserved tissues can maintain the ability to resume meiosis (Luvoni et. al, 2012). Vitrification is technically simpler and may be more effective than slow freezing. In one study by Cao slow freezing and vitrification were compared with evaluation of survival, early embryonic development, fertilization, meiotic spindle assembly and chromosome alignment in

frozen thawed human oocytes (Cao et. al, 2009). The oocyte survival in the slow cooling group was significantly lower than those in the vitrification group and the fertilization rate was the same for both groups. When looking at the cleavage rate the vitrified zygotes had a significantly higher proportion than the slow cooling. Even when looking at the high quality embryos the post-vitrification survival was double that seen with slow cooling, including the percentage of blastocyst development. There was a much higher percentage of oocyte abnormalities in terms of spindle assembly and chromosome alignment in the slow freezing group. Overall vitrification was superior to the slow freezing method in terms of survival fertilization and developmental rate for human matured oocytes in this study (Cao, et al. 2009). In comparison Prentice (2011) found that maturation rate was low in vitrified immature

Table 2.2 Survival and development rate of human pronuclear embryos cryopreserved by slow cooling or vitrification using Cryotop method. (Derived from Valojerdi et al., 2009)

	Slow Rate Freezing	Vitrification
Survived/ cryopreserved rate	1730/1944 (89%)	5881/5881 (100%)
Cleaved/surviving rate	1557/1730 (90%)	5469/5881 (93%)
Blastocyst/cleaved rate	7961/1557 (51%)	3058/5469 (56%)
Blastocyst/cryopreserved rate	7961/1944 (41%)	3058/5881 (52%)

bovine cumulus cell complexed oocytes compared to slow freezing controls, with cleavage and blastocyst rates being higher in controls. Her results showed that the vitrification procedure damaged the bovine cumulus cells oocyte complexes resulting in poor nuclear maturation and embryo development (Prentice et. al, 2011). At present, approximately equal numbers of

babies have been born from both techniques (Noyes et. al, 2009). Table 2.2 shows the differences between slow rate freezing and vitrification in Valojerdi (2009) study showing the survival and development rate of human pronuclear embryos. These results showed that vitrification may be the superior cryopreservation technique with human embryos (Valojerdi, 2009). It would appear that there is a role for both procedures today and in the future. Vitrification is a fast, efficient, and economical method for oocyte cryopreservation providing a new alternative for the management of female infertility and fertility preservation options.

### **CRYOPRESERVATION TOOLS**

When vitrifying, it is ideal to minimize sample volume in order to achieve very rapid cooling and warming rates. Cryopreservation would not be possible without a safe, secure container for oocytes and embryos to be frozen in. There are many possible carriers to minimize the volume of vitrification solution that is used today. The most common are open pulled straw, cryoloop, cryotop, and cryolock. Cryotop systems consist of a fine, transparent polypropylene film attached to a plastic handle and equipped with a cover straw, in which cells can be loaded in a very small volume. Cryotops have been successfully used to cryopreserve cow and buffalo embryos (Laowtammathron et. al, 2005) and oocytes (Chian et. al, 2004). Open Pulled Straws (OPS) were especially designed for vitrification, and are an open system allowing for even faster cooling rates. Techniques are categorized as either 'open' or 'closed' according to occurrence of direct contact between the medium and liquid nitrogen during cryopreservation. Scientists who use open systems refer to the lack of evidence of disease transmission in an open system and regard their systems as more consistent and efficient (Vajta et. al, 2015). Cells can also be packaged into straws, which are then sealed on both ends

before plunging into liquid nitrogen to ensure that contamination is prevented. When comparing cryotops to open pulled straws a lower percentage of those oocytes vitrified in the open pulled straw had a normal spindle configuration compared to fresh oocytes, while normal spindle and chromosome configurations were observed more consistently in the cryotop vitrified bovine oocytes. This suggests that the cryotop system is a more efficient carrier for vitrification of bovine oocytes than open pulled straws (Morato et. al, 2008). The cryoloop method is a new technique where a thin nylon loop is used to suspend a film of cryoprotectant containing the oocytes to directly submerge them in liquid nitrogen. The loop shows minimal diffraction, is thin for fast freezing and is aerodynamic. In one experiment the cryoloop vitrification method produced a survival rate of 90.5% compared to the traditional slow freezing technique that yielded a rate of 54.4% (Mavrides and Morroll, 2002). Cryolock is a square shaped stick, with four flat surfaces. It has a concave tip, where the cells, oocytes or embryos are placed, it provides protection to the cell against contact with other surfaces, avoiding loss or damage of samples. During slow rate freezing there are many tools needed that include a secure container for oocytes and embryos to be frozen in. This is normally an 0.25mL straw that contains a plug, as a straw is needed for the programmable freezer control unit. In most programmable freezers, the straws are cooled by nitrogen vapor. The temperature inside the cooling chamber can be accurately controlled and the time course of the temperature can be programmed. The rate of heat exchange is governed by the temperature difference between the inside and the outside of the straw (Sansinen et. al, 2010) . When using both techniques the tools are very important aspects that need to be evaluated before cryopreservation. The freezing process is not the only concern for harmful

effects on the cell, there are many factors that can change the viability. One main issue with vitrification and slow rate freezing is the thawing process after vitrification and slow rate freezing.

## **WARMING**

Freezing cells allows them to be stored for years in a state where their normal metabolic activity is suspended to protect them from damage from chemical reactivity and time (Simione, 2009). The cells can then be warmed and resuscitated as needed. The thawing and rewarming process after the storage of oocytes and/or embryos also has a very important role for the survival rates (Schellander et. al, 1994). Time is the most important factor when warming cells. These cells are extremely fragile and need gentle handling and immediate settlement into a pre-warmed culture medium. Some cell types are more sensitive than others to the freezing process (Felipe de Lara Janz et. al, 2011). Not only do the cells need to be handled properly to ensure consistency and sterility but also to ensure a correct choice of type and amount of cryoprotective agent added (Tucker, 2007). On thawing, oocytes have to be rehydrated and the intracellular cryoprotectant needs to be removed. The extracellular agents, like sucrose, have been shown to have a beneficial influence during removal of the cryoprotectants (Yokoyama et. al, 2012). As explained previously, freezing can cause cellular injury, resulting in cell death, especially from -15°C to -30°C (Djuwantono et. al, 2011) and cells will pass through this critical range twice, once during freezing and again at thawing. Depending on what cryopreservation tool is used, the thawing protocol will be altered. When using straws, they will be immediately submerged in a 37°C water bath and then oocytes will be removed into a dilution solution. If on a cryolock or cryotop, the cap will be removed and

the stick itself will be placed into a warmed or room temperature dilution solution. Generally, cells should be thawed quickly to avoid ice crystal formation but diluted slowly to remove the intercellular cryoprotectant while preventing osmotic shock (Tucker, 2007).

## **EMBRYOS**

The preservation of female genetics can be done through the preservation of the germplasm. Germplasm refer to the tissues maintained for the purpose of animal breeding, preservation, and other research uses. These resources can be animal breeding lines maintained in a breeding program. Gametes can be collected at different developmental stages using many different techniques including post ovulation flushing, transabdominal laparoscopic surgery, transvaginal uterine flushing, or trans rectal ovum pick up (Arav, 2014). These procedures may be performed during a natural estrous cycle, following chemical stimulation to achieve superovulation, or after fertilization at various developmental stages before embryo implantation. The first reports on successful embryo cryopreservation were published in 1971 by Whittingham (Whittingham, 1971) more than two decades after Polge reported success in freezing spermatozoa in 1949 (Polge and Parkes, 1949). Embryo freezing has the ability of preserving both parents genetic background. In wild animals, especially with endangered species, this is often nearly impossible, and the opportunity to collect oocytes or embryos is very rare, such that researchers find it helpful to use laboratory, farm, or companion animals as model species during the process of developing the necessary reproductive techniques associated with embryo cryopreservation. According to a recent report by the International Embryo Transfer Society, over 300,000 frozen thawed bovine embryos were transferred in 2008 worldwide (Thibier, 2009). This success was motivated by

three primary factors, the availability of financial resources to support an increasing number of studies, the availability of an unlimited flow of oocytes from slaughterhouses that make studies possible, and the fact that non-surgical collection of embryos is possible in cattle via flushing (Saragusty, 2011). Regardless of many advances in the field of embryo cryopreservation, there is still no agreement as to the ideal developmental stage for embryo cryopreservation. Factors that have an impact on fresh embryo transfer have a comparable effect on the transfer of frozen thawed embryos. In humans, patient age at the time of freezing influences pregnancy outcome due to poorer quality of oocytes collected from aged females, often due to aneuploidy (Sherbahn, 2011). The number of embryos transferred and the quality of embryos impact the outcome, in both frozen and fresh embryos (Burks et. al, 2015). When research on human embryos is performed, the ethical questions arise whether we are doing research on unborn children and killing them (Beca et. al, 2014). To reduce any religious or moral issues the cryopreservation of oocytes was evaluated and has been shown to be successful.

## **MATURE OOCYTES**

Maturation of the oocyte in a follicle depends on the progress of the maturation of the surrounding follicle cells. The oocyte is arrested for an extended time at the diplotene stage of the first meiosis and now will prepare itself for meiosis completion due to the triggered luteinizing hormone peak and the dominance of the follicle (Senger, 2005). Within the oocyte, the completion of the first meiosis is initiated by the ejection of the first polar body and the beginning of the second meiosis starts and arrests in the metaphase stage. The start of meiosis II is also known as the equational division (Senger, 2005). The end result is the production of

four haploid cells from the two haploid cells produced in meiosis one. First they go through prophase II, the nucleoli and nuclear envelope start to disappear, along with the shortening and thickening of the chromatids (Senger, 2005). Centrosomes move to the polar regions and arrange on the spindle fibers (Raven et. al, 2016). Centromeres attach to the spindle fibers from the centrosome at the opposite poles in metaphase II and will not continue until fertilization occurs (Raven et. al, 2016). The most characterized cytoskeletal damage by cryopreservation via slow cooling and vitrification concerns the disruption of sub-cortical actin network, microtubule depolymerization, abnormal spindle configuration and chromosome scattering within mature oocytes and therefore compromising the ability to develop into a viable embryo (Ferris et. al, 2015). One experiment, published by Huang, demonstrated that there is no significant difference in post-warming survival, normal spindle morphology, chromosome alignment and incidence of aneuploidy between in-vitro and in-vivo matured murine oocytes (Huang, 2008). Nonetheless, he did display that oocyte in vitro maturation and vitrification did decrease the cleavage and blastocyst formation rates compared to in vivo matured mouse oocytes after vitrification (Huang et. al, 2008). The reduced development is most likely an effect of an increase in DNA fragmentation occurring in the in-vitro matured oocytes, likely caused by suboptimal culture conditions (Huang et. al, 2008). Mature metaphase II mouse oocytes were divided into two groups and cryopreserved via slow freezing and vitrification in a study completed in early 2015. After thawing, the oocytes were observed for survival and fertilization, and were also compared to investigate the effect of both protocols on spindle reassembly and chromosome configuration at different times during warming. Survival rates were higher for vitrified oocytes as compared to slow frozen oocytes.

The morphology of the meiotic spindle was found to be in a disorganized configuration in slow frozen oocytes at all time points whereas in vitrified oocytes the spindles were found to be aligned at all-time points. Chromosomes were seen to be displaced from equatorial region in both groups (Jadoon, et al. 2015). This shows that vitrification may be a better protocol when using mature metaphase II oocytes but can the same be said to be the same with immature germinal vesicle oocytes?

## **IMMATURE OOCYTES**

In immature oocytes the microtubules are not yet organized in the metaphase two spindle formations. Therefore, cryopreservation at this stage bypasses the risk of chromosome abnormalities as the chromatin is decondensed and is still protected by the nuclear envelope (Senger, 2005). Female gametes are normally cryopreserved as cumulus oocyte complexes (COC), with the surrounding cumulus cells which are small and numerous and are easy to freeze (Schellander et. al, 1994). In the late 1980s, it appeared that the oocyte was too vulnerable to cryopreserve at the metaphase II stage and animal work shifted to evaluating slow rate freezing of germinal vesicle oocytes, but little was published (Jain and Paulson, 2006). High survival could be accomplished with mouse germinal vesicle oocytes (Van der Elst, 1992) but some studies observed reduced overall development (Schroeder et. al, 1990), this suggested that there was no total benefit to cryopreserving at the germinal vesicle compared with the metaphase II stage. In many studies it has been shown that cryopreserving immature oocytes harms the ooplasm (Kim et. al, 2014), potentially due to inadequate conditions of in vitro maturation or basic oocyte defects. In a study led by Coticchio, they explored the possibility that the DNA of immature oocytes may be damaged and that the

inability to trigger a repair action is associated with the germinal vesicle arrest. They found double strand DNA breaks in these oocytes, and hypothesized that this may be an important factor affecting the meiotic resumption (Coticchio et. al, 2015). It was demonstrated that immature bovine oocytes are very sensitive to low temperatures from 0 to 4°C, with an abnormal meiotic spindle formation that inhibits the normal development of oocytes after in vitro fertilization (Wu and Leibo, 1999). There are still many flaws with the experimental designs when using immature oocytes for patients undergoing fertility treatments. Such that, with current protocols it may be best to cryopreserve mature oocytes after in vitro maturation to metaphase II (Combelles et al. 2012). Cryopreserving after maturation to metaphase II still leaves us with the issue of the spindle fibers being damaged during cryopreservation by ice crystal formation. Oocyte freezing is more advisable for patients with personal, moral, or religious objections to the cryopreservation of embryos as oocyte cryopreservation would eliminate many of these ethical and legal implications. It is much more acceptable to discard frozen oocytes rather than frozen embryos.

## **IN VITRO EMBRYO PRODUCTION**

In vitro maturation and in vitro fertilization are both steps in the in vitro embryo production process. In vitro maturation is a precursor to in vitro fertilization when using immature oocytes. In vitro maturation is an optional step depending on the state of the oocytes at retrieval: with in vitro fertilization, the oocytes are normally matured inside the ovaries, while in in vitro maturation the immature oocytes are removed and then are matured outside the body. In vitro maturation requires no hyper stimulation of the ovaries. Once the immature oocytes are removed, they are placed in a petri dish and exposed to a combination

of luteinizing hormones and follicle stimulating hormones. The oocytes are usually left in this medium for 22 to 24 hours; once they have reached maturation, they are then inseminated normally (by IVF) or by intracytoplasmic sperm injection (ICSI) for fertilization of the matured oocytes (Lim et. al, 2013). The developing embryos are then transferred back into the uterus within four to seven days. While the success rates for in vitro maturation are better than in the past, they are still lower than those for in vitro fertilization following in vivo maturation. Pregnancy rates differ based on a few factors, the most important being the age of the females' oocytes (Sherbahn, 2011). In humans, a typical 35-year-old woman, with no major health problems, can normally have a 40% success rate for a single cycle of in vitro fertilization, compared to 32% with in vitro maturation (Abma et. al, 1997). A full cycle of in vitro fertilization can cost as much as \$30,000, while a full cycle of in vitro maturation is closer to \$6,000 and often both procedures need to be repeated before pregnancy occurs (Abma et. al, 1997). In vitro maturation is clearly a safer alternative than using in vivo matured oocytes for in vitro fertilization in patients' sensitive to gonadotropins. In vitro maturation may be the procedure of choice not only for infertile couples but also for obtaining oocytes for preservation of fertility. In vitro maturation is a good alternative in fertilization preservation in cancer patients. The process of in vitro maturation can be conducted safely even with patients who are hormone sensitive. In vitro maturation could be a key method used for patients who are soon to be using cancer therapy since there is no interruption to treatment for ovarian stimulation (Lim et. al, 2013).

In vitro fertilization is the combination of an oocyte with a sperm cell in a petri dish to achieve successful fertilization. In vitro fertilization is a common assisted reproductive

technology, the first successful in vitro fertilization and birth of live offspring using frozen thawed mouse oocytes was reported in 1976 (Parkening and Chang, 1976). The process of in vitro fertilization is very basic; often beginning with super ovulation of the female to obtain mature oocytes. This can be done with hormonal injections; follicle stimulating hormone, luteinizing hormone, prostaglandin and others (Yenkie and Diwekar, 2014). Super ovulation helps the female mature more than one oocyte in a cycle to optimize retrieval rates.

The second step is harvesting the oocytes which can be done through multiple methods; surgically exposing the ovary and aspirating the follicles, non-surgically aspirating the follicles utilizing ultrasonography and a hypodermic needle, and aspirating follicles postmortem (Lai et. al, 2015). The most common technique for recovery of oocytes by aspiration in equids involves inserting a needle through the vaginal wall and the use of an ultrasound. This helps identify the dominant follicles before aspirating the oocytes into a special apparatus, such as a filter (Ulloa et. al, 2015). With humans it is done through a minor surgery of inserting a thin needle through the vagina and into the ovary and follicles that contain the oocytes. This needle is connected to a suction device which then pulls the oocytes and the fluid out of each follicle, one at a time (Ulloa et. al, 2015). The procedure with bovine is very similar, it is done the same way but investigators go rectally to grasp the ovary and then go in vaginally with the ultrasound and follicle aspirator to remove the oocytes from the ovaries (Ulloa et. al, 2015).

The next step is insemination and fertilization; sperm cells that have good motility and morphology are selected and then placed with the best quality oocytes. To isolate the best quality bovine sperm cells they are put into a percoll gradient and centrifuged down multiple

times. There is two-layer density gradient which separates the motile and non-motile sperm cells. The fast and well moving sperm cells will be able to swim down through the lower gradient, but the poor cells will be trapped in the gradient interface (Guimaraes et. al, 2014). To determine the best quality oocytes, the amount of cumulus cell layers is evaluated. More layers of cumulus present are associated with increased transfer of necessary factors to the oocytes to fertilize and grow throughout the in vitro fertilization process (Tetsuka et. al, 2015).

The fourth step consists of monitoring the embryo, to make sure it is growing properly. The embryo will be dividing rapidly at this time and by day seven the embryo will be ready for transfer (Moreno et. al, 2015). To transfer an embryo a catheter containing the embryos is inserted into the vagina, through the cervix, and up into the uterus. Pregnancy occurs and is successful if an embryo sticks and is implanted in the lining of the uterus and grows (Moreno et. al, 2015). Choosing embryos with the highest developmental potential is one key step to overcoming the hurdle of early embryonic mortality. The success of the development of the embryo is highly complex and depends on both the embryo quality and the maternal receptivity. (Tanabe and Hasler, 1985) Oocyte quality is another important factor to the health of the embryo (Dumesic, 2015). Regardless of these factors, the survival depends on the delicate placement of the embryos at the proper location. The first successful embryo transfer was done in a rabbit in 1890 by Walter Heape (1890), and began in food animals in the 1930s with sheep and goats (Warwick and Horlacher, 1934) and later in cattle (Chang, 1951) and swine (Day and Melampy, 1959) in the 1950s. Commercial and non-invasive embryo transfers in the United States began in the early 1970s and continues to thrive (Moffit, 1979). Pregnancy rates are generally around 60% with fresh embryo transfer and 50-60% with frozen thawed

embryo transfers (Galliano et. al, 2015). In cattle there are about 150,000 potential oocytes (Selk, 2016) in the female but in natural breeding only a fraction of the reproductive potential of a single cow can be realized. Embryo transfer is a technique that can greatly increase the number of offspring that a genetically superior cow can produce.

#### **CURRENT ISSUES FOR FUTURE RESEARCH**

The global diversity of domestic animals is considered to be under threat, a large number of domestic animals worldwide are endangered or in critical status. In farm animals, trends in within-breed diversity are as significant as between-breed diversity that are in demand to be able to manage with changing requirements. These would include the requirements in breeding and the selection process. Cryopreservation of germplasm is a smart strategy to conserve existing diversity for future use commercially and in rare breeds. There are studies (Baka et. al, 1995) (Combelles and Chateau, 2012) (Fasano et. l, 2014) that not only affect our domestic and wild species but humans as well. Although over one hundred babies have been born from oocyte storage, pregnancy rates still remain low (Cobo et. al, 2015). Others problems beyond intracellular ice formation and osmotic effects need to be identified. The ability to cryopreserve human oocytes and store them would be beneficial for cancer patients, since there is a risk of these patients becoming sterile after therapy (Barcroft et. al, 2013). It would also be beneficial for women who want to delay reproduction and to alleviate religious concerns dealing with embryo storage. Studying and experimenting with domestic animal species' oocytes and sperm cells will help us in the future with our own reproduction issues.

New methods that include vitrification have proven useful in increasing the survival and pregnancy rates of oocytes and embryos. There are other methods that have not been tested yet, including different protocols, processes and solutions, cryoprotectant concentrations and others with both immature and mature oocytes. The study and research of vitrification protocols has led to the formation of new ideas and has demonstrated the flexibility of cells to survive cryopreservation. In general, further advances are needed to improve the storage of human oocytes before a widespread routine is used in clinics worldwide. The use of animal oocytes and embryo will help resolve these issues and find an optimal cryopreservation protocol when utilizing immature and mature cumulus cell complex oocytes.

## **CHAPTER III**

### **VITRIFICATION OF BOVINE IMMATURE AND MATURE CUMULUS CELL OOCYTE COMPLEXES**

#### **INTRODUCTION**

In vitro production can be maximized through the use of cryopreservation for long term oocyte storage (Shaw 2000). Cryopreserving of mature oocytes can result in damage to the metaphase spindle due to the temperature sensitivity of the microfilaments and microtubules as well as other morphological issues (Baka et. al, 1995). In 2008 Huang observed a decrease in cleavage and blastocyst formation of murine IVM oocytes after vitrification compared to in vivo oocytes (Huang et. al, 2008). In addition, Jadoon and others concluded the meiotic spindle of mature metaphase II mouse oocytes after cryopreservation were disorganized and displaced (Jadoon, et al. 2015). Cryopreservation of immature oocytes may circumvent this problem because these structures have not yet formed and the genetic material is enclosed within a nuclear envelope (Benson, 2008). However, Kim demonstrated that cryopreserving immature rat oocytes may harm the ooplasm leading to reduced development and compromise cell cycle status compared to fresh control oocytes (Kim et. al, 2014). The use of different cryoprotectants can also affect the viability of each specific cell type for the freezing and warming process. DMSO and glycerol are the most commonly used cryoprotectants but have mixed results of specific cell types and sizes. Si and colleagues found that glycerol at concentrations below 5% in comparison to 15% dimethyl sulfoxide led to improved post-thaw survival (Si, 2004). However, Lim and colleagues showed that IVM bovine oocytes were more morphologically normal, had higher spermatozoa penetration rate and higher percentage

developed to 2-cell stage at 48 h postinsemination when cryopreserved with 1.0 M DMSO versus 1.0 M glycerol (Lim et. al, 1999)

The aim of these experiments was to determine if immature and mature cumulus cell complexes cryopreserved via vitrification with dimethyl sulfoxide and glycerol solutions would remain viable following thawing and in vitro fertilization, as indicated by pronuclei formation. The specific aim was to vitrify immature germinal vesicle stage COC in different cryoprotectants to determine differences in survival, as assessed through post thaw oocyte maturation and fertilization rates with mature oocytes after in vitro fertilization. The second aim was to vitrify mature M2 stage COC in different cryoprotectants with an endpoint including post thaw oocyte fertilization rates in mature oocytes after in vitro fertilization. We hypothesized that DMSO would yield higher survival and fertilization rates than glycerol for both immature and mature bovine oocytes. We also hypothesized that immature bovine oocytes would fertilize at a higher rate than mature oocytes after vitrification and IVF.

An experiment conducted in 2013 determined if immature oocytes cryopreserved by vitrification and slow rate freezing would resume meiosis upon thawing (Hardin et. al, 2013). There were two separate experiments in this study, comparing vitrification and slow rate to one another with controls. Within the first experiment oocytes were cryopreserved via slow rate freezing process using 10% ethylene glycol. The second experiment was subjected to vitrification in a three step process using glycerol and ethylene glycol (10% glycerol for 5 minutes, 10% glycerol and 20% ethylene glycol for 5 minutes then 24% glycerol and 26% ethylene glycol for 45 seconds). Upon thawing and IVM the results in the slow rate freezing group yielded oocytes matured to the metaphase two stage at the same rate as the controls

(45% and 55% respectively  $P = 0.44$ ). Within the vitrification group the cryopreserved oocytes matured to the metaphase two stage at a lower rate than the controls (49% and 79%  $P = 0.01$ ). This experiment indicated that immature cryopreserved oocytes would resume meiosis following warming while vitrified immature oocytes matured at a lower rate than controls under these conditions. These unexpected results gave us motivation to further investigate vitrification on immature bovine oocytes.

## **MATERIALS AND METHODS**

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

### **EXPERIMENTAL DESIGN**

Oocytes from Angus cross cows located in St. Gabriel, LA at the LSU Reproductive Biology Center were collected by transvaginal ultrasound-guided follicular aspiration (TUGA) and vitrified immediately as immature oocytes (experiment one). On a separate collection day oocytes were placed into maturation immediately after OPU and prior to manipulation (experiment two). A total of 200 mature oocytes and 200 immature oocytes were collected and vitrified in DMSO and glycerol treatment groups in both experiments.

### **EXPERIMENT ONE DESIGN**

Oocytes were vitrified using two cryoprotectants solutions on cryolocks immediately after collection and stored in liquid nitrogen for at least seven days (Table 3.1). After vitrification and storage, oocytes were thawed in the dilution solution for 5 minutes (Table 3.4), washed with HEPES-TALP and placed in 40  $\mu$ L droplets of standard IVM medium consisting of M199 supplemented with 10% fetal bovine serum (FBS), 0.2 mM sodium

pyruvate, 1% pen/strep, 2 mM glutamine, and FSH (5.0 µg/mL) (Appendix B) covered with mineral oil. Following maturation for 22 hours within the 5% CO<sub>2</sub> incubator, 10% of the oocytes were evaluated for maturation rate by fixation in 3:1 methanol acetic acid for at least 48 hours and stained with aceto orcein. The remaining oocytes were placed into standard IVF for fertilization for 18 hours in a 5% CO<sub>2</sub> incubator. After 18 hours they were vortexed in 1 mg/mL hyaluronidase solution and put into global 35+ droplets covered with oil and cultured for 24 hours. They were then stained with Hoechst 33342 and evaluated to determine their pronuclei formation, then fixed and stained in acetic orcein for further evaluation.

## **EXPERIMENT TWO DESIGN**

Aspirated oocytes were put into 40 µL droplets of standard IVM medium covered with mineral oil immediately after collection and prior to vitrification in 5% CO<sub>2</sub> incubator for 22 hours. Following maturation, 10% of the oocytes were evaluated to determine maturation rate by fixation in 3:1 methanol acetic acid for at least 48 hours and staining with aceto Orcein (Appendix A and B). The remaining oocytes were then vitrified using DMSO and Glycerol cryoprotectant solutions, shown in table 3.1 (5 minutes in the first solution and 45 seconds in the second solution) (Appendix A and B) on cryolocks and stored in liquid nitrogen for at least seven days. After vitrification and storage, all oocytes in both cryoprotectant groups were thawed in an 0.025M sucrose dilution solution shown in table 3.2 for 5 minutes and washed with HEPES-TALP (Appendix B). Oocytes were then placed into standard IVF (see Appendix A and B) for fertilization in 5% CO<sub>2</sub> incubator for 18 hours. After 18 hours they were then vortexed in 1 mg/mL hyaluronidase solution (Appendix B) and put into global 35+, embryos culture media (Life Global) droplets covered with oil for 24 hours. They were then stained and

evaluated using Hoechst 33342 (Appendix A and B) to determine pronuclear formation and then fixed to be stained in acetic orcein for further evaluation.

Table 3.1 Composition of Cryoprotectant Solutions for both Treatments

Vitrification Solutions for Glycerol Treatment		Vitrification Solutions for DMSO Treatment	
Vitrification One	Vitrification Two	Vitrification One	Vitrification Two
PBS	PBS	PBS	PBS
20% Calf Serum	20% Calf Serum	20% Calf Serum	20% Calf Serum
10% Ethylene Glycol	20% Ethylene Glycol	10% Ethylene Glycol	20% Ethylene Glycol
10% Glycerol	20% Glycerol	10% DMSO	20% DMSO
0.5 M sucrose	0.5 M sucrose	0.5 M sucrose	0.5 M sucrose

Table 3.2 Dilution Solution for both Experiments for both Treatments

PBS	16 mL
20% Calf Serum	4 mL
0.025 M Sucrose	0.855g

### **IVF VALIDATION**

As validation of the IVF system, we received matured oocytes from a commercial source DeSoto Biosciences (TN). Oocytes went into IVF-TALP (Appendix A and B) 20-30 per well with percoll separated sperm cells and incubated in 5% CO<sub>2</sub> for 18 hours. After 18 hours they were vortexed in hyaluronidase and put into 50 µL droplets of global 35+ medium covered in oil until the next morning. All oocytes were then stained and evaluated using Hoechst 33342 to

determine pronuclei formation and then fixed and stained in acetic orcein for further evaluation.

### **PREPARATION**

Required media included Dulbecco's Phosphate Buffered Saline (D-PBS), Hepes TALP, standard maturation medium, dimethyl sulfoxide vitrification solution, glycerol vitrification solution, dilution solution, standard fertilization medium and culture medium (Appendix B) was prepared the day prior to use. On the afternoon before oocyte collection, D-PBS was placed in the incubator to warm overnight. 10 mL of bovine calf serum (BCS) and 1 mL of Heparin (Sagent Pharmaceuticals, Schaumburg, IL, USA) were added to the D-PBS the morning of collection. HEPES-TALP holding media for the control and treatment groups consisted of 20 mL HEPES-TL (Caisson Labs, North Logan, UT, USA) with 60 mg BSA and 200  $\mu$ L sodium pyruvate and pen/strep (Gibco, No.15140).

On the afternoon before oocyte collection, all necessary media dishes were prepared with premade solutions and equilibrated for at least two hours in the incubator before being used. Petri dishes (35mm, BD Falcon) of HEPES-TALP and D-PBS were placed in a non-CO<sub>2</sub> incubator at 39°C to warm 2 hours prior to oocyte collection, for washing, and sorting into treatment groups. 500  $\mu$ L of fertilization media was put into a 4 well dish and water put into the center on the dish. Both maturation and fertilization media were then placed into a 37°C 5% CO<sub>2</sub> incubator for at least 2 hours prior to adding oocytes, to allow for equilibration. Fixation media was a 3:1 solution of methanol acetic acid and was added before staining in aceto orcein.

### **OOCYTE COLLECTION**

These experiments consisted of 9 collection days, four for experiment one and five for experiment two. Oocytes were collected from follicles three to eight mm in diameter by transvaginal ultrasound-guided follicular aspiration. Oocytes from all aspirations on a given day were pooled and transferred to a 35 mm dish containing pre-warmed HEPES-TALP holding medium. Oocytes were randomly assigned to one of the two treatment groups: dimethyl sulfoxide or glycerol cryoprotectant solution. Each collection day was pre-determined to be an immature or mature oocyte group. Following collection oocytes were either matured or vitrified following above outlined procedures in accordance with the allocated groups for that specific day.

### **TRANSVAGINAL ULTRASOUND-GUIDED FOLLICULAR ASPIRATION**

Oocytes were obtained with TUGA (Figure 3.1) at the LSU AgCenter, Reproductive Biology Center in St. Gabriel, LA. Each cow was held within a holding chute and given an epidural injection of 5 mL lidocaine (Vet One, Product No. 510212) between vertebrae near the base of the tail. An ultrasound probe with a 7.5 MHz transducer was inserted into the vaginal canal, and the ovary was held through the rectum and placed against the vaginal wall along the transducer at the end of the ultrasound probe. The ovary was observed on an ultrasound monitor and the follicles were identified. An 18-gauge disposable collection needle inside the ultrasound probe was connected to a suction pump via polyethylene tubing, after good visualization of the follicle with the ultrasound, the collection needle was advanced through the vaginal wall and into the follicle, the follicular fluid along with the oocytes were aspirated via suction. The tubing and needles were rinsed with collection media after each

ovary and the needle changed after each cow, the follicular fluid and oocytes were deposited into an embryo collection filter (Agtech Inc., Manhattan, KS, USA). All media were maintained at 39°C during collection with a heated tube holder. After collection of two cows the tubing and needles were rinsed with collection media (D-PBS with heparin and BCS) and all fluid filtered and poured into a search dish. Oocytes were located and placed into a warmed 35 mm dish containing HEPES-TALP.

Following collection, all oocytes were then placed into a 1mL micro centrifuge tube containing warmed HEPES-TALP and transferred to the Animal and Food Science Laboratory on Louisiana State University campus.

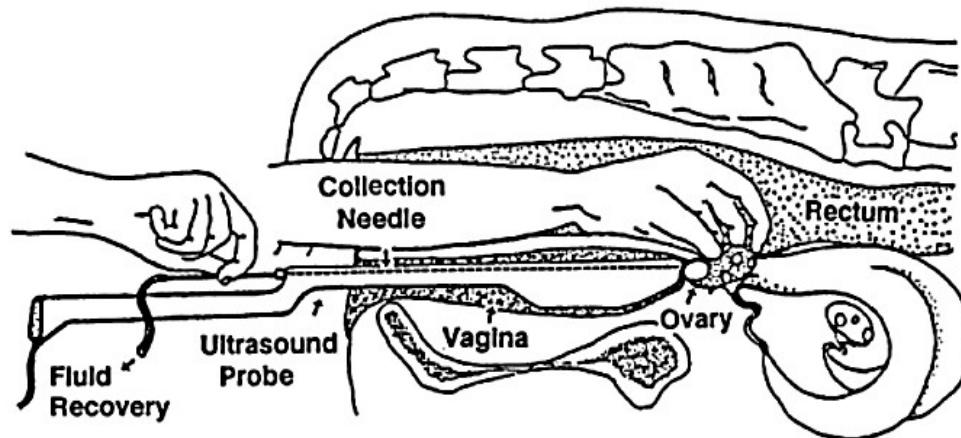


Figure 3.1 Diagram of transvaginal ultrasound-guided follicular aspiration. (Drawing by E. Meintjes, 1995)

### **VITRIFICATION AND WARMING PROCEDURES**

Oocytes in both maturation groups were vitrified in both dimethyl sulfoxide and glycerol cryoprotectant groups. Oocytes, within each day were randomly assigned to glycerol or DMSO group for vitrification. Groups of two to five oocytes would be placed into the designated equilibration solution for 5 minutes (Figure 3.2). They were then transferred to the

higher concentration vitrification solution, placed on the cryolock device (Figure 3.3), excess media removed and plunged into liquid nitrogen within 45 seconds (Figure 3.2). The tops to the cryolocks were put on with forceps after being plunged into liquid nitrogen. The immature group of oocytes would go through the same procedure without maturation beforehand. They were transferred to the Food and Animal Science Laboratory Building in HEPES-TALP and vitrified immediately.

After the oocytes were stored in the liquid nitrogen for at least seven days, the cryolocks were removed from the canisters, the tops removed and the vitrified oocytes on the cryolocks were immersed directly into a petri dish containing the warmed dilution solution and were kept there for 5 minutes to insure all cryoprotectants were removed from the ooplasm. Oocytes that had been thawed were then rinsed in HEPES-TALP. Matured oocytes were then washed in IVF-TALP and then placed into 48  $\mu$ L droplets of IVF-TALP. The oocytes were in groups of 10-12 per droplet, in a 35mm dish that was being held within the 5% CO<sub>2</sub> incubator at 37°C two hours prior to fertilization. Immature oocytes went through maturation prior to IVF.

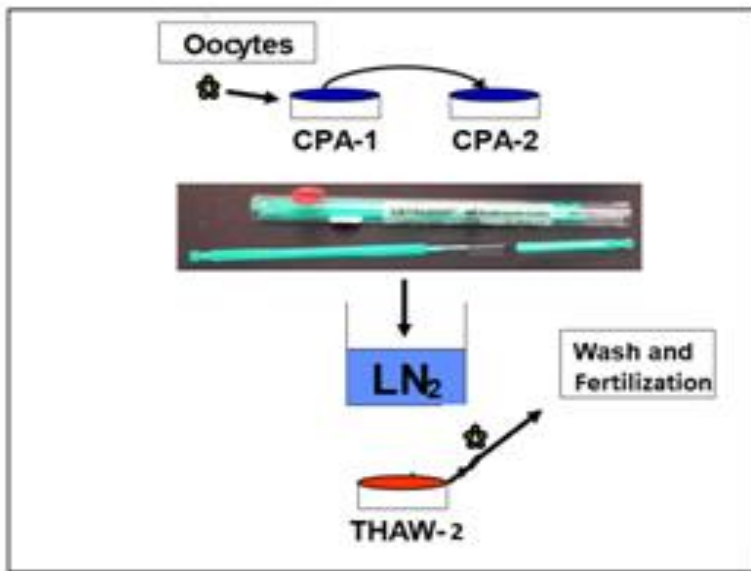


Figure 3.2 Cryolock vitrification and warming procedure for both experiments.

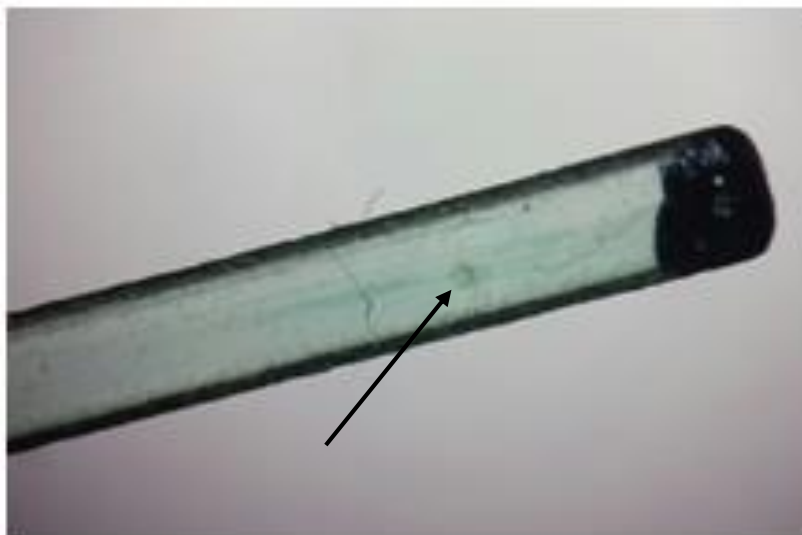


Figure 3.3 One denuded bovine oocyte loaded onto Cryolock device (indicated by arrow)

### **IN VITRO FERTILIZATION**

In vitro fertilization was performed using spermatozoa from multiple batches of semen from a single Angus bull of proven fertility that had been frozen in straws, from Genex. One

0.25 mL straw of frozen semen was thawed in a 39°C water bath for 30 seconds. Semen was separated on percoll gradient (Irvine Scientific 99264) and washed three times using IVF- TALP by centrifugation at 400 x g for 20 minutes starting at 37°C and reducing to 25°C, 10 minutes and 5 minutes consecutively. Each time the sperm pellet was re-suspended with a reducing amount of IVF-TALP. After the last suspension, the IVF-TALP was aspirated down to the sperm pellet and the appropriate amount of IVF-TALP was added to reach a final concentration of  $1 \times 10^6$  sperm/mL. 20 µL of the final sperm suspension was added to each well of oocytes and IVF-TALP within a 5% CO<sub>2</sub> incubator at 39°C for 18 hours.

Example:  $7500/X = \mu\text{L of sperm}$ ;  $300 - \mu\text{L of sperm} = \text{amount of IVF-TALP}$

Sperm morphology was evaluated by adding 95 µL of water to 5 µL of the sperm pellet to a micro centrifuge tube and gently mixing. 10 µL of sperm dilution was added to each side of the hemocytometer and sperm was counted. Sperm mobility was also estimated post thaw, by looking under a microscope.

### **STAINING AND DATA COLLECTION**

All oocytes were placed into Hoechst 33342 (5 µL with 500 µL HEPES) for 10 minutes. They were then rinsed in HEPES-TALP and 8-10 embryos were transferred to a microscope slide with minimal media, and mounted on the slide with a cover slip. They were observed with an epi-fluorescent microscope equipped with UV excitation and appropriate barrier filters using Hoechst 33342, 42 hours after IVF.

### **STATISTICAL ANALYSIS**

This experiment consisted of 9 replicates, 5 mature oocyte groups and 4 immature oocyte groups. Assessment of oocyte viability was based on morphology, the fertilization rate

as indicated by cleavage, and development rate was compared within respective parameters using Fisher Exact Test. Means with a probability value of  $P < 0.05$  were considered significantly different.

## RESULTS

### EXPERIMENT ONE

IVF results of immature oocytes after vitrification in the DMSO treatment group had 8.49% fertilization rate compared with 0% oocytes in the glycerol group, seen in Table 3.3. There was no statistical difference between DMSO and glycerol fertilized oocytes with the P-value of  $P = 0.0056$  due to low numbers of oocytes fertilized (DMSO 9/106). The results for both treatment groups did show a proportion of the oocytes having polyspermy fertilization (3.77% and 3.70%).

Table 3.3 Nuclear status of Immature Oocytes after IVF with DMSO (N=106) and Glycerol (n=81)

	DMSO % (no.)	Glycerol % (no.)	P value
Matured <sup>A</sup>	12.26% (13/106)	2.47% (2/81)	0.0149
Fertilized	8.49% (9/106)	0%	0.0056
Immature	0%	1.23% (1/81)	0.4332
Polyspermy	3.77%(4/106)	3.70% (3/81)	1.0000
Degenerate	74.04% (77/106)	80.49% (66/81)	0.1688
Indeterminate	11.54%(12/106)	7.32% (9/81)	1.0000

<sup>A</sup> Oocytes matured includes oocytes fertilized

\*Proportions with a probability value of  $P < 0.05$  were considered significantly different

Results also demonstrated that some proportion of oocytes became matured (12.26% and 2.47%) after vitrification in both treatment groups and showed a significant difference the two ( $P=0.0149$ ) but were not able to become fertilized. Results also show a small proportion of oocytes still immature (1.23% Glycerol) after vitrification, IVM and IVF, signifying that vitrification could be interfering with the resumption of meiosis. The majority amount of the oocytes was degenerate (74.04% DMSO 80.49% glycerol) and some were indeterminate (11.54% DMSO 7.32% glycerol) (as described in oocyte analysis). The numbers, proportions and P-values can be seen in Table 3.3. Pictures taken after Hoechst 33342 and Acetic Orcein staining can be seen in Figure 3.4 of both treatments groups within the first experiment illustrating different nuclear maturation observed after IVF.

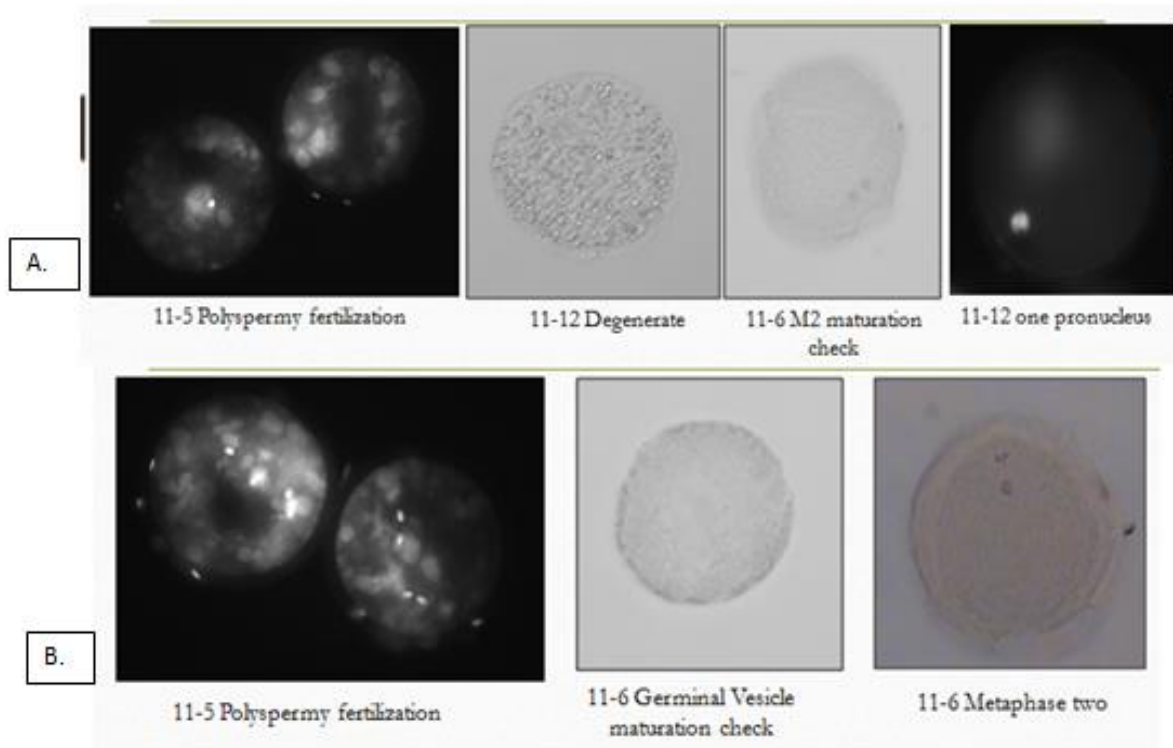


Figure 3.4 Different stages of nuclear maturation of Immature DMSO (A) and Glycerol (B) cryoprotectant treatments.

Oocytes were analyzed (Table 3.3), for nuclear status and development following fertilization, pictures as followed in Figure 3.4. The indication of fertilization included formation of two pronuclei. Matured oocytes had either one pronucleus, a metaphase two plate or were anaphase stage (detailed tables found in Appendix C). Immature group indicated oocytes in metaphase one, and polyspermy means polyspermy fertilization occurred. The degenerate variable means that the oocytes were dead. The indeterminate variable means that the oocytes were unknown, had sperm heads and/or cumulus cells interfering with visualization (detailed tables found in Appendix C).

## EXPERIMENT TWO

Mature oocytes had fertilization in both cryoprotectant treatment groups after vitrification and IVF, but with low proportions in both treatments, DMSO 12.62% fertilized and glycerol 3.4% seen in Table 3.4. There is a statistically significant difference between the two cryoprotectants with a P-value of 0.0335. The results for the glycerol treatment group did show a proportion of the oocytes having polyspermy fertilization (3.4%) but none in the DMSO group. The results also revealed that a decent portion of oocytes were still mature after IVF in both treatments (29.12% DMSO and 13.63% glycerol) and showed a significant difference ( $P=0.0136$ ). There were also some oocytes that did not mature after IVM and prior to vitrification that were designated as immature oocytes. The majority of the oocytes were degenerate or indeterminate (as described in oocyte analysis). Pictures taken after Hoechst 33342 and Acetic Orcein staining can be seen in Figure 3.5 with treatments groups within the second experiment illustrating different nuclear maturation observed after IVF.

Oocytes were analyzed (Table 3.4), for nuclear status and development following fertilization. The indication of fertilization included formation of two pronuclei and included three cell and four cell embryo development as seen in Figure 3.5. Matured oocytes had either one pronucleus, a metaphase two plate or were anaphase stage (detailed tables found in Appendix C). Immature group indicated oocytes in metaphase one or germinal vesicles being present, and polyspermy means polyspermy fertilization occurred. The degenerate variable means that the oocytes were dead. The indeterminate variable means that the oocytes were unknown, had sperm heads and/or cumulus cells interfering with visualization (Appendix C).

Table 3.4 Nuclear status of Mature Oocytes after IVF with DMSO (N=103) and Glycerol (n=88)

	DMSO % (no.)	Glycerol % (no.)	P value
Matured <sup>A</sup>	29.12% (30/103)	13.63% (12/88)	0.0136
Fertilized	12.62% (13/103)	3.41% (3/88)	0.0335
Immature	4.85% (5/103)	1.14% (1/88)	0.2201
Polyspermy	0% (0/103)	3.4% (3/88)	0.0960
Degenerate	45.63% (47/103)	56.82% (50/88)	0.1470
Indeterminate	20.39% (21/103)	25.00% (22/88)	0.4895

<sup>A</sup>Oocytes matured includes oocytes fertilized

\*Proportions with a probability value of  $P < 0.05$  were considered significantly different

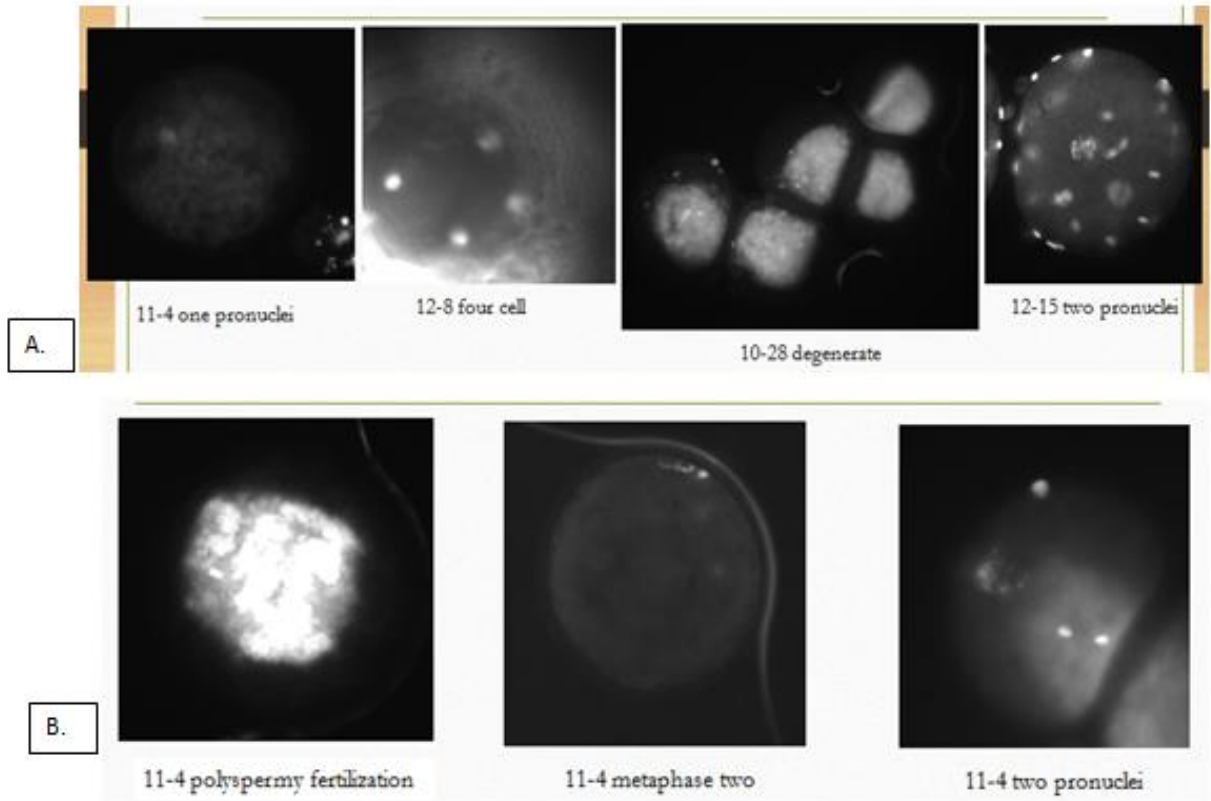


Figure 3.5 Different stages of nuclear maturation of Mature DMSO (A) and Glycerol (B) cryoprotectant treatments.

### IVF VALIDATION RESULTS

To validate IVF procedure, in vitro fertilization procedure was done with matured cumulus cell complexed bovine oocytes from a commercial source for the sole purpose of validating IVF. 63.88% were fertilized (46/72). In conclusion our IVF methods could be utilized to analyze the pronuclear formation with bovine cumulus cell complex oocytes.

### MATURATION CHECK

Maturation rate was evaluated in a subset of oocytes from each group, in the first experiment maturation was evaluated after IVM and vitrification. The first experiment, immature oocytes showed that 2/10 oocytes advanced to metaphase two stage in the glycerol

treatment group, 4 were lost or crushed and 4 were immature. Within in the DMSO treatment group 3/10 showed metaphase one maturation, 2 exhibited germinal vesicles and 1 confirmed germinal vesicle breakdown, and 3 were degenerate. Therefore 20% showed maturation prior to IVF in the immature glycerol group and 30% showed maturation prior to IVF in the immature DMSO group.

In experiment two after IVM and prior to vitrification 61.5% attained a metaphase two plate indicating maturation. After vitrification and warming of matured oocytes in the second experiment maturation in both cryoprotective groups was evaluated prior to IVF and 0% showed a metaphase two plate, all were degenerate regardless of treatment.

## **DISCUSSION**

The aim of these experiments was to determine if immature and mature cumulus cell oocyte complexes cryopreserved via vitrification with dimethyl sulfoxide or glycerol solutions would illustrate pronuclear formation following warming and in vitro fertilization, indicating viability. Discovering an optimal vitrification, warming, IVF, and culture procedure for bovine immature and mature cumulus cell complexes would be beneficial for infertility issues and improving gene pools. Within these experiments we made some progress towards optimizing the conditions of vitrification with bovine oocytes.

In the first experiment, fertilization occurred within the DMSO treatment group but not the glycerol treatment group, however, there was statistically significant difference ( $P=0.0056$ ) between treatment groups, due to the proportions of oocytes in DMSO treatment group being fertilized (Table 3.3). The low fertilization rates in the first experiment could be affected by the maturation rate of the oocytes. After vitrification the oocytes most likely did not mature

properly or were morphologically altered leading to the low fertilization rates (8.49% DMSO) but the high number of oocytes that were still in the metaphase two stage (12.26% DMSO 2.47% glycerol) could indicate issues with the formation of metaphase plates after vitrification and upon warming and IVM. These results showed a significant difference between DMSO and glycerol ( $P=0.0149$ ) suggesting that DMSO cryoprotectant is significantly superior for cryopreservation followed by maturation of oocytes when combined with ethylene glycol. Specifically, a possible interruption of the tubulin formation. The spindle apparatus is a dynamic conglomerate of microtubules and associated structural proteins, acting to coordinate cytokinetic and karyokinetic events essential for normal chromosome segregation (Jung, 2014). First meiotic division is completed by cooperation of cytoskeletons, especially microfilaments and microtubules. The cytoskeleton is a key factor to successful competent oocytes. Matured oocytes stay in metaphase block until the entry of spermatozoa (Pickering, 1987). This block is accomplished by stable spindle and other regulators for cell cycle. As oocytes transit into metaphase, microtubules change from radial arrays to an organized barrel shaped structure containing dense material at either pole known as microtubules organizing centers (MTOCs) (Aman, 1994). The polyspermic fertilization in the first experiment (3.77%DMSO, 3.70% glycerol) may indicate a possibility that vitrification of oocytes is affecting the zona pellucida's ability to become hardened after fertilization possibly disrupting the cortical granule reaction. The cortical reaction is a process initiated during fertilization by the release of cortical granules from the oocyte which prevents polyspermic fertilization. In contrast to the fast block of polyspermy which immediately but temporarily blocks additional sperm from fertilizing the oocyte, the cortical reaction gradually establishes a permanent

barrier. Another hypothesis is that the cryoprotectants are not penetrating the cell membrane creating osmotic stress on the oocytes and ice crystals are forming. These ice crystals can puncture holes through the cell membrane allowing multiple spermatozoa to enter the oocytes cytoplasm and causing polyspermic fertilization. If the spermatozoa are not penetrating the membrane and entering through possible holes formed by ice crystals then oocyte activation is not occurring, producing multiple sperm pronuclei but not the maternal pronucleus. Polyspermic fertilization of oocytes could also be affecting the intracellular calcium concentration resulting in an elevation after cryopreservation, which could trigger cortical granule release and cause premature hardening of the zona pellucida and parthenogenetic activation. These results can be compared to the fact that our controls had no sign of polyspermic fertilization. There was also a trend of degeneration in experiment one that points to an influence of deleterious trends of vitrification and possibly from the cryoprotectants used that effect the immature oocytes ability to become matured and/or affect the oocytes that did become mature to become fertilized.

In the second experiment we saw a statistically significant difference between treatments of DMSO and glycerol when looking at the fertilization rate after IVF, DMSO being 12.5% fertilized (13/103) and glycerol being 3.4% fertilized (3/88) with a P value of 0.0335 indicating a significant difference between the two cryoprotectants. These results indicate that both combinations of cryoprotective agents in vitrification can be used successfully for both mature bovine oocyte cumulus cell complexes but that DMSO cryoprotectant may be a more suitable agent for matured bovine oocytes. A large portion of oocytes remained in the metaphase two stage (29.12% DMSO 13.63% glycerol) with a P value of 0.0136 after IVF

leading us to believe that vitrification is affecting the viability and altering the oocytes prior to IVF. These results also suggest that DMSO cryoprotectant is more ideal for recovering the metaphase plate of oocytes after cryopreservation. It has been well documented that oocytes suffer from reversible and irreversible damage during cryopreservation. This damage includes hardening of the zona pellucida, premature cortical granules release, depolymerization of the microtubules, and misalignment of the chromosomes. Vitrification has in the past negatively affected spindle morphology and other organelle morphology in mature oocytes (Purohit, 2012) and could possibly be affecting mature oocytes after vitrification in this experiment as well. Importantly, the mechanism of meiotic spindle regeneration after oocyte cryopreservation is essentially unknown (Ma, 2010) In Table 3.3 and Figure 3.5 there were oocytes showing maturation after IVF indicating that spindle repair occurred but fertilization could not, possibly be effecting the tubulin or pre-hardening of the zona pellucida. Studies with mice demonstrated that cryopreservation induced hardening of the zona pellucida and premature release of the cortical granule content (Al- Hasani, 1987, Gook, 1993) inhibiting sperm penetration and fertilization. During the freezing process, fracture of the zona pellucida and ooplasmic membrane disruption could also occur. In a past experiment taxol was used to help re-polymerize disrupted microtubules of somatic cells (Jordan et al. 1993) and oocytes (Morato et al. 2008) and is a cytoskeleton stabilizer. Taxol has been shown to improve vitrification of human immature oocytes and bovine, ovine and murine mature oocytes (Shi et al. 2006, Morato et al. 2008, and Zhang, 2009) Cytochalasin B is also a cytoskeletal stabilizer and has also been suggested to improve post- thaw development of mouse and porcine oocytes (Park, 2001, Dobrinsky, 2000). There was also a trend of degeneration in experiment

two that points to an influence of deleterious trends of vitrification and possibly from the cryoprotectants used that effect the mature oocytes ability to repair the metaphase spindles or other organelle disruption or can affect the matured oocytes after vitrification to become fertilized.

In both experiments during warming, the dilution solution consisted of PBS, calf serum, and sucrose. Vitrified oocytes are sensitive to osmotic changes during warming. Many studies have a warming procedure in a series of steps, starting at a high concentration of an extracellular agent like sucrose and reduce it over a short period of time. In the book titled Human Assisted Reproductive Technology, Gardner explains that warming human embryos first go into a sucrose solution of 0.65M for 30seconds, then into 0.325M sucrose for 1 minute, 0.125M sucrose for 2 minutes then 0.0M sucrose for 5 minutes. The results yielded a 92.1% cryosurvival rate (Gardner, 2011). As early as in 1993, mouse embryos were vitrified with 2.75 M dimethyl sulfoxide and 2.75 M propylene glycol supplemented with 1.0 M sucrose after 30 second exposure, which revealed a significantly higher in vitro survival rate of 82% than the solution without 1.0 M sucrose, 44% (Tada, 1993). Additionally, it has been reported that vitrification solution with sugars could improve the survival rate of the vitrified bovine blastocysts (Saito, 1994). In another study the use of sucrose in the warming solutions had no significant effect on expansion or hatching rates on vitrified mouse embryos, results yielded 76-82% with 0.0M sucrose compared to 40-54% with 0.8M sucrose. It was concluded that vitrification solutions should contain a moderate concentrations of sucrose, however in dilution medium sucrose is unnecessary (Al Yacoub, 2013). In the two experiments of this thesis the warming solution consisted of 0.025M sucrose and oocytes were washed for 5

minutes. Originally the protocol called for 0.5M sucrose for the vitrification solutions and the dilution solution, however the warming solution contained a reduced amount of sucrose to observe the cryosurvival of a lower concentration. In reported studies it has been demonstrated that a high concentration of sucrose is not needed for warming solution after vitrification and a lower amount has beneficial factors on cryosurvival (Al Yacoub, 2013). The results in the present study had a low survival rate in both experiments indicating that there could be a possibility of reduced cryosurvival with the vitrification process or the warming process. This could be a possible future experiment, comparing a low sucrose concentration with a higher sucrose level in the warming solution and in the vitrification solutions to see possible morphology changes.

There were trends that focused our attention to the cryoprotectant DMSO showing more promising results in fertilization rates in both experiments, 8.49% fertilization in DMSO to 0% in glycerol and 12.62% in DMSO and 3.4% in glycerol in the second experiment. We cannot say that there was a statically significant difference between the two experiments, but what we observed we can say that dimethyl sulfoxide may have a better potential in vitrification of both immature and mature bovine oocyte and bears further investigation. Previous studies demonstrated the beneficial effect of DMSO in spindle polymerization (Kuleshova, 1999). Therefore, the use of DMSO for oocyte cryopreservation might have a protective effect during vitrification (Kuleshova, 1999, Katayama, 2003) These results provide the opportunity to explore other variables with optimizing the vitrification procedure or protocol for a particular cell type, or size. A good place to start would be assessing epigenetic changes. In a recent study, acetylation of H4 and methylation of H3K9 were altered by

vitrification that may lead to abnormal epigenetic presentation of female chromatin to fertilization and may be responsible for the reduction of developmental competence of vitrified pig oocytes (Spinaci, 2012). Both of these histone modifications could also be useful markers to monitor epigenetic perturbations induced by various experimental vitrification protocols and eventually for optimizing the cryopreservation of human oocytes (Yan, 2010).

During these two experiments, many obstacles occurred and trouble shooting was necessary. During both experiments there were possible problems with different protocols and media solutions. There are many protocols with different additives that contribute to the growth and development of oocytes and embryos. The exploration for a perfect maturation media for bovine oocytes alone could be an experiment and the search for the optimal culture for bovine embryos could also be extensively explored, including in vitro fertilization procedure. Amino acids, growth factors, different serum, a base buffer all could be altered and produce different results.

In our isolate we found that it could be possibly causing DNA damage to sperm cells due to contamination with ions during sperm separation. The production of nitric oxide, is regarded as a sperm toxicant that reduces motility (Rosselli et. al, 1995). This could be due to an activation of guanylyl cyclase, that increases cGMP production, which inhibits sperm motility (Chan et. al, 1990). On the other hand, nitric oxide is also known to be a physiologic mediator for vasodilatation, immunosuppression, neurotransmission and cytotoxicity (Anggard et. al, 1994). This could cause poor fertility rates with the IVF protocol and embryo development (Nathan et. al, 1992). One of the main factors known to influence quality and fertility of bovine cryopreserved semen is the extender used. Despite the benefits of egg yolk

on semen cryopreservation, components of animal origin may represent a potential microbiological risk, compromising the quality of cryopreserved semen and standardization (Bousseau et. al, 1998). The egg yolk extender within the freezing media during this experiment could be resulting in complications in spermatozoa attaching and entering the zona pellucida for fertilization. In a recent study, lecithin, can be used in substitution for egg yolk as an extender (Forouzanfar et. al, 2010). We did see a significant difference between the IVF validation experiment and both treatments in both experiments in regard to fertilization despite using semen from the same bull. The quality of the sperm, extender and isolate used could produce lower results but was not observed during the results.

Temperature sensitivity is always a problem that needs to be avoided during cryopreservation and thawing of cells, but it also can be a problem during collection of the bovine oocytes (Mortimer et. al, 2015). During collection the filter that holds the follicular fluid and oocytes are washed in a warmed holding media but are at room temperature for the collection period. The collection room is indoors and the room's temperature is controlled. This change in temperature from the ovary to the warm media to cooling within the filter then back into warmed searching media could cause a problem with maturation and/or fertilization or aneuploidy due to chilling factors. In regard to the IVF validation oocytes, they were also collected but from a slaughter house. These procedures were done elsewhere so comparison with temperature sensitivity cannot be fairly measured to one another. The problem of maintaining temperature throughout the OPU and oocyte search procedure is an excellent illustration that not only do perceived problems represent opportunities for improvement.

There also is a possibility that the age of the cows can affect the quality of the collected oocytes within each experiment and the opportunity to become viable embryos. In one study results suggested that the fertilization ability of oocytes from older cows is low and that premature progression of meiotic division in oocytes is partly due to impaired oocyte-CC gap junction communication (Yamamoto et. al, 2010). Synchronizing the herd could have also helped retrieve larger and better quality oocytes within the follicles. During synchronization procedure the herd is reproductively synchronized to one another. This produces a new follicular wave to be created and releases a new dominant follicle. The better quality the oocytes the greater the chance they would become fertilized (Yamamoto, 2010). After collection, the filter was searched for the oocytes and removed. During this experiment we used no scale or judgment on collected oocytes quality. No morphology features were evaluated to choose one oocyte over the other. Lonergan and colleagues demonstrated the effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization and culture in vitro for porcine (Lonergan et. al, 2005) and concluded that oocytes from >6mm follicles yielded better results (Lonergan et. al, 2005). All oocytes collected were used in this experiment and this could have caused lower maturation rates and lower fertilization rates.

Another option was to use a pre-IVM media. This may have increased the quality of maturation rather than holding them within HEPES-TALP after collection of oocytes for both experiments. Mitochondrial activity can be increased as well as the nuclear maturation and developmental competence, the amount of ICM cells increase, and the percentage of embryo development increases when using a pre-IVM culture (Huang et. al, 2014). In a recent study

Pre-IVM with FSK + IBMX increased subsequent blastocyst formation rate and quality compared with standard IVM (Li et. al, 2016). Pre-IVM could have been an advantage during these experiments and may have improved our fertilization results.

Overall, fertilization rates were uniformly low across treatment groups. While this could be due to the vitrification procedure, there are many other aspects that may have contributed to low fertilization. Initially this study faced many obstacles, which were overcome in a series of pre-trials to optimize the in vitro maturation and fertilization (as proven with our IVF validation experiment). As such, we believe that our results further show that vitrification still needs improvement to be efficient for bovine oocytes cryopreservation. However, results indicate that the best course would be to focus on mature bovine oocyte vitrification.

## **CHAPTER IV**

### **SUMMARY AND CONCLUSION**

#### **SUMMARY**

Oocyte cryopreservation is a reproductive technique that has been a useful tool for a number of industries, including livestock production, human infertility treatment, endangered species, preservation and stem cell research. Commercial and research industries depend on producing viable and developmentally competent oocytes and embryos for optimal success. It has been found that in vitro culture systems need to be improved to optimize oocyte and embryo quality and pregnancy rates and allowing for the wide spread of genetic resources (Rodriguez et. al, 2012) within cryopreservation. One example includes bovine zygotes cultured in vitro are capable of rates of development similar to those of in vivo cultured counterparts (in terms of day 8 blastocyst yield, cell number and early pregnancy rate), there were significant differences in embryo cryosurvival. This suggests that current in vitro culture systems need to be improved to optimize embryo quality and pregnancy rates (Enright et. al, 2000). It has been demonstrated that there is a dramatic effect on blastocyst quality with those blastocysts produced following in vivo culture surviving vitrification at significantly higher rates than in vitro cultured counterparts (Rizos et. al, 2002). Successful cryopreservation of spermatozoa and embryos made assisted reproductive technologies more practical and available for commercial use, because of the potential advantages to allow long distance transportation and to avoid estrous synchronization. This reduces the size of the recipient female population necessary to be maintained. Cryopreservation of oocytes has short and less successful history when compared to the other reproductive cells such as spermatozoa and embryos. Cryopreservation of unfertilized oocytes can be combined with

these advanced reproductive technologies, in addition to its potential advantage as oocyte banking for preserving female genetic resources. During maturation of an oocyte centromeres attach to the spindle fibers from the centrosome at the opposite poles in metaphase II and will not continue until fertilization occurs (Raven et. al, 2016). The most characterized cytoskeletal damage by cryopreservation concerns the disruption of sub-cortical actin network, microtubule depolymerization, abnormal spindle configuration and chromosome scattering within mature oocytes and therefore compromising the ability to develop into a viable embryo (Ferris et. al, 2015). Cryopreservation of germinal vesicle immature oocyte bypasses the risk of chromosome abnormalities as the chromatin is decondensed and is still protected by the nuclear envelope (Senger, 2005). However, cryopreservation of immature oocytes at the GV stage is also the subject for challenging endeavor. In many studies it has been shown that cryopreserving immature oocytes harms the ooplasm (Kim et. al, 2014), potentially due to inadequate conditions of in vitro maturation or basic oocyte defects. To date, with current protocols it may be best to cryopreserve mature oocytes after in vitro maturation to metaphase II (Combelles et al. 2012). Cryopreserving after maturation to metaphase II still leaves us with the issue of the spindle fibers being damaged during cryopreservation by ice crystal formation.

Cryoprotectant is the functionally derived term created to describe any additive which can be provided to cells before freezing to yield a higher post thaw survival and help reduce the amount of ice crystals formed within the cell. Cryoprotectants also help to ensure high levels of cell viability post thaw, and lower freezing temperatures allowing for greater dehydration of cells before water freezes (Watson, 1995). The most common cryoprotectants

used with embryos and oocyte cryopreservation are dimethyl sulfoxide and glycerol. Both of these cryoprotective agents are comparable to one another in many respects. In recent studies dimethyl sulfoxide has been used more often for oocytes and embryos (Schellander et. al, 1994). In one particular study dimethyl sulfoxide treatment led to significantly better cleavage and development up to 4-cell stage in oocytes cryopreserved at the MII stage (Schellander et. al, 1994). In another study it was found that when using glycerol and 1,2- propanediol in vitrification of bovine blastocysts the success following the 16 step equilibration minimized the ultrastructural damage to the plasma membrane during freezing and therefore ice crystal formation (Kuwayama et. al, 1994). Depending on the source consulted, there have been variable results on the relative toxicity of the different cryoprotectants, with disagreement on which is the least or most toxic (Lawson and Sambanis, 2011). Many factors for optimizing cryopreservation are dependent on the cell type and species including cryoprotectants used experimental protocols and thawing time.

## **CONCLUSION**

The results of the first experiment show a decrease in survival and development after vitrification and IVF. Fertilization rate in the first experiment of 8.49% DMSO vs 0% glycerol, were low and did have a significant difference between the two ( $p=0.0056$ ) due to fertilization in the DMSO group and not within the glycerol treatment group. The results showing polyspermic fertilization in the first experiment (3.77%DMSO, 3.70% glycerol) may indicate a possibility that vitrification of oocytes is affecting the zona pellucida ability to become hardened after fertilization possibly disrupting the cortical granule reaction. Reiterating that cryopreservation of immature oocyte still has a way to go in optimizing protocols with the

majority degenerate after IVF (74.04% DMSO, 80.49% glycerol). In the second experiment we did have some progress in optimizing the vitrification protocol for bovine mature oocytes with the results showing DMSO having a significant difference in fertilization rate compared with glycerol ( $P=0.0056$ ). Results also demonstrated that DMSO did recover the metaphase plate within the oocytes after vitrification at a higher rate than glycerol (29.12%, 13.63%,  $P$  value= 0.0136) Overall, the majority of oocytes were degenerate like in the first experiment (45.63% DMSO, 56.82% glycerol) indicating vitrification and warming could be damaging oocytes.

Future research is needed on the varying levels of cryopreservation via vitrification of immature and mature bovine oocytes. Specifically, on mature oocytes, while they demonstrated higher survival and fertilization rates in both treatment groups. For example, allowing vitrified mature oocytes to repair the spindles for a limited time after warming may allow for higher fertilization rates after IVF or the additional of calcium. DMSO induced calcium increases solely from the internal calcium pool was found that zona hardening significantly reduces and subsequent fertilization and development to the two cell stage significantly increased (Larman, 2006). Since zona hardening is one of the early activation events normally triggered by the sperm induced calcium increases observed at fertilization, it is possible that other processes are negatively affected by the calcium rise caused by cryoprotectants used during oocyte freezing, which might explain the current poor efficiency of this technique.

There are still many aspects of oocyte cryopreservation that are not fully understood yet. Cryoprotectants are just one small factor in the big picture that involves many other pathways. Further research into the understanding of details of oocyte cryopreservation is necessary. This may help understand different protocols or factors influencing

cryopreservation and warming to increase the overall development and competence of oocyte survival and fertilization of vitrified oocytes.

## LITERATURE CITED

- Abma, Mosher, Peterson, Piccinino. 1997. Fertility, Family Planning and Women's Health: New Data from the 1995 National Survey of Family Growth, In: Statistics, C.f.D.C.a.P.N.C.f.H. (Ed.), Vital and Health Statistics. 23:19.
- Adams, L. 1957. An egg transfer experiment in sheep. Vet Rec. 69:849.
- Agca, JL, Peter, AT, Critser, ES, Crister, JK. 1998. Effect of Developmental Stage on Bovine Oocyte Plasma Membrane Water and Cryoprotectant Permeability Characteristics. Molecular Reproduction and Development 49:408-415.
- Ahmad, Sayeed. 2013. In Vitro Production of Alkaloids: Factors, Approaches, Challenges and Prospects. Pharmacognosy Reviews 7.13: 27–33.
- Alberts, Lewis. 2002. The Genetic System of Mitochondria and Plastids. Molecular Biology of the Cell 4th edition
- Alberts, B, Lewis, J. 2002. Molecular Biology of the Cell. The Genetic Systems of Mitochondria and Plastids. 4th edition
- Al-Hasani, S, Tolksdorf, A, Diedrich, K, Van der Ven, H, Krebs, D. 1986. Successful in-vitro fertilization of frozen–thawed rabbit oocytes. Hum. Reprod 1:309-312.
- Al-Hasani, S, Diedrich, K, Van der Ven, A, Reineke, M, Harjte, M, Krebs. 1987. Cryopreservation of human oocytes. Hum Reprod; 2:695–700.
- Al-Yacoub, Gauly, Holtz. 2013. Is sucrose required in Open Pull Straw vitrification of mouse embryos. 2013. Cryo Letters. 34.5:466-470.
- Alink, VC, Offerigns, FGJ. 1977. Isolation and low temperature preservation of adult rat heart cells. Cryobiology 14:399-408.
- Aman, R, Parks, J. 1994. Effects of cooling and rewarming on the meiotic spindle and chromosomes of in vitro matured bovine oocytes. Biol Reprod; 50:103–110.
- Anggard, E. 1995. Nitric oxide: Mediator, murderer, and medicine. Open Journal of Molecular and Integrative Physiology 343:1199–1206.
- Arav, A. 2014. Cryopreservation of oocytes and embryos. Theriogenology 81.1:96-102

- Atabay, EB Atabay, EC, Aquino, FP, Duran, DH, De Vera, RV, Cruz, LC. 2010. Cryopreservation of in vitro matured buffalo (*Bubalus bubalis*) oocytes by slow freezing or vitrification. *Philippine Journal of Veterinary Medicine*. 27:2
- Albrecht, BA. 1982. Reversible compression of cytoplasm. *Exp. Cell Res* 140:173-189.
- Aye, CDG, De Mo, A, Botta, A, Perrin, J, Courbiere, B. 2010. Assessment of the genotoxicity of three cryoprotectants used for human oocyte vitrification: dimethyl sulfoxide, ethylene glycol and propylene glycol. *Food Chem Tox* 48:1905-1912.
- Baka, SG, Veek, LL, Jones, HW, Lanzendorf, SJ. 1995. Evaluation of spindle apparatus of in vitro matured human oocytes following cryopreservation *Hum Reprod* 10:1816-1820.
- Baldassarre, CK. 2004. Advanced assisted reproduction technologies in goats. *Animal reproduction science* 82-83:255-266.
- Barcroft, J, Dayoub, N, Thong, KJ. 2013. Fifteen-year follow-up of embryos cryopreserved in cancer patients for fertility preservation. *Journal of assisted reproduction and genetics* 30:1407-1413.
- Batista, M, Torres, A, Mateus, P, Lopes-da-Costa, L. 2012. Development of a bovine luteal cell in vitro culture system suitable for co-culture with early embryos. *In vitro cellular & developmental biology*. *Animal* 48:583-592.
- Beca, J, Lecaros, A, Gonzalez, P, Sanhueza, P, Mandakovic, B. 2014. Medical, ethical and legal issues in cryopreservation of human embryos. *Revista medica de Chile* 142:903-908.
- Benson, E. 2008. *Cryopreservation Theory, Conservation and Environmental Science*. Springer Chapter 2:1-19.
- Bianchi, V, Lappi, M, Bonu, MA, Borini, A. 2012. Oocyte slow freezing using a 0.2-0.3 M sucrose concentration protocol: is it really the time to trash the cryopreservation machine? *Fertil Steril* 97:1101-1107.
- Bousseau, JP, Brillard, B, Marquant-Le-Guienne, B, Guérin, A, Camus, M, Lechat. 1998. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology*, 50:699–706
- Bowen, R. 2004. Gonadotropins: Luteinizing and Follicle Stimulating Hormones. *The Hypothalamus and Pituitary Gland Index*. Colorado State University Biomedical Sciences, Endocrine System.
- Bunge, G, Sherman K. 1954. Clinical use of frozen semen. *Fertil Steril* 5:520-529.

- Burks, H, Buckbinder, J, Francis-Hernandez, M, Chung, K, Jabara, S, Bendikson, K, Paulson, R. 2015. Developmentally delayed cleavage-stage embryos maintain comparable implantation rates in frozen embryo transfers. *J Assist Reprod Genet* 32.10:1477-81.
- Cao, YX, Xing, Q, Li, L, Cong, L, Zhang, ZG, Wei, ZL, Zhou, P. 2009. Comparison of survival and embryonic development in human oocytes cryopreserved by slow-freezing and vitrification. *Fertil Steril* 92:1306-1311.
- Carroll, J, Warnes, GM, and Matthews, CD. 1989. Increase in digyny explains polyploidy after in-vitro fertilization of frozen-thawed mouse oocytes. *Reprod. Fertil* 85:489-494.
- Chan, YM, Chan, SY, Tucker, MJ, Wong, CJ, Leung, CK, Leong, MK. 1990. Effects of dibutyryl cyclic guanosine monophosphate on human spermatozoal motility and penetration of zona-free hamster oocytes. *Hum Reprod* 3:304-308.
- Chang, M. 1951. The problems of superovulation and egg transfer in cattle. *Foundation for Applied Research* 39.
- Chen, SU, Yang, YS. 2009. Slow freezing or vitrification of oocytes: their effects on survival and meiotic spindles, and the time schedule for clinical practice. *Taiwanese Journal of Obstetrics & Gynecology* 48:15-22.
- Chian, MK, Tan, J, Tan, O, Kato, T, Nagai. 2004. High survival rate of bovine oocytes matured in vitro following vitrification. *J Reprod Fertil* 50:685-696.
- Cobo, A, Garrido, N, Pellicer, A, Remohi, J. 2015. Six years' experience in ovum donation using vitrified oocytes: report of cumulative outcomes, impact of storage time, and development of a predictive model for oocyte survival rate. *Fertil Steril* 104.6:1426-1434.
- Combelles, CM, Chateau, G. 2012. The use of immature oocytes in the fertility preservation of cancer patients: current promises and challenges. *The International journal of developmental biology* 56:919-929.
- Comizzoli, DW, Pukazhenth, BS. 2004. Effect of 1,2-propanediol versus 1,2-ethanediol on subsequent oocyte maturation, spindle integrity, fertilization, and embryo development in vitro in the domestic cat. *Biology of reproduction* 71:598-604.
- Coticchio, G, Dal Canto, M, Guglielmo, MC, Albertini, DF, Mignini Renzini, M, Merola, M, Lain, M, Sottocornola, M, De Ponti, E, Fadini, R. 2015. Double-strand DNA breaks and repair response in human immature oocytes and their relevance to meiotic resumption. *Journal of assisted reproduction and genetics* 32.10:1509-1516.

- Da Costa, NN, Brito, KN, Santana, PD, Cordeiro, MD, Silva, TV, Santos, AX, Ramos, PD, Santos, SD, King, WA, Miranda, MD, Ohashi, OM. 2015. Effect of cortisol on bovine oocyte maturation and embryo development in vitro. *Theriogenology* 85.2:323-329.
- Dalvit, G, Gutnisky, C, Alvarez, G, Cetica, P. 2012. Vitrification of bovine oocytes and embryos. *Cryobiology* 65:341-342.
- Day, LA, R Melampy. 1959. Synchronization of oestrus and ovulation in swine. *J Animal Sci* 18:909.
- Dobrinsky, Pursel, Long. 2000. Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification *Biol Reprod* 62:564–570.
- Do VA, WS, Taylor-Robinson AW. 2014. Benefits and contrants of vitrification technologies for cryopreservation of Bovine in Vitro Fertilized Embryos. *J Vet Science Anim Husb* 2:401.
- Dumesic, DA, Meldrum, DR, Katz-Jaffe, MG, Krisher, RL, Schoolcraft, WB. 2025. Oocyte environment: follicular fluid and cumuls cells are critical for oocyte health. *Fertil Steril* 103.2: 303-316.
- Dutta DJ, D.H.a.R.H. 2013. In vitro blasotcyst development of post thaw vitrified bovine oocytes. *Veterinary World* 6:730-733.
- Djuwantono, Wirakusumah, Achmad, Sandra, Halim, Faried. 2011. A comparison of cryopreservation methods: Slow-cooling vs. Rapid-cooling based on vell viability, oxidative stress, apoptosis, and CD34+ enumeration of human umbilical cord blood mononucleated cells. *BMC Research Notes* 4:1-9.
- Enright, BP, Lonergan, P, Dinnyes, A, Fair, T, Ward, FA, Yang, X, Boland, MP. 2000.Culture of in vitro produced bovine zygotes in vitro vs in vivo: implications for early embryo development and quality. *Theriogenology* 54.5:659-673.
- Fahy, GM, Wowk, B. 2015.Principles of cryopreservation by vitrification. *Methods in Molecular Biology* 1257:21-82.
- Fasano, FN, Vannin AS. 2014. A randomized controlled trial comparing two vitrification methods versus slow-freezing for cryopreservation of human cleavage stage embryos. *Journal of Assisted Reproduction and Genetics* 31.2:241-247.
- Felipe de Lara Janz, Rita de Cássia Cavaglieri, Sérgio Aloísio Duarte, Carolina Martinez Romão, Antonio Fernandes Morón, Marcelo Zugaib, and Sérgio Paulo Bydlowski. 2011. Evaluation of Distinct Freezing Methods and Cryoprotectants for Human Amniotic Fluid Stem Cells Cryopreservation. *Journal of Biomedicine and Biotechnology* 2012:10.

- Ferris, J, Favetta, LA, King, WA. 2015. Bisphenol A Exposure during Oocyte Maturation in vitro Results in Spindle Abnormalities and Chromosome Misalignment in *Bos taurus*. *Cytogenetic and genome research* 145:50-58.
- Forouzanfar, M, Sharafi, M, Hosseini, SM, Ostadhosseini, S, Hajian, M, Hosseini, L, Abedi, P, Nili, N, Rahmani, HR, Nasr-Esfahani, MH. 2010. In vitro comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology* 1.73.4:480-487.
- Franciosi, F, Coticchio, G, Lodde, V, Tessaro, I, Modina, SC, Fadini, R, Dal Canto, M, Renzini, MM, Albertini, DF, Luciano, AM. 2014. Natriuretic peptide precursor C delays meiotic resumption and sustains gap junction-mediated communication in bovine cumulus-enclosed oocytes. *Biology of reproduction* 91:61.
- Fuller, B. 2004. Cryoprotectants: the essential antifreezes to protect life in the frozen state. *Cryo letters* 25:375-388.
- Fusi, FM, Arnoldi, M, Bosisio, C, Lombardo, G, Ferrario, M, Zanga, L, Galimberti, A, Capitanio, E. 2015. Ovulation induction and luteal support with GnRH agonist in patients at high risk for hyperstimulation syndrome. *Gynecological endocrinology: the official journal of the International Society of Gynecological Endocrinology* 31:693-697.
- Galliano, D, Garrido, N, Serra-Serra, V, Pellicer, A. 2015. Difference in birth weight of consecutive sibling singletons is not found in oocyte donation when comparing fresh versus frozen embryo replacements. *Fertil Steril* 104.6:1411-1418.
- Gardner, Rizk, Falcone. 2011. *Human Assisted Reproductive Technology; Future Trends in Laboratory and Clinical Practice*. Cambridge University Press. 28:316.
- Gook, D, Osborn, S, Johnston, W. 1993. Cryopreservation of mouse and human oocytes using 1,2 propanediol and the configuration of the meiotic spindle. *Hum Reprod* 8:1101-1109.
- Gook, D.H.E.a.D.A. 2012. A critical appraisal of cryopreservation (slow cooling versus vitrification) of human oocytes and embryos. *Oxford Journals* 18:536-554.
- Guimaraes, AC, Leivas, FG, Santos, FW, Schwengber, EB, Giotto, AB, Machado, CI. 2014. Reduction of centrifugation force in discontinuous percoll gradients increases in vitro fertilization rates without reducing bovine sperm recovery. *Anim Reprod Sci* 146.3-4:103-110.
- Goncalves, CG, Folchini, NP, Brum, DS. 2014. Reduction of centrifugation force in discontinuous percoll gradients increases in vitro fertilization rates without reducing bovine sperm recovery. *Animal reproduction science* 146:103-110.

- Hajarian. 2011. Cryotop Device Enhances Vitrification Outcome of Immature Bovine Oocytes. *Journal of Animal and Veterinary Advances* 10.19:2541-2545.
- Hanson, PJ. 2013. In Vitro Production of Bovine Embryos. Department of Animal Sciences, University of Florida. Version 10.16.
- Hardin, P, Bondioli, K. 2013. Cryopreservation of Immature Bovine Cumulus-Oocyte Complexes by Slow Rate Freezing and Vitrification. *Reproduction Fertility and Development* 26.1:225.
- Hashimoto, RT, M, Kishi, T, Sudo, N, Minami, M, Yamada. 1999. Ultrasound-guided follicle aspiration: the collection of bovine cumuls-oocyte complexes from ovaries of slaughtered or live cows. *Theriogenology* 51:757-765.
- Heape, W. 1890. Prelimiary note on the transplantation and growth of mammalian ova within a uterine foster-mother. *Biol Sci* 48:457-459.
- Hiroyoshi. 2002. In vitro production of bovine embryos and their application for embryo transfer. *Theriogenology* 59.2:675-685.
- Hsieh, YY, Tsai, HD, Chang, CC, Lo, HY, and Lai, AC. 1999. Ultrarapid cryopreservation of human embryos: experience with 1582 embryos. *Fertil Steril* 72:253-256.
- Huang, JY, CH, Park, JY, Tan, SL, Chian, RC. 2008. Comparison of spindle and chromosome configuration in in vitro- and in vivo-matured mouse oocytes after vitrification. *Fertil Steril* 90:1424.
- Huang, JYJ, CH, Tan, SL, Chian, RC. 2006. Effects of osmotic stress and cryoprotectant toxicity on mouse oocyte fertilization and subsequent embryonic development in vitro. *Cell Preserv Technol* 4:149-160.
- Hung, WT, Hong, X, Christenson, LK, McGinnis, LK. 2015. Extracellular Vesicles from Bovine Follicular Fluid Support Cumulus Expansion. *Biology of Reproduction* 93.5:117.
- Hurt, FLA, GE, Seidel Jr, EL Squires. 2000. Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. *Theriogenology* 54:119-128.
- Hwang, IS, Hochi, S. 2014. Recent progress in cryopreservation of bovine oocytes. *Biomed Research International* 2014.11.
- Ishimori, H, Saeki, K, Inai, M. 1993. Vitrification of bovine embryos in a mixture of ethylene glycol and dimethyl sulfoxide. *Theriogenology* 40:427-433.

- Jadoon, S, Adeel, M. 2015. Cryopreservation of Oocytes. *Journal of Ayub Medical College* 27:22-28.
- Jain, JK, Paulson, RJ. 2006. Oocyte cryopreservation. *Fertility and Sterility* 86:1037-1046.
- Jordan, MA, Toso, RJ, Thrower, D, Wilson, L. 1993. Mechanism of mitotic block and inhibition of cell-proliferation by taxol at low concentrations. *Proc. Natl. Acad. Sci. U. S. A.* 90:9552-9556.
- Jin, HX, Song, WY, Xin, ZM, Dai, SJ, Chen, ZJ, Sun, YP. 2012. Effects of cumulus cells on vitreous cryopreservation of human mature oocytes and clinical pregnancy outcomes. *Reproductive sciences* 19:216-220.
- Jung and Cheon. 2014. Improvement of the Vitrification method Suppressing the Disturbance of Meiotic Spindle and Chromosome System in Mature Oocytes. *Development & Reproduction* 18.2:117-125.
- Katayama, KP, Stehlik, J, Kuwayama, M, Kato, O, and Stehlik, E. 2003. High survival rate of vitrified human oocytes results in clinical pregnancy. *Fertil Steril* 80:223-224.
- Kim, SS, Olsen, R, Kim, DD, Albertini, DF. 2014. The impact of vitrification on immature oocyte cell cycle and cytoskeletal integrity in a rat model. *Journal of assisted reproduction and genetics* 31:739-747.
- Kuleshova, L, Gianaroli, L, Magli, C, Ferraretti, A, Trounson. 1993. Birth following vitrification of a small number of human oocytes: case report. *Hum Reprod* 14:3077–3079.
- Kuleshova, L, Gianaroli, L, Magli, C, Ferraretti, A, Trounson, A. 1999. Birth following vitrification of a small number of human oocytes: case report. *Hum Reprod* 14:3077-3079.
- Kuleshova, LL, MacFarlane, DR, Trounson, AO, Shaw, JM. 1999. Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. *Cryobiology* 38:119-130.
- Kuwayama, SF, Nagai, T. 1994. Ultrastructure of IVM-IVF bovine blastocysts vitrified after equilibration in glycerol 1,2-propanediol using 2-step and 16-step procedures. *Cryobiology* 5:415-422.
- Lai, SW, Roberts, DJ, Rabi, DM, Winston, KY. 2015. Diagnostic accuracy of fine needle aspiration biopsy for detection of malignancy in pediatric thyroid nodules: protocol for a systematic review and meta-analysis. *Systematic reviews* 4:120.
- Laowtammathron, CL, Ketudat-Cairns, M, Hochi, S, Parnpai, R. 2005. Factors affecting cryosurvival of nuclear-transferred bovine and swamp buffalo blastocysts: effects of

- hatching stage, linoleic acid-albumin in IVC medium and Ficoll supplementation to vitrification solution. *Theriogenology* 64:1185-1196.
- Lawson, A, Sambanis. 2011. Cytotoxicity effects of cryoprotectants as single-component and cocktail vitrification solutions. *Cryobiology* 62:115-122.
- Li, HJ, Sutton-McDowall, ML, Wang, X, Sugimura, S, Thompson, JG, Gilchrist, RB. 2016. Extending prematuration with cAMP modulators enhances the cumulus contribution to oocyte antioxidant defence and oocyte quality via gap junctions. *Hum Reprod.* 4:810-821.
- Lim, Ko, Hwang, Chung, and Niwa. 1999. Development of in vitro matured bovine oocytes after cryopreservation with different cryoprotectants. *Theriogenology.* 51.7:1301-1310.
- Lim, KS, Chae, SJ, Choo, CW, Ku, YH, Lee, HJ, Hur, CY, Lim, JH, Lee, WD. 2013. In vitro maturation: Clinical applications. *Clinical and Experimental Reproductive Medicine* 40:143-147.
- Luvoni, GC, Tessaro, I, Apparicio, M, Ruggeri, E, Luciano, AM, Modina, SC. 2012. Effect of vitrification of feline ovarian cortex on follicular and oocyte quality and competence. *Reproduction in domestic animals. Zuchthygiene* 47:385-391.
- Ma, W, Baumann, C, Viveiros. 2010. MM NEDD1 is crucial for meiotic spindle stability and accurate chromosome segregation in mammalian oocytes. *Dev Biol* 339.2:439-50.
- Manik, SS, P, Palta. 2003. Collection of oocytes through transvaginal ultrasound-guided aspiration of follicles in an Indian breed of cattle. *Animal reproduction science* 76:155-161.
- Larman, G, Sheehan, B, Gardner, K. 2006. Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. *Reproduction* 10.1530.
- Mavrides, A, Morroll, D. 2002. Cryopreservation of bovine oocytes: is cryoloop vitrification the future to preserving the female gamete? *Reproduction, nutrition, development* 42:73-80.
- Meintjes, Bellow, Broussard, Paul, Godke. 1995. Transvaginal aspiration of oocytes from hormone treated pregnant beef cattle for in vitro fertilization. *J Anim Sci* 73.4:967-974.
- Meryman, H. 1971. Cryoprotectants agents. *Cryobiology* 8:173-183.

- Moffit, J. 1979. World Situation with regard to embryo transfer. *Cattle Breed Club* 34:5.
- Morato, R, Izquierdo, D, Paramio, MT, Mogas, T. 2008. Cryotops versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: effects on spindle and chromosome configuration and embryo development. *Cryobiology* 57:137-141.
- Morató, R, Izquierdo, D, Albarracín, JL, Anguita, B, Palomo, MJ, Jiménez-Macedo, AR, Paramio, MT, Mogas. 2008. Effects of pre-treating in vitro-matured bovine oocytes with the cytoskeleton stabilizing agent taxol prior to vitrification. *Mol. Reprod. Dev* 75: 191-201.
- Morató, R, Izquierdo, D, Paramio, MT, Mogas. 2008. Embryo development and structural analysis of in vitro matured bovine oocytes vitrified in flexipet denuding pipettes. *Theriogenology*. 70: 1536-1543.
- Morató, R, Mogas, T, Maddox-Hyttel. 2008. Ultrastructure of bovine oocytes exposed to taxol prior to OPS vitrification. *Mol. Reprod. Dev* 75:1318-1326.
- Moreno, D, Neira, A, Dubreil, L, Liegeois, L, Destrumelle, S, Michaud, S, Thorin, C, Briand-Amirat, L, Bencharif, D, Tainturier, D. 2015. In vitro bovine embryo production in a synthetic medium: Embryo development, cryosurvival, and establishment of pregnancy. *Theriogenology* 84:1053-1060.
- Mortimer S, Mortimer D. 2015. *Quality and Risk Management in the IVF Laboratory*. Cambridge University Press 6:135
- Nathan C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 6:3051–3064.
- Noyes, N, Knopman, J, Labella, P, McCaffrey, C, Clark-Williams, M, and Grifo. 2009. Oocyte cryopreservation outcomes including pre-cryopreservation and post-thaw meiotic spindle evaluation following slow cooling and vitrification of human oocytes. *Fertil Steril* 94.6:2078-2082.
- Oktay, AC, H, Bang. 2006. Efficiency of oocyte cryopreservation: a meta-analysis. *Fertil Steril* 1:70-80.
- Park, Chung, Cha. 2001. Cryopreservation of ICR mouse oocytes: improved post thawed pre implantation development after vitrification using taxol, a cytoskeleton stabilizer *Fertil Steril* 75:1177–1184.
- Park, MJ, Lee, SE, Kim, EY, Lee, JB, Jeong, CJ, Park, SP. 2015. Effective Oocyte Vitrification and Survival Techniques for Bovine Somatic Cell Nuclear Transfer. *Cellular reprogramming* 17:199-210.

- Parkening, TA, TY, Chang MC. 1976. Effects of various low temperatures, cryoprotective agents and cooling rates on the survival, fertilizability and development of frozen-thawed mouse eggs. *J Exp Zool* 197:369-374.
- Pieterse, Kappen, Kruip, Taverne. 1988. Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries. *Theriogeneology* 30.4:751-762.
- Peiterse, PV, AM, Kruip, TA, Wurth, H, Beneden, AH, Willemse, MAM, Taverne. 1991. Transvagnial ultrasound guided follicular aspiration of bovine oocytes. *Theriogenology* 35:857-862.
- Pickering, S. 1987. The influence of cooling on the organization of the meiotic spindle of the mouse oocyte. *Hum Reprod* 2:207-216.
- Polge C, SA, Parkes S. 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature* 164:666.
- Prentice, JR, Singh, J, Dochi, O, Anzar, M. 2011. Factors affecting nuclear maturation, cleavage and embryo development of vitrified bovine cumulus-oocyte complexes. *Theriogenology* 75:602-609.
- Purohit, GN, Meena, H, Solanki, K. 2012. Effects of Vitrification on Immature and in vitro Matured, Denuded and Cumulus Compact Goat Oocytes and Their Subsequent Fertilization. *Journal of reproduction & infertility* 13:53-59.
- Rall, GF. 1985. Ice-free cryopreservation of mouse embryos at 7196 C by vitrification. *Nature* 313:573-575.
- Rall, WF, Wood, MJ, Kirby, C, Whittingham, DG. 1987. *Reprod. Fertil* 80:499-504.
- Raven, Johnson, Losos, Mason, Singer. 2016. Chapter 11 Sexual Reproduction and Meiosis, *Biology*, Mc Graw Hill. Eighth edition.
- Rodriguez Villamil, D, Lozano, JM, Oviedo, FL, Ongaratto, GA, Bó. 2012. Developmental rates of in vivo and in vitro produced bovine embryos cryopreserved in ethylene glycol based solutions by slow freezing or solid surface vitrification. *Anim. Reprod* 9.2:86-92.
- Rossell,i M, Dubey, RK, Imthurn, B, Macas, E, Keller, PJ. 1995. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. *Hum Reprod.* 10.7:1786-1790.
- Rowley, SD. 1992. Hematopoietic stem cell cryopreservation: a review of current techniques. *Journal of hematotherapy* 1:233-250.

- Rizos, D, Ward, F, Duffy, P, Boland, MP, Lonergan, P. 2002. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev.* 61.2:234-248.
- Saito, Imai, Tomizawa. 1994. Effect of sugars-addition on the survival of vitrified bovine blastocysts produced in vitro. *Theriogenology* 41.5:1053-1060.
- Sansinen, Santos, Zaritzky, Baez, Chirife. 2010. Theoretical prediction of the effect of heat transfer parameters on cooling rates of liquid-filled plastic straws used for cryopreservation of spermatozoa. *Cryo Letters* 31.2:120-129.
- Saragusty, Joseph, AA. 2011. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction* 141:1-19.
- Schellander, K, Peli, J, Schmoll, F, Brem, G. 1994. Effects of different cryoprotectants and carbohydrates on freezing of matured and unmaturred bovine oocytes. *Theriogenology* 42:909-915.
- Schroeder, AC, Champlin, AK, Mobraaten, LE, and Eppig, JJ. 1990. Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro. *J Reprod Fertil* 89:43-50.
- Selk, G. 2016. Embryo Transfer in Cattle, Oklahoma State Cooperative Extensive Service, Animal Reproduction, Oklahoma.
- Senger, PL. 2005. Pathways to Pregnancy and Parturition. 2:164-175.
- Shaw, JM, OA, Troson, AO. 2000. Fundemental Cryobiology of Mammalian oocytes and ovarian tissue. *Theriogenology* 53:59-72.
- Sherbahn, R. 2016. Embryo freezing after IVF: Human blastocyst and embryo cryopreservation and vitrification, Advanced Fertility Center of Chicago.
- Sherman, J. 1959. Temperature shock and cold storage of unfertilized mouse eggs. *Feril Steril* 10:384-387.
- Shi, WQ, Zhu, SE, Zhang, D, Wang, WH, Tang, GL, Hou, YP, Tian, SJ. 2006. Improved development by taxol pretreatment after vitrification of in vitro matured porcine oocytes. *Reproduction* 131:795-804.
- Si, W, Zheng, P, Li, Y, Dinnyes, A, Ji, W. 2004. Effect of glycerol and dimethyl sulfoxide on cryopreservation of rhesus monkey (*Macaca mulatta*) sperm. *American journal of primatology* 62:301-306.

- Sigma. 2010. Cryopreservation and storage of cells. Sigma-Aldrich. Fundamental Techniques in Cell Culture Laboratory Handbook 2:12.
- Simione, F. 2009. Cryopreservation Guide, American Type Culture Collection with Thermo Fisher Scientific 1-14.
- Singleton, EF. 1970. Field collection and preservation of bovine semen for artificial insemination Australian Veterinary Journal 46:160-163.
- Sinha, R, Kharche, SD, Sinha, NK, Goel, AK, Jindal, SK, Saraswat, S. 2014. Effect of vitrification and slow freezing on in-vitro matured prepubertal goat oocytes. Indian Journal of Animal Sciences 84:289.
- Spinaci, Vallorani, Bucci, Tamanini, Porcu, Galeati. 2012. Vitrification of pig oocytes induces changes in histone H4 acetylation and histone H3 lysine 9 methylation (H3K9). Vet Res Commun 36.3: 165-171.
- Tada, Sato, Amann, Ogawa. 1993. A simple and rapid method for cryopreservation of mouse 2-cell embryos by vitrification: Beneficial effect of sucrose and raffinose on their cryosurvival rate. Theriogenology 40.2:333-344.
- Tanabe, HH, J, Hasler. 1985. Comparative fertility of normal and repeat-breeding cows as embryo recipients. Theriogenology 23:687-696.
- Tetsuka, M, Takagi, R, Ambo, N, Myat, TS, Zempo, Y, Onuma, A. 2015. Glucocorticoid Metabolism in the Bovine Cumulus-Oocyte Complex matured in vitro. Reproduction 151.1:73-82.
- Thibier, M. 2009. The worldwide statistics of embryo transfers in farm animals. International Embryo Transfer Society Newsletter 27:13-19.
- Todorow, SJ, Siebzehnruhl, ER, Koch, R, Wildt, L, Lang, N. 1989. Comparative results on survival of human and animal eggs using different cryoprotectants and freeze–thawing regimens: I. Mouse and hamster. Hum. Reprod 4:805-811.
- Djuwantono, FFW, Achmad, H, Sandra, F, Halim, D, Faried, A. 2011. A comparison of cryopreservation methods: Slow-cooling vs. rapid-cooling based on cell viability, oxidative stress, apoptosis, and CD34+ enumeration of human umbilical cord blood mononucleated cells. Biomed Research International 4:371.
- Tucker, JL. 2007. Vitrification in Assisted Reproduction. Informa Healthcare United Kingdom.

- Ulloa, SM, Heinzmann, J, Herrmann, D, Timmermann, B, Baulain, U, Grossfeld, R, Diederich, M, Lucas-Hahn, A, Niemann, H. 2015. Effects of different oocyte retrieval and in vitro maturation systems on bovine embryo development and quality. *Zygote* 23:367-377.
- Vajta, G, Holm, P, Kuwayama, M. 1998. Open Pulled Straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol. Reprod. Dev* 51: 53-58.
- Vajta, G, Rienzi, L, Ubaldi, FM. 2015. Open versus closed systems for vitrification of human oocytes and embryos. *Reproductive biomedicine online* 30:325-333.
- Valojerdi, M, Eftekhari-Yazdi, P, Karimian, L, Hassani, F, Movaghar, B. 2009. Vitrification versus slow rate freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. *J Assist Reprod Genet.* 26.6:347-354.
- Van der Elst, J, Nerinckx, S, Van Steirteghem, AC. 1992. In vitro maturation of mouse germinal vesicle-stage oocytes following cooling, exposure to cryoprotectants and ultrarapid freezing: limited effect on the morphology of the second meiotic spindle. *Hum Reprod* 7:1440-1446.
- Vanderhyden, BC, Armstrong, DT. 1989. Role of cumulus cells and serum on the in vitro maturation, fertilization, and subsequent development of rat oocytes. *Biology of reproduction* 40:720-728.
- Wahid, Hajarian, H. 2012. Structural Changes in Cattle Immature Oocytes Subjected to Slow Freezing and Vitrification. *Pakistan Veterinary Journal* 32.2:188-192.
- Warwick, LB, W, Horlacher. 1934. Results of mating rams to Angora female goats. *Amer. Soc. Anim. Prod* 225-227.
- Watson, P. 1995. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of thier post-thawing function. *Reproduction, Fertility and Development* 7: 871-891.
- Whittingham. 1971. Survival of mouse embryos after freezing and thawing. *Nature* 233:125-126.
- Wu, JT, SP, Leibo. 1999. Effects of cooling germinal vesicle-stage bovine oocytes on meiotic spindle formation following in vitro maturation. *Mol. Reprod. Dev* 54:388-395.
- Yamada, C, Caetano, HV, Simoes, R, Nicacio, AC, Feitosa, WB, Assumpcao, ME, Visintin, JA 2007. Immature bovine oocyte cryopreservation: comparison of different associations with ethylene glycol, glycerol and dimethylsulfoxide. *Animal reproduction science* 99:384-388.

Yan, LY, Yan, J, Qiao, Liu. 2010. Effects of oocyte vitrification on histone modification. *Repro Fertil Dev.* 22.6: 920-925

Yang, CY, Chen, MC, Lee, PT, Lin, TT. 2012. Cryopreservation of germinal vesicle stage porcine oocytes based on intracellular ice formation assessment. *Cryo letters* 33:349-362.

Yenkie, KM, Diwekar, U. 2014. Optimal control for predicting customized drug dosage for superovulation stage of in vitro fertilization. *Journal of theoretical biology* 355:219-228.

Yokoyama, WM, Thompson, ML, Ehrhardt, RO. 2012. Cryopreservation and thawing of cells. *Current protocols in immunology* 99:3G.

Zhang, J, Nedambale, TL, Yang, M, Li, J. 2009. Improved development of ovine matured oocyte following solid surface vitrification (SSV): effect of cumulus cells and cytoskeleton stabilizer. *Anim. Reprod. Sci* 110: 46-55.

Graziano da Silva, J. 2016. Basic principles of cryopreservation. *Food and Agriculture Organization. Cryoconservation of animal genetic resources.* 7: 85-94.

## **APPENDIX A PROTOCOLS**

### **Vitrification for Bovine Oocytes**

1. Prepare equilibration and vitrification solutions before beginning (Appendix B).
2. Place 3-5 oocytes in equilibration solution (solution one) 50uL drop for 5 minutes, pipette oocytes up, down and around dish for continued time during 5 minute interval.
3. After 5 minutes in first solution move oocytes to second solution (50uL drop) for 35-40 seconds, pipetting them around the dish.
4. In the last 15 seconds of a total of 45 seconds in the second solution, pipette oocytes up and down and place on cryolock with minimal media.
5. After oocytes are placed on the tip of the cryolock and media removed, plunge cryolock into liquid nitrogen and repeat until all oocytes are vitrified.
6. With forceps, gently secure the top onto the cryolock device under the liquid nitrogen.
7. Move all cryolock with tops into canister for storage.

### **In Vitro Fertilization for Bovine Oocytes**

#### **Preparation:**

1. Place two centrifuge carriers into non CO<sub>2</sub> incubator (39°C).
2. Prepare IVF-TALP. Check that there is warmed mineral oil in a CO<sub>2</sub> incubator.
3. Prepare 500μl of IVF-TALP in four well dish with water surrounding each well. Prepare enough drops for the number of oocytes to be fertilized using 1 well per 15-20 oocytes.

Add 500 ul IVF-TALP in each well of a four well plate for use for washing. Equilibrate in CO<sub>2</sub> incubator for at least 2-3 hours.

4. Place tube with remaining IVF-TALP into the CO<sub>2</sub> incubator (loosened cap).
5. Prepare Percoll (Isolate) gradient. Place 1.5 ml 90% lower layer in 15 ml centrifuge tube. Carefully add 1.5 ml of 45% upper layer on top in a manner that does not cause mixing of the two layers.
6. Transfer gradient to a pre-warmed centrifuge carrier in the non CO<sub>2</sub> incubator.
7. Add 95 ul water to a small centrifuge tube or use later with sperm counting (keep at room temperature).
8. Wash hemacytometer and cover slip with alcohol and place on microscope.
9. Clean microscope slides and coverslips and place on warming plate to use for checking motility later. Place a microcentrifuge tube on warming plate to use for the sperm pellet later.

#### Semen Thaw and Preparation of Sperm Suspension:

1. Thaw semen in water bath at 39°C for 30 seconds being careful not to raise remaining straws in LN tank above the frost line.
2. Dry straw and keep it warm. Cut the sealed end off and, using a plunger carefully layer the semen on top of the percoll gradient in a manner that does not cause mixing.
3. Check for motility of the thawed semen by placing a drop remaining in the straw on the pre-warmed slide. View at 20X or 40X magnification to confirm motility. If motility is not present prepare a new gradient and thaw another unit of semen.

4. Return the gradient with semen to the pre-warmed centrifuge carrier and centrifuge at 400 xg for 20 minutes at 37°C.
5. While the sperm is in the centrifuge, use this time to move the oocytes from maturation medium into IVF-TALP. Wash oocytes through the IVF-TALP using the prepared wash dish from the CO<sub>2</sub> incubator.
6. Transfer 15-20 oocytes to each 500 ul drop of IVF-TALP in the fertilization dish and return fertilization dish to the CO<sub>2</sub> incubator.
7. With 2 minutes remaining on the centrifuge time, place 3 ml of IVF-TALP in a 15 ml centrifuge tube and return to CO<sub>2</sub> incubator.
8. When centrifugation is complete carefully remove carrier with gradient from the centrifuge. There should be a sperm pellet in the bottom of the tube. If not start over.
9. With a 5 ml pipet, remove the extender (top layer) and the dead sperm (at the interphase).
10. Collect 200 ul of sperm pellet from the bottom of the centrifuge tube and add slowly to the 3 ml of IVF-TALP.
11. Centrifuge 400 xg for 10 minutes. During this time add 2 ml of IVF-TALP to a new 15 ml centrifuge tube and return to the CO<sub>2</sub> incubator.
12. When centrifugation is complete remove ~ 3 ml of the medium from the top of the tube.
13. Collect 200 ul of sperm pellet from the bottom of the centrifuge tube and add slowly to the 2 ml of IVF-TALP.
14. Centrifuge 400 xg for 5 minutes.

15. Remove ~ 1 ml of medium from the top of the tube.
16. With a 200 ul pipettor collect the pellet at the bottom of the tube. Collect no more than the pellet and place in the pre-warmed sperm pellet tube. Keep this tube on the warming plate until ready for fertilization.
17. Determine the sperm pellet concentration:
  - a. Gently mix the sperm pellet and transfer 5 ul into the 95 ul water prepared earlier (1:20 dilution).
  - b. load each side of the hemacytometer with 10 ul of the diluted sperm. Count the sperm heads within five double-ruled squares on each side of the hemacytometer. These two counts should not vary more than 10%. If they do clean the hemacytometer, reload and count again.
18. Prepare the final sperm suspension:
  - a. Average the two counts from above. This number (x) will be divided into 7500 to determine the  $\mu\text{L}$  of sperm pellet required to prepare the final suspension. Subtract this number from 300 to obtain the  $\mu\text{L}$  of IVF-TALP for the suspension.  
  
Eg:  $7500/x = \_\_\mu\text{L sperm pellet}$  ;  $300 - \_\_\mu\text{L sperm pellet} = \_\_\mu\text{L of IVF-TALP}$ .
  - b. Add the calculated amount of IVF-TALP to the pre-warmed micro centrifuge tube and then add the calculated amount of sperm pellet. The final sperm suspension will be 300  $\mu\text{L}$ . Mix gently by pipetting.

#### Fertilization:

1. To each fertilization drop with oocytes add 20 µl of Heparin stock.
2. To each fertilization drop with oocytes add 20 µl of Penicillamine/Hypotarine (PH) stock.
3. To each fertilization drop with oocytes add 20 µl of final sperm suspension for a final concentration of  $1 \times 10^6$  sperm/ml in the fertilization drops.
4. These last three steps should be done rapidly and the fertilization dishes returned to the CO<sub>2</sub> incubator as rapidly as possible.

#### Culture:

1. Prepare culture dishes with 40µL droplets of global 35+ covered with mineral oil and wash droplets and place into 5% CO<sub>2</sub> incubator at least 2 hours prior for equilibration.
2. After 18 hours within 5% CO<sub>2</sub> incubator, remove oocytes and place into 1mL centrifuge tube of hyaluronidase and vortex for 5-7 minutes to remove cumulus cells.
3. When all cumulus cells removed, wash presumptive embryos in culture droplets and then transfer into fresh droplets for an additional 24 hours in 5% CO<sub>2</sub> incubator.

#### **Aceto-Orcein Staining Protocol for Oocytes or Embryos**

1. Prepare aceto-orcein stain and methanol/acetic acid solution before beginning (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.

Place  $\approx 10$  oocytes/embryos onto the microscope slide, making sure none are on top of each other. Draw off excess medium as possible to ensure that they stick to the slide.

4. Quickly place the coverslip onto the slide to prevent dehydration.

5. 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 secured to the slide.

5. Place rubber cement on two corners of the cover slip.

6. 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.

7. Stain and view after at least 48 hours of fixing. Slides can safely be stored in the coplin jars for up to 7 days.

#### 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 Kim wipe 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.

### **Hoechst 33342 Staining Protocol for Oocytes or Embryos**

1. Prepare Hoechst 33342 stain solution before beginning (Appendix B).
2. Wash presumptive embryos 1x in PBS.
3. Incubate for 30 minutes in 10  $\mu$ L/ mL Hoechst 33342 in a 5% CO<sub>2</sub> incubator at 37°C.
4. Rinse in Dulbecco's Phosphate-Buffered Saline.
5. Use a pencil to label the top of a microscope slide with the date, number of embryos to be mounted, and any other relevant information.
6. Transfer presumptive embryos in a minimal amount of media, 8-10 embryos per slide.
7. Add a 10  $\mu$ L drop of Molecular Probes Prolong Gold Antifade (P36930) on top of presumptive embryos and gently place coverslip on top.
8. Use microscope to view embryos. Record observations and take photographs if necessary.

## APPENDIX B MEDIA FORMULATIONS AND STOCK SOLUTIONS

### Media Formulations:

#### HEPES-TALP Medium

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

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

#### Maturation Media

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 uL
Na Pyruvate	Stock solution	P-4562	100 uL
Glutamine	100x stock solution	G-8540	100 uL
FSH (Folltropin)	1000x stock solution	Bioniche	10 uL

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

#### IVF-TALP Medium

Component	Source	Product Number	Amount
TL-FERT	Caisson	IVL02	22.5 mL
PH	Stock solution	P-4875 / H-1384	1 mL
1Heparin	Stock solution	H-3149	1 mL
Pen/Strep	Gibco	15140	250 uL
Na Pyruvate	Stock solution	P-4562	250 uL
BSA	Sigma	A-6003	150 mg

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

**Vitrification Solution One for Glycerol**

PBS	3 mL
20% Calf Serum	1.0 mL
10% Ethylene Glycol	0.5 mL
10% Glycerol	0.5 mL
0.5 M Sucrose	0.855g

**Vitrification Solution Two for Glycerol**

PBS	2 mL
20% Calf Serum	1.0 mL
20% Ethylene Glycol	1.0 mL
20% Glycerol	1.0 mL
0.5 M Sucrose	0.855g

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

**Vitrification Solution One for DMSO**

PBS	3 mL
20% Calf Serum	1.0 mL
10% Ethylene Glycol	0.5 mL
10% DMSO	0.5 mL
0.5 M Sucrose	0.855g

**Vitrification Solution Two for DMSO**

PBS	2 mL
20% Calf Serum	1.0 mL
20% Ethylene Glycol	1.0 mL
20% DMSO	1.0 mL
0.5 M Sucrose	0.855g

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

**Dilution Solution for Warming**

PBS	16 mL
20% Calf Serum	4 mL
0.025 M Sucrose	0.855g

Sterile, filter. 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  $\approx 500$   $\mu$ 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  $\mu$ L aliquots.
6. **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.
7. **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.
8. **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).
9. **Hoechst 33342:** Dissolve 25 mg of Hoechst in 25 mL distilled water in a 50 mL centrifuge tube. Aliquot in 1 mL microcentrifuge tubes (1 mL / tube). Store at -20°C for several months. For working solution in embryo culture or holding media dilute to 1  $\mu$ g/mL.
10. **Heparin:** Dissolve H-3149 1 mg Heparin into 20 mL of 0.9% saline and filter. Store -20°C
11. **PH:** Combine 5 mL of 1 mM of Hypotaurine and 5 mL of 2 mM Penicillamine to 8 mL of 0.9% saline. Aliquot into 100  $\mu$ L, store at -20°C.

## APPENDIX C

### DETAILED OOCYTE ASSESSMENT BREAKDOWN

Nuclear Maturation Breakdown of Oocytes/Embryos in Experiment One

	<b>DMSO % (no.)</b>	<b>Glycerol % (no.)</b>	<b>P value</b>
<b>Pronucleus</b>	3.23%(6/104)	0%(0/82)	0.0352
<b>Two Pronuclei</b>	2.15%(4/104)	0%(0/82)	0.1315
<b>Metaphase One</b>	0%(0/104)	0.54%(1/82)	0.4409
<b>Metaphase Two</b>	1.8%(2/104)	1.08%(2/82)	1.00
<b>Anaphase</b>	0.54%(1-104)	0%(0/82)	1.00
<b>Polyspermy</b>	2.15%(4-104)	3.23%(6/82)	0.3402
<b>Degenerate</b>	41.40%(77/104)	35.48%(6/82)	0.3814
<b>Sperm and CC</b>	6.45%(12/104)	3.23%(6/82)	0.4551

Comparison of Nuclear Maturation in Exp. One, Immature Group with DMSO and Glycerol

\* Proportion with a probability value of  $P < 0.05$  were considered significantly different

Oocytes were analyzed in the following tables for nuclear status and development following fertilization. For experiment one, the indication of fertilization included formation of two pronuclei. Matured oocytes had either one pronucleus, a metaphase two plate or were anaphase stage. Immature group indicated oocytes in metaphase one, and polyspermy means polyspermy fertilization occurred. The degenerate variable means that the oocytes were dead. The indeterminate variable means that the oocytes were unknown, had sperm heads and/or cumulus cells interfering with visualization.

### Nuclear Maturation Breakdown of Oocytes/Embryos in Experiment Two

	<b>DMSO % (no.)</b>	<b>Glycerol % (no.)</b>	<b>P value</b>
<b>Pronucleus</b>	4.71%(9/103)	2.62%(5/88)	0.5794
<b>Two Pronuclei</b>	4.19%(8/103)	1.57%(3/88)	0.2292
<b>Metaphase One</b>	0.52%(1/103)	0%(0/88)	1.00
<b>Metaphase Two</b>	4.19%(8/103)	1.57%(3/88)	0.2292
<b>Anaphase</b>	0%(0/103)	0.52%(1/88)	0.4607
<b>Polyspermy</b>	0%(0/103)	1.57%(3/88)	0.0960
<b>Degenerate</b>	24.61%(47/103)	26.18%(50/88)	0.1470
<b>Sperm and CC</b>	10.99%(21/103)	11.52%(22/88)	0.4895
<b>Germinal Vesicle</b>	2.09%(4/103)	0.52%(1/88)	0.3762
<b>Three Cell</b>	1.56%(3/103)	0%(0/88)	0.2501
<b>Four Cell</b>	1.05%(2/103)	0%(0/88)	0.5005

Comparison of Nuclear Maturation in Exp. Two, Mature Group with DMSO and Glycerol

\* Proportion with a probability value of  $P < 0.05$  were considered significantly different

Within the second experiment the categorization of oocyte development followed as fertilization included formation of two pronuclei, three cell and four cell embryo development. Matured oocytes had either one pronucleus, a metaphase two plate or were anaphase stage. Immature group indicated oocytes in metaphase one and germinal vesicles being present, and polyspermy means polyspermy fertilization occurred. The degenerate variable means that the oocytes were dead. The indeterminate variable means that the oocytes were unknown, had sperm heads and/or cumulus cells interfering with visualization.

### Nuclear Maturation Breakdown of Oocytes by Replicate (Day) in Experiment One

	5-NOV		6-Nov		12-Nov		24-Nov	
	DM SO (no.) )	Glycer ol (no.)	DMS O (no.)	Glycer ol (no.)	DMS O (no.)	Glycer ol (no.)	DMS O (no.)	Glycer ol (no.)
<b>1pronuclei</b>	0	0	0	0	5	0	1	0
<b>2pronuclei</b>	0	0	3	0	1	0	0	0
<b>Met1</b>	0	0	0	1	0	0	0	0
<b>Met 2</b>	0	0	2	2	0	0	0	0
<b>Anaphase</b>	0	0	1	0	0	0	0	0
<b>Polyspermy</b>	3	6	0	0	1	0	0	0
<b>Deg/unknown</b>	2	4	14	10	35	33	27	19
<b>CC/Sperm</b>	12	6	0	0	0	0	0	0

Comparison of Nuclear Maturation in Exp. One, Immature Group with DMSO and Glycerol  
seperated by each replicate in numbers D(DMSO=104) G(Glycerol=82)  
Met=Metaphase, Deg=Degenerate, CC=Cumulus Cell

### Nuclear Maturation Breakdown of Oocytes by Replicate (Day) in Experiment Two

	4-Nov(no.)		28-Oct(no.)		8-Dec(no.)		10-Dec(no.)		15-Dec(no.)	
	D	G	D	G	D	G	D	G	D	G
<b>1pronuclei</b>	4	2	0	0	1	1	1	1	3	1
<b>2pronuclei</b>	0	0	0	0	1	1	3	0	4	2
<b>3 Cell</b>	1	0	0	0	0	0	0	0	1	0
<b>4 Cell</b>	1	0	0	0	1	0	0	0	0	0
<b>Met 1</b>	0	0	0	0	0	0	1	0	0	0
<b>Met 2</b>	4	3	0	0	1	0	3	0	0	0
<b>Anaphase</b>	0	1	0	0	0	0	0	0	0	0
<b>Polyspermy</b>	0	3	0	0	0	0	0	0	0	0
<b>Deg/unknown</b>	13	7	19	17	7	10	4	11	4	5
<b>CC/Sperm</b>	0	3	0	0	8	8	12	7	1	4
<b>GV</b>	3	0	1	1	0	0	0	0	1	0

Comparison of Nuclear Maturation in Exp. Two, Mature Group with DMSO and Glycerol  
seperated by each replicate in numbers D(DMSO=103), G(Glycerol=88)  
Met=Metaphase, Deg=Degenerate, CC=Cumulus Cell GV=Germinal Vesicle

## **VITA**

Paige Taylor Hardin was born in Pembroke Pines, Florida to John Hardin and Jorja Renwick in June, 1990. She has three siblings, David Renwick who holds a degree in Environmental Science with an emphasis on Toxicology from Western Washington University, Chad Wood who holds a degree in Geology from the University of Tennessee at Chattanooga and a sister who is in pursuit of a degree from the University of Montevallo in Alabama for Business and a minor in Art. Paige attended high school in Chesapeake Virginia her first year and finished her high school diploma in Mandeville Louisiana. She graduated high school in 2009.

Paige attended Louisiana State University at Baton Rouge from 2009 to 2013. While at LSU, she was involved with the Pre-Vet Club and was a member of the National Society of Leadership. She also worked at the LSU Vet School and Acadian Oaks Pet Clinic during her undergraduate degree. Paige also started working in the lab of Dr. Kenneth Bondioli. She entered and won first place in the International Embryo Transfer Society undergraduate poster competition in 2013. She graduated in December 2013 from Louisiana State University with a B.S. in Animal, Dairy, Poultry Science.

Paige began graduate school at the Louisiana State University in the fall of 2014. 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.