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EFFECTS OF DIMETHYL SULFOXIDE AND GLYCEROL BASED VITRIFICATION PROTOCOLS ON ZONA PELLUCIDA HARDENING IN 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, Dairy, and Poultry Sciences

by
Kaci Denise Rogers
B.S., Louisiana State University, 2016
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TABLE OF CONTENTS

LIST OF TABLES……………………………………………………………..…..……v

LIST OF FIGURES…………………………………………………………….…...vi

LIST OF ABBREVIATIONS……………………………………………………..…vii

ABSTRACT……………………………………………………………………….viii

CHAPTER

I. INTRODUCTION…………………………………………………………...1

II. LITERATURE REVIEW……………………………………………………3
HISTORY…………………………………………………………………3
OOGENESIS……………………………………………………………...4
COLLECTION……………………………………………………………6
CRYOPRESERVATION…………………………………………………8
CRYOPROTECTANTS…………………………………………………10
SLOW RATE FREEZING………….………………………………...13
VITRIFICATION………………………………………………………..14
WARMING…………………………………………………………....17
ZONA PELLUCIDA/ ZONA HARDENING……..……………………………18
CURRENT ISSUES FOR FUTURE RESEARCH……………………………...21

III. VITRIFICATION OF MATURE BOVINE OOCYTES………………………..22
INTRODUCTION…………………………………………………..…...22
MATERIALS AND METHODS…………………………………...…………23
RESULTS……………………………………………………………..…28
DISCUSSION……………………………………………………………....32

IV. SUMMARY AND CONCLUSION………………………………………….37
SUMMARY………………………………………………………………...37
CONCLUSION……………………………………………………………..38

LITERATURE CITED……………………………………………………………..40

APPENDIX A: PROTOCOLS……………………………………………………..49

APPENDIX B: MEDIA FORMULATIONS AND STOCK SOLUTIONS…………………….52

VITA……………………………………………………………………………..55

LIST OF TABLES

2.1 A Comparison of Vitrification with Slow-Freezing Procedures…………………...16

3.1 Composition of Cryoprotectant Solutions for Fresh and Vitrified Oocytes……..26

3.2 Zona Pellucida Mean Protease Digestion Time using Fresh Oocytes………..…29

3.3 Comparison of Zona Pellucida Protease Digestion Time between Treatments using Fresh Oocytes…………………………………………………………...29

3.4 Zona Pellucida Mean Protease Digestion Time using Vitrified Oocytes………..31

3.5 Comparison of Zona Pellucida Protease Digestion Time between Treatments using Vitrified Oocytes……………………………………………………………..31

LIST OF FIGURES

3.1 Flowchart of Experimental Design....……………………………………………24

3.2 Stages of Zona Pellucida Digestion by Protease………………………………....30

3.3 Protease Digestion of the Zona Pellucida using Fresh and Vitrified Oocytes…...32

LIST OF ABBREVIATIONS

ART	assisted reproductive technology
BCS	bovine calf serum
CCs	cumulus cells
COC	cumulus-oocyte-complex
CPA	cryoprotectant
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EG	ethylene glycol
EGF	epidermal growth factor
GCs	granulosa cells
GLY	glycerol
ICM	inner cell mass
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
LH	luteinizing hormone
MI	metaphase I
MII	metaphase II
OPU	oocyte-pick up
PRT	protease
TUGA	transvaginal ultrasound-guided aspiration

ABSTRACT

Zona pellucida hardening is a natural process that occurs after oocyte fertilization to prevent polyspermic fertilization and to protect embryonic development. Pre-fertilization hardening of the zona pellucida however, decreases fertilization rates. Cryoprotectants have also been shown to negatively affect fertilization rates, one possible mechanism of which being through zona hardening. This experiment was conducted to determine the effect of different cryoprotectants on hardening of the zona pellucida using fresh mature bovine oocytes and vitrified mature bovine oocytes. Oocytes were post-slaughter from mixed breed cows. After collection, oocytes were randomly assigned to three cryoprotectant treatment groups: dimethyl sulfoxide (DMSO), glycerol or phosphate buffered saline (PBS-control). Drops (50 µl) of each vitrification solution were placed under mineral oil. Vitrification solution 1 (VS1) contained 10% ethylene glycol (EG), either 10% DMSO or glycerol, and 0.5 M sucrose. Vitrification solution 2 (VS2) contained 20% EG, 20% DMSO or glycerol, and 0.5 M sucrose. All oocytes were held in VS1 for 5 minutes before being transferred to VS2 for 45 seconds. All oocytes were washed in a common dilution solution (80% PBS, 20% calf serum, 0.025 M sucrose) for 5 minutes. Next, oocytes were moved to drops (50µl) of protease solution (0.1% protease) under mineral oil. Control oocytes were held in PBS for 5 minutes and 45 seconds before entering the dilution solution for 5 minutes, to represent the same time period as the vitrification procedure. The oocytes were observed until the zona pellucidae were completely digested and times were recorded for each oocyte. This protocol was repeated with the vitrified oocytes, which were vitrified in liquid nitrogen after exposure to the vitrification solutions. The first experiment using fresh oocytes included five replicates with a total of 117 oocytes used, 41 each in DMSO and glycerol and 35 in PBS. The data were analyzed using LSM and contrasts. The differences between DMSO and

glycerol were significant (p = 0.0238) but, the differences between DMSO and PBS were not significant (p = 0.3325). However, both the differences between glycerol versus PBS and the average of DMSO and glycerol compared to PBS was significantly different (P-value = 0.0058 and P-value = 0.0262, respectively). The second experiment using vitrified ‘oocytes included three replicates with a total of 114 oocytes used, 37 in DMSO, 41 in glycerol and 36 in PBS. The data were analyzed using LSM and contrasts. In all of the comparisons, no statistical significance was observed (DMSO vs. Glycerol- P=0.5301; DMSO vs. PBS- P=0.1699; Glycerol vs. PBS- P= 0.3818; and DMSO and Glycerol vs. PBS- P=0.2004). These results show that with fresh oocytes, glycerol hardens the zona pellucida more than DMSO or PBS but, there is not enough evidence to determine if DMSO hardens the zona pellucida more than PBS. However, with vitrified oocytes, cryoinjury proved to be too great to accurately measure zona hardening. These data suggest that, in relation to zona hardening, and ensuring proper fertilization, glycerol-based cryoprotectants may be a better option than DMSO-based ones. Further, these results may be important in embryo vitrification as zona hardening may prevent blastocyst hatching, suggesting that glycerol-based cryoprotectants should be investigated as the optimal cryoprotectant here also.

CHAPTER I
INTRODUCTION

In vitro embryo production, or IVP, is an inclusive term used to describe all the steps used to produce embryos. However, *in vitro* fertilization, IVF, is frequently used as a general term for the process of creating embryos outside of the body, which includes *in vitro* maturation (IVM) of oocytes, *in vitro* insemination and fertilization of oocytes (IVF) and *in vivo* culture (IVC) of resulting embryos. The steps of IVP are conducted in the sequence IVM-IVF-IVC (Hasler and Barfield, 2015). Assisted reproductive technologies, or ART, encompasses all the procedures that could be used to complete IVP. *In vitro* production is an invaluable tool for human fertility clinics, as these ART help to increase the chances of subfertile men and women having children of their own (Hansen, 2013). Also, in the commercial livestock industry, IVP has had many beneficial applications including, but not limited to, improving genetic selection, allowing gender selection, and increasing the number of potential offspring per individual.

Cryopreservation is the use of very low temperatures to preserve structurally intact living cells and tissues (Pegg, 2015). Cryopreservation allows the transfer of a limited number of fresh embryos back to the uterus and the storage of the remaining embryos for future use, thus maximizing the cumulative effectiveness of IVP (Loutradi et. al, 2008). Although there are definite benefits to cryopreservation, there are also negative effects. The various steps required for cryopreservation may contribute individually or cumulatively to oocyte damage which in turn, decrease fertilization and developmental rates (Saunders and Parks, 1999). Preventing ice crystal formation during cryopreservation is critical to the developmental capacity of the oocyte/embryo. Cryoprotective agents (CPAs) are used to eliminate ice crystal formation but, toxicity of CPAs limits the concentrations that can be used (Best, 2015).

Vitrification, a form of cryopreservation, has become the industry standard. It does not require expensive equipment, uses small amounts of liquid nitrogen, and is not time-consuming. However, vitrification requires high concentrations of CPAs that can prevent the oocyte/embryo from achieving its full developmental capacity. There is now significant evidence that CPAs, despite their benefits, can actually play a direct role in producing cryoinjry (Fahy, 1986). One such form of cryoinjury is zona pellucida hardening.

The overall goal of this research is to determine which cryoprotectant, dimethyl sulfoxide (DMSO) or glycerol, has the greatest effect on zona pellucida hardening. Zona pellucida hardness, after exposure to cryoprotectant, will be determined by protease digestion. This will help determine which cryoprotectant has less of an impact on the zona pellucida.

CHAPTER II
LITERATURE REVIEW

HISTORY

The accidental discovery by Polge, Smith, and Parkes in 1949, that glycerol would enable fowl spermatozoa to survive freezing to -70°C, making the fowl spermatozoa the first mammalian reproductive cells to be successfully frozen, initiated a phase of dramatic development in the techniques we now know as cryopreservation (Polge et al., 1949). Prior to Polge, Smith, and Parkes discovery, the field of cryopreservation had been limited to descriptions of cellular and tissue behavior of many different cell types at subphysologic temperatures (Walters et al., 2009). The first human birth from frozen sperm was reported in 1953 (Sherman, 1973). Improvements in the efficiency of cryoprotectants and discoveries of new ones, led to the first human birth from a frozen embryo in 1984 (Trounson et al., 1983) and the first human birth from a frozen oocyte in 1986 (Chen, 1986). Subsequent modifications of cryopreservation techniques resulted in the introduction of cryopreservation as a standard method offered by virtually every full-service IVF program worldwide (Valojerdi et al., 2009). The most common method during that time period of cryopreservation was slow rate freezing. Currently, cryopreservation via vitrification is considered the only alternative to long-term preservation of large-sized biological specimens (Eisenberg et al., 2014). Since such high concentrations of CPAs are required for cryopreservation, cryoinjury occurs at high rates. Studies have raised concerns regarding potential fertilization of cryopreserved mature oocytes following observations of physical damage (Gook, 2011) and structural modifications to the zona pellucida (Todorow et al., 1989). Incidence of cryoinjuries depends on the size and shape of the cell, the permeability of the cell membranes, and the quality of the cells. However, these factors

differ between species, developmental stage and origin (Vajta and Kuwayama, 2006). Successful cryopreservation of spermatozoa and embryos has made ART more practical and available for commercial use (Baldassarre and Karatzas, 2004).

OOGENESIS

Oogenesis, the differentiation of the ovum, is a complex process that starts during fetal development (Harper, 2001). Primordial germ cells develop from the inner lining of the fetal yolk sac and migrate to the genital ridge (Senger, 2005). In the developing ovary, these primordial germ cells divide by mitosis, or rapid proliferation, to form a population of oogonia (Feher, 2012). At the end of mitotic proliferation, the oogonia enter meiotic prophase and differentiate into primary oocytes (Miyamoto and Gurdon, 2013). Development of the primary oocyte is arrested in prophase I of meiosis and remains in this state until the onset of ovulatory cycles at puberty (Szmelskyj and Aquilina, 2015). During this period, oocytes accumulate a stockpile of macromolecules and organelles that are required to support early embryonic development (Miyamoto and Gurdon, 2013). Meiotic progression seems to be an integral part of oogenesis, since a number of proteins are required for both meiotic progression and for the development of functional oocytes (Maine, 2001). Each oocyte is contained within a cellular follicle that grows concomitantly with the oocyte, from a single layer of a few epithelial-like cells to three layers of cuboidal granulosa cells by the time the oocyte has completed its growth (Wassarman, 2001). A relatively high degree of synchronization appears to be a general feature of mammalian oocytes since the germ cells mature in groups, each of which consists of the elements at an identical stage of differentiation (Miyamoto and Gurdon, 2013). This synchronization is made possible by the presence of cytoplasmic bridges connecting the cells in each group to one another and bringing about a syncytial organization. The frequency of which

these bridges are found in the thin sections of embryonic ovarian tissue in electron microscopy indicates that their actual number is very high and that they form a network of intracellular connections which results in the organization of the germ cells into multiples syncytial groups (Zamboni, 1972). During all developmental stages, oogonia, oocytes and follicles may become atretic and die (Byskov and Anderson, 2013). At birth, the human ovaries contain in excess of 2 million primary oocytes which enlarge gradually to become the single largest unicellular cell in the body. However, over the reproductive lifespan, a woman will typically only ovulate 400-500 of those primary oocytes due to atresia of the remainder (Feher, 2012).

Once puberty, the ability to accomplish reproduction successfully, has been reached (Senger, 2015), a gonadotropin surge stimulates a luteinizing hormone (LH) surge. The completion of meiotic maturation results in response to the LH surge, emitting the first polar body, subsequently becoming an unfertilized egg. Upon fertilization by sperm, the ovulated egg completes the second meiotic division, with separation of chromatids and extrusion of a second polar body (Wassarman, 2001).

Two types of cells in particular play major roles in oogenesis: mural granulosa cells and cumulus cells. Apart from anatomical differences, mural granulosa cells and cumulus cells are functionally distinct (Rong et al., 2000). Granulosa cells line the follicle and support the growth of the follicle. They are responsible for the conversion of androgens to estrogens, as well as progesterone synthesis (Havelock et al., 2004). The granulosa cells of the ovulatory follicle are the major and virtually only source of estradiol in the follicular phase of the ovarian cycle and secrete estrogens in response to follicle stimulating hormone (FSH) (Goodman, 2009). After the LH surge, the granulosa cells produce epidermal growth factors that act on cumulus cells to induce cumulus cell expansion and oocyte maturation (Hung et al., 2015). Conversely, cumulus

cells surround the oocyte and are in intimate metabolic contact with the oocyte (Rong et al., 2000). Cumulus cells regulate oocyte growth, oocyte maturation and the fertilization process (Da Costa et al., 2015). Also, expansion of the cumulus-oocyte complex is necessary for meiotic maturation and acquiring developmental competence (Nevoral et al., 2015). The cumulus cells and the oocyte make up the cumulus-oocyte complex (COC). The COC is connected by gap junctions between the cumulus cells and also between the cumulus cells and the oocyte. Gap junctions between cumulus cells and oocytes are thought to be essential for oocyte maturation and fertilization (Zhou et al., 2016) and they allow a rapid transfer of small metabolites and regulatory molecules from the cumulus cells into the oocyte (Van Soom et al., 2002).

COLLECTION

In the history of oocyte collection, several methods have been employed: surgical, laparoscopic, abattoir ovaries, and oocyte pick-up via transvaginal guided follicular aspiration. Surgical and laparoscopic methods have become outdated in cattle due to their high cost and low oocyte recovery numbers (Pawshe et al., 1994), long recovery period for the animal, and overall time it takes to complete these procedures. In human fertility, the main disadvantage of laparoscopic collection lies in the necessary use of general anesthesia, the limitation of access to adherent or covered ovaries and its common post-operative abdominal discomfort and hospitalization stay of 6-24 hours (Leeton, 1989). Oocyte collection from an abattoir has become the most common collection method. The oocytes can be obtained relatively easily and inexpensively (Das et al., 1996) and it produces the most abundant source of primary oocytes (Wang et al., 1996). Neglia and colleagues determined that a higher proportion of Grade A oocytes are recovered from abattoir derived ovaries compared to transvaginal ultrasound guided aspirated oocytes, possibly due to the fact that greater mechanical damage is induced to

granulosa cells during the oocyte pick-up procedure (Neglia et al., 2003). In a study conducted by Souza-Fabjan and colleagues, they determined that oocyte pick-up derived oocytes require more time to mature when submitted to maturation media, have lower cleavage potential, and were less advanced in meiosis than abattoir derived oocytes (Souza-Fabjan et al., 2014). However, due to collection from abattoirs' terminal nature, this method cannot be repeated (Hashimoto et al., 1999).

Oocyte pick-up via transvaginal ultrasound guided follicular aspiration (TUGA) has shown to be a reliable method for oocyte retrieval (Bols et al., 1996). It can be performed twice weekly without side effects on the animals' reproductive career (Galli et al., 2013). To begin TUGA, the cow must be contained in a chute to ensure as little movement as possible, with an epidural anesthesia administered to prevent abdominal straining (Pieterse et al., 1991) and ease of handling the ovaries through the rectum (Galli et al., 2013). After emptying the rectum and a thorough cleaning of the vulva and perineal area (Garcia and Salaheddine, 1998), the vaginal probe is inserted into the vagina adjacent to the cervix, and after retraction of the uterus, the right or left ovary is placed against the head of the transducer. The antral follicles appear as hypoechoic round structures on the ultrasound monitor and an ultrasound needle guide may or may not be used to aspirate the imaged follicles (Pieterse et al., 1991). The transducer is positioned so that the needle guide on the ultrasound monitor transects the follicle to be aspirated and the needle is pushed through the vaginal wall into the follicle (Pieterse et al., 1988). The follicular fluid is aspirated using continuous negative pressure applied by an electronic suction pump. The needle itself is connected to silastic tubing so that the contents of the aspirated follicle are collected in a tube vial (Garcia and Salaheddine, 1998). After the needle is withdrawn from the ovary and the probe from the vagina, the needle and silastic tubing is flushed with phosphate-

buffered saline (PBS). The fluid aspirated from the follicles is filtered and inspected for oocytes (Pieterse et al., 1991). The combination of TUGA and IVP enables the acceleration of genetic improvement for production traits and provides the basis for more advanced technologies (Aller et al., 2010). By employing TUGA heavily in livestock species, it has provided decades of knowledge for assisted reproduction techniques that have been translated to innumerable advancements in the human fertility field (Galli et al., 2013).

CRYOPRESERVATION

Cryopreservation is the process of freezing biological material at extremely low temperatures; most commonly -196 degrees Celsius in liquid nitrogen. At these low temperatures, all biological activity ceases, including the biochemical reactions that lead to cell death and DNA degradation. During freezing, all cells go through the steps of temperature reduction, cellular dehydration, freezing and thawing, respectively. The accidental discovery, made by Polge, Smith, and Parkes, of the cryoprotective properties of glycerol revolutionized the cryopreservation field and sparked an abundance of further research (Polge et al., 1949). This landmark study also identified the fine balance between the protection and toxicity associated with the use of glycerol and other cryoprotectants such as propylene glycol and ethylene glycol (Gook, 2011). However, regardless of cryoprotectant use, some cryoinjury is still unavoidable due to the fact that each cell type responds differently to cryoprotectants and cryopreservation. Optimal protocols have not been established for each. The extent of the injury depends on factors including size and shape of the cells, the permeability of the membranes and the quality and sensitivity of the cells, oocytes, and embryos. All these factors may be highly variable depending on species, developmental stage and origin (Vajta and Kuwayama, 2006). Things that also must be considered during cryopreservation are transmembrane fluxes associated with the addition

and removal of CPAs, the change in chemical potentials during cooling and warming, both intracellular and extracellular ice formation, the effects of cooling and warming rates and storage temperatures, and heat transfer in solutions and tissues (Walters et al., 2009). Nonetheless, the main key to successful cryopreservation of any cell type is to avoid ice formation inside cells (Godsen, 2011). Understanding and controlling water permeability during cooling and thawing is crucial to minimizing cryoinjury. To prevent intracellular ice formation, or minimize the damage it can cause, all commonly used cryopreservation protocols for oocytes are designed to dehydrate them (Shaw et al., 2000). Cells have osmotic tolerance limits to which they can shrink or swell during cryopreservation, without significant damage. The formation of ice crystals hinders the cell from being able to shrink (Walters et al., 2009). At temperatures at or below 0 degrees Celsius, water resists the breaking of hydrogen bonds and the molecules lock together in a lattice-like symmetry, creating pockets of "open space", allowing ice crystal formation. Ice nucleation, the point at which ice crystal growth is initiated, has the ability to grow and spread rapidly throughout the cell (Benson, 2008). Cryoprotectants, acting through increasing the total concentration of all solutes in the system, reduce the amount of ice formed at any given temperature. Providing water can leave the cells rapidly to maintain thermodynamic equilibrium across the cell membrane, the cytoplasm will not cool below its freezing point and all the ice will be external to the cells (Pegg, 2007). The probability of intracellular freezing is minimized by the cooling rate and the permeability of the cells to water (Pegg, 2010). Two types of cryopreservation exist: slow-rate freezing and vitrification. Slow rate freezing allows the cell to be able to lose water by exosmosis, avoiding intracellular freezing, while in vitrification, the cell is not able to lose water at a rate that maintains osmotic equilibrium. The cell becomes

supercooled in vitrification, eventually reaching equilibrium by forming a glass-like structure intracellularly (Alink and Offerings, 1977).

Cryopreservation of oocytes and embryos has become an integral part of improving the success rate of infertility treatment (Kuleshova and Lopata, 2002). It has increased the efficiency of ART procedures, allowing for the conservation of biodiversity, and providing a way to preserve rare and valuable biological material. The acceptance of oocyte and embryo cryopreservation in general assisted reproduction practice has finally come to fruition and has now arrived at a stage where it is considered to be routine (Gook, 2011).

CRYOPROTECTANTS

Cryoprotective agents (CPAs) are small, water-soluble molecules that serve as colligative antifreeze agents by disrupting hydrogen bonds between water (Godsen, 2011). In 1949, Polge and colleges discovered that the use of glycerol as a cryoprotectant greatly increased the proportion of human spermatozoa that could be revived after cryopreservation. Their subsequent experiments all suggested that a high proportion of water could be withdrawn from cells at low temperatures with ensuing restoration of structure and function of the cell (Polge et al., 1949). The effectiveness of a cryoprotectant depends on a number of properties: 1) the compound must be highly soluble in water and remain so at low temperatures in order to produce a profound depression of the freezing temperature; 2) it must be able to penetrate into the cells; and 3) must have low toxicity so that it can be used in the high concentrations that are required to produce the cryopreservative effects (Pegg, 2007). Cryoprotectants are divided into two main classes: permeating and non-permeating. Permeating CPAs penetrate the cell membrane and prevent ice crystal formation and membrane rupture, whereas non-permeating CPAs do not penetrate the cell

membrane and act by reducing the hyperosmotic effect during the freezing procedure (Janz et al., 2012).

In cryopreservation protocols, commonly used permeating cryoprotectants are dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG) and glycerol (GLY), with glycerol being the most frequently used. They are small, cell membrane penetrating molecules that act by increasing membrane fluidity through rearrangement of membrane lipid and proteins, resulting in greater dehydration at lower temperatures and reduced intracellular ice formation (Holt et al., 2000). The criteria for a permeating cryoprotectant is that the compound must penetrate the cell, otherwise it will dehydrate the cell osmotically and stimulate freezing injury, and it must be non-toxic in the multimolar concentrations that are necessary to prevent excessive ice formation. The rate at which these compounds enter cells varies not only with the agent, but with the type and origin of the cell (Meryman, 1971). For example, a study conducted by Hober and colleges showed that the penetration rate of glycerol into the red blood cells of a variety of mammals, is highest among rats and men, and 200 times slower in the sheep (Hober et al., 1945). The permeating cryoprotective agent is traditionally associated with protection against slow freezing injury; it prevents injury simply by reducing, on a purely colligative basis, the amount of ice formed, thereby reducing the concentration of non-permeating solute needed (Meryman, 1971). The potential for adverse events or reactions is heavily skewed towards permeating CPAs, since these are more able to interact directly with a multitude of cellular activities, such as enzymatic processes, transporter mechanisms, ion exchanges and the like (Elliot et al., 2017).

Non-permeating cryoprotectants are the second class of cryoprotectants. Some of the frequently used non-permeating cryoprotectants are sucrose, trehalose, dextrose, and polyvinylpyrrolidone, with sucrose being the most commonly used. These cryoprotectants do not

penetrate the cell membrane, are high in molecular weight, hydrophilic, and act by helping a cell stabilize its concentration of internal solutes under osmotic stress (Cleland et al., 2004). Because of their inability to diffuse across the plasma membrane, non-permeating cryoprotectants create an osmotic pressure that lowers the freezing temperature of the medium and decreases extracellular ice formation (Aisen et al., 2002). Osmotic stress occurs when the extracellular solute concentration is too high and water rapidly leaves the cell, causing harm to the structure and function of the cell. However, if not enough water is removed from the cell, intracellular ice formation will occur and possibly cause the cell to lyse during the thawing process.

The processes of diffusion and osmosis have important effects during the introduction of cryoprotectants, the removal of cryoprotectants, the freezing process and during thawing (Pegg, 2007). Depending upon which type of cryoprotection used, either slow rate freezing or vitrification, the effects of diffusion and osmosis are different. In 1963, Mazur discovered that the rate of change of temperature was also important because it controlled the transport of water across the cell membrane, and hence, indirectly, the probability of intracellular freezing (Mazur, 1963). The toxicity of cryoprotectants to cells is another major concern. The toxicity of cryoprotectants limits the concentration of additive that can be used before freezing and therefore, limits the cryoprotective efficacy of these agents. With high enough concentrations of cryoprotectants, damaging factors can be suppressed (Fahy, 1986). However, the concentration needed for complete suppression is often too high, causing injury rather than protection (Fahy, 2010). Therefore, cryoprotectant toxicity is the single most limiting factor for the success of cryopreservation by both slow rate freezing and vitrification and for the scope of cryobiological protocols (Fahy, 1987). Typically, to limit toxicity and to best cryopreserve cells, a combination of permeating and non-permeating cryoprotectants are used in most protocols. However, the

ways in which the various cryoprotectants, and their combinations, affect a given cell type cannot be predicted, and hence, the selection of cryoprotectants for each type of cell is still largely dependent on an empirical and experimental approach (Fan et al., 2009).

SLOW RATE FREEZING

Slow rate freezing, which is considered an equilibrium approach, allows cells to be cooled to very low temperatures while minimizing intracellular ice formation, and at the same time, attempting to minimize detrimental influences of increased solute concentrations and osmotic stress (Friedler et al., 1988). Most importantly, it attempts to control the biophysical properties of freezing, like cooling and warming rates (Smith et al., 2010). The success of slow rate freezing depends on achieving the optimal balance between the rate at which water can leave a cell and the rate at which it is converted to ice (Shaw et al., 2000). Through the use of a programmable cooling machine that is controlled by a computer program, the rate at which the cells are cooled is controlled within an internal chamber. The exact freezing rate and temperature can be programmed by the researcher, providing optimal species specific cellular dehydration. Instead of intracellular ice formation, slow rate freezing leads to extracellular ice formation. A consensus has developed that intracellular freezing is dangerous, whereas extracellular ice is harmless (Pegg, 2007). After cells are placed in a permeating cryoprotectant solution, the temperature is then lowered by the cooling machine and ice crystal growth is initiated. As the ice crystals grow, water in the solution is converted from its liquid state to a solid state, which increases the concentration of solution, and therefore, draws water out of the cells (Shaw et al., 2000). Water migrates out of the cell in efforts to maintain osmotic equilibrium. After the cooling temperature has been lowered to freezing range, cells are plunged into liquid nitrogen and stored until thawing. A commonly used protocol, used in a study by Cao and colleagues, for

slow rate freezing begins at 25° C. Cooling is started at a rate of -2° C per minute. At -7° C, seeding is performed by touching the side of each straw with forceps that have been cooled in liquid nitrogen. The straws are held at -7°C for 10 minutes and are cooled at a rate of -0.3°C per minute to -33°C before being plunged into liquid nitrogen and stored for later use at -196°C (Cao et al., 2009). The consequences of slow rate freezing have been documented to include cellular injury, resulting in cell death, especially from -15°C to -30°C (Djumwantono et al., 2011). However, other studies, like the study by Sinha and colleagues, show approximately 85% of slow rate cryopreserved mature oocytes had normal morphological characteristics after thawing (Sinha et al., 2014). Slow freezing has the advantage of using low concentrations of cryoprotectants, which are associated with chemical toxicity and osmotic shock (Arav, 2014). While many consider slow rate cryopreservation an acceptable approach to cryopreservation, recent advances in vitrification have made it the method of choice when cryopreserving oocytes and embryos.

VITRIFICATION

Vitrification is the production of a glassy state that is defined by the viscosity reaching a sufficiently high value to behave like a solid, but without any crystallization (Pegg, 2007). Vitrification was first proposed for cryobiology applications by Luyet in 1937 (Luyet, 1937), but the avoidance of ice in tissues were based on the early work of Rall, Fahy and colleagues (Fahy et al., 1984; Rall and Fahy, 1985; and Rall, 1987). Unlike slow rate freezing, vitrification offers the ability to control the solute penetration, the dehydration rate, and the maintenance at physiological temperature during the non-equilibrium procedure (Al-Azawi et al., 2013). The use of non-permeating cryoprotectants is very useful because the shrinkage of the oocyte and consequently the amount of water inside the cell that may crystallize during rapid cooling and

warming is lower (Rall et al., 1987) and because this reduces the amount of the cryoprotectant that penetrates the cell thus reducing the possible toxic effect (Szell and Shelton, 1987). Most vitrification protocols use solutions with high concentrations of solutes, which rapidly draw water out of cells. Usually, vitrification requires molar concentrations of threefold or fourfold higher than slow rate freezing, and a very rapid rate of cooling cells below the glass transition temperature (T_g) (Godsen, 2011). Both the cooling and warming rates, as well as the composition and concentration of the solution will influence how much, if any, ice will form (Kuleshova et al., 1999, Shaw et al., 1999, and Vajta et al., 1998). The high cooling rates applied during vitrification provide a unique benefit compared to traditional slow rate freezing: the possible partial and sometimes total elimination of chilling injury, as the sample passes through the dangerous temperature zone quickly enough to disallow sufficient time for damage to develop. Vitrification is the most appealing cryopreservation protocol because it takes very little time and requires no expensive equipment (Walters et al., 2009). As stated before, most vitrification protocols combine the use of both permeating and non-permeating cryoprotectants to achieve the best results. The combination of ethylene glycol and dimethyl sulfoxide with sucrose was reported for mouse and bovine embryos in 1993 and formed the basis of vitrification protocols (Ishimori et al., 1993). Before entering the protocol, oocytes are typically placed in holding medium of HEPES-buffered TCM 199, with bovine serum albumin (BSA) or bovine calf serum (BCS), and an antibiotic. Gentamicin is routinely used because it is a broad-spectrum antibiotic (Dowglish et at., 2017). Many protocols commonly use a stepwise addition of cryoprotectants in efforts to slowly equilibrate oocytes and embryos. The first solution, the equilibration solution, contains low concentrations of cryoprotectants, usually 10%. With each solution thereafter, the concentration of cryoprotectants are gradually increased. The amount of time the oocyte spends

in each vitrification solution is dictated by the concentrations of the solution. The higher the concentration, the less time the cells are exposed to the solution and vice versa. Exposure for 5 minutes in equilibrium solution, or vitrification solution 1, then 45 seconds for vitrification solution 2, is a protocol used to vitrify bovine oocytes and embryos. Next, the oocytes are loaded on a cryopreservation tool. Of the tools used, the most common are open pulled straw, cryoloop, cryotop, and cryolock. The cryolock has become the preferred tool because its rectangular, four flat surfaces, and concave tip make it much easier to handle and takes fewer steps to load or remove oocytes than the other tools. Also, the cap of the cryolock makes it a

Table 2.1- A Comparison of Vitrification with Slow-Freezing Procedures (From Kuleshova, 2002).

Accessibility and regulation	**Vitrification**	**Slow freezing**
Can be observed	yes	no
Can be analyzed	yes	no
Interaction with the oocyte or embryo	yes	no
Control of solute penetration	yes	no
Control of dehydration rate	yes	no
Maintenance of physiological temperature during equilibration procedure	yes	no
Duration out of incubator	~10 min	~3 h
Prolonged temperature shock	no	yes
Interference with oocyte or embryo	low	high
Fracture of the zona pellucida	no	possible
Capture by growing ice crystals	no	possible

closed system, preventing pathogen transfer.

If performed correctly, vitrification does not have any of the biologically damaging effects associated with freezing because no appreciable degradation occurs over time in living matter trapped within a vitreous matrix. Complete vitrification should eliminate concerns for the known damaging effects of intra- and extracellular crystallization that leads to loss of cell viability, tissue morphology, extracellular matrix integrity, and RNA degradation (Brockbank et al., 2014). When comparing the advantages and disadvantages of vitrification with slow rate freezing, vitrification is clearly the superior method. In one study by Li and colleagues, the results showed that more human blastocysts survived with higher DNA-integrity index after vitrification/warming than after slow freezing/thawing (Li et al., 2012). Cao and colleagues conducted a study where 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. In all areas of evaluation, better results were achieved with vitrification (Cao et al., 2009). Even though both procedures are still used today, vitrification appears to be the fastest, most efficient and economical approach for oocyte cryopreservation.

WARMING

The thawing and rewarming process after the storage of oocytes and/or embryos is critical to the survival rate (Schellander et al., 1994). As previously mentioned, when going through the process of freezing/cooling, cells shrink due to osmotic dehydration. Upon thawing/rewarming, water must reenter the cells and cryoprotectants must be removed, causing the cell to swell. However, if intracellular ice formed during cryopreservation, upon thawing, it will return to water and the cell with have a greater water volume than its normal physiological

volume, and the cell could swell to the point to lysing. Because cells are generally more sensitive to swelling than to shrinking, removal of cryoprotectants tends to be more hazardous than their addition (Pegg, 2007). Diluting cells directly into isotonic solutions inevitably results in a significant uptake of water by the cells, which, in extreme cases, can destroy homeostatic mechanisms, including membrane barrier characteristics (Elliot et al., 2017). Because of this, as in vitrification, a stepwise removal of cryoprotectants is preferred. Agents such as sugars can assist in mitigating influx of water into CPA loaded cells upon dilution (Elliot et al., 2007). Rewarming must also be ultra-rapid to avoid ice nucleation (Godsen, 2011). Samples are transferred from storage in liquid nitrogen (LN_2) to a holding reservoir filled with LN_2 in preparation for the warming procedure. A thawing solution is warmed to 37°C either in an incubator without CO_2 or on a warming plate. A commonly used protocol for warming oocytes and/or embryos begins with removal of the samples from the holing reservoir of LN_2 and immediately plunging them into the warmed thawing solution, where it remains for 1 minute. The oocytes are then transferred to a dilution solution for 4 minutes and then to two separate wash solutions for 4 minutes each (Irvine Scientific). A rule of thumb is that cells should be thawed quickly and diluted slowly.

ZONA PELLUCIDA/ ZONA HARDENING

The zona pellucida is a thick, translucent, extracellular matrix that surrounds the developing oocyte and early embryo. The zona pellucida supports communication between oocytes and follicle cells during oogenesis, protects oocytes and embryos during development, and regulates interactions between ovulated oocytes and free-swimming sperm during and following fertilization. Also, the zona pellucida serves as a barrier to polyspermic fertilization: the fertilization of the oocyte with more than one sperm (Wassarman et al., 1999). Three

glycoproteins make up the zona pellucida: zona proteins 1, 2, and 3 (ZP1, ZP2, and ZP3). Zona proteins 1 and 2 are structural proteins providing structural integrity to the zona. ZP3 is much like a receptor for a hormone: it binds to proteins on the spermatozoal membrane (Senger, 2005). The zona pellucida is composed of long interconnected filaments that are polymers of ZP2 and ZP3, crosslinked by ZP1 to create a three-dimensional matrix (Wassarman et al., 1999).

A current problem with oocyte cryopreservation is the induction of a primary activation event, zona hardening, which specifically inhibits IVF (Matson et al., 1997) and may affect subsequent implantation. The process of zona hardening, which is essential for normal development after fertilization, can prevent fertilization when it occurs prematurely (Larman et al., 2006). Zona hardening is brought about by the fusion of cortical granules to the plasma membrane and the release of their contents into the zona pellucida layers (exocytosis) (Sun, 2003). The membrane fusion event is calcium-dependent and is normally triggered by the increase in intracellular calcium initiated by sperm-egg fusion (Kline and Kline, 1992). However, it has been documented that oocyte and/or embryo exposure to cryoprotectants during cryopreservation causes a rise in intracellular calcium, prematurely inducing the cortical granule reaction. Morley and Whitfield reported that DMSO caused an intracellular calcium rise in various cell lines (Morley and Whitflield, 1993). Premature cortical release has been observed after cryopreservation in both mouse and human oocytes (Mavrides and Morroll, 2005), but this phenomenon has not been studied in the bovine (Fuku et al., 1995). The premature release ultimately disrupts the timing of the process and increases the rate of polyspermy and subsequently of polyploidy (Fuku et al., 1995). Zona hardening can be overcome in most species by intracytoplasmic sperm injection (ICSI) (Porcu et al., 2000) but the fact that the oocyte has undergone the cortical granule reaction, indicates that the oocyte has been artificially activated

prior to actual fertilization and possible down-regulation of cell cycle control proteins has occurred (Larman et al., 2006). If fertilization is successful after zona hardening, many embryos encounter implantation failure. Failure of the embryonic zona pellucida to rupture following blastocyst expansion is regularly attributed to premature zona hardening. In order to help embryos escape from their zonae, different types of assisted hatching have been developed, with laser-assisted hatching being the most favored. In vitro studies with both mouse and human embryos have indicated that an artificial gap in the zona pellucida significantly improves the hatching ability of blastocyst grown in vitro as compared to non-micromanipulated embryos (De Vos and Steirteghem, 2000).

In many agricultural species, ICSI is not a feasible option to overcome zona pellucida hardening. ICSI of bovine oocytes is not effective, unlike in human and mouse oocytes, because bovine oocytes are not activated easily by the injection procedure, and therefore, successful fertilization rates can be as low as 3%. Bovine oocytes can be activated by ionomycin or 6-dimethylaminopurine (DMAP) to improve cleavage rates up to 70% (Mavrides and Morroll, 2005). However, a signification problem arises when activating the oocyte with ionomycin or DMAP: these compounds induce parthenogenetic activation, the growth and development of the embryo without any actual paternal contribution, which can be as high as 40%, and is a source of error in studies (Chung et al, 2000). Overall, IVF yields better cleavage rates than ICSI for bovine studies (Mavrides and Morroll, 2005). Zona hardening is often overlooked in many species where ICSI can circumvent the hardening. However, since ICSI is time consuming, requires expensive equipment and a high level of skill to master the technique, and has low success rates in bovine, zona hardening has a more significant impact on the overall fertilization process and should be studied in greater detail.

CURRENT ISSUES FOR FUTURE RESEARCH

Successful cryopreservation of bovine oocytes has major implications in agriculture for commercial use. It allows long-distance transportation, omission of estrous synchronization, and adds advantages to oocyte banking to preserve valuable female genetic resources. However, in bovine species, the low fertilization rates and developmental competence of cryopreserved oocytes still needs to be improved (Hwang and Hochi, 2014). Since the efforts to combat zona pellucida hardening of cryopreserved oocytes by intracytoplasmic sperm injection in the bovine have been mostly unsuccessful, more research needs to be conducted in the area of the causation of zona hardening, in particular, the premature intracellular calcium rise and subsequent cortical granule reaction due to the exposure of oocytes to cryoprotectants.

CHAPTER III
VITRIFICATION OF MATURE BOVINE OOCYTES

INTRODUCTION

Cryopreservation is an integral component of the cattle embryo transfer industry and is used to transport genetically superior breeds internationally. Vitrification has become a competitive alternative to slow rate freezing (Vajta and Kuwayama, 2006). It is most appealing because it is so fast and requires no expensive equipment (Walters, 2009). However, the high concentrations of cryoprotectants necessary for vitrification can be toxic to the oocytes, therefore, causing it to fail to resume normal structure and function upon thawing. Special cytological features of mature oocytes, namely the meiotic spindle, cortical granules and cytoskeleton, are thought to be susceptible to damage during cooling and exposure to cryoprotectants (Rall, 1992). Dimethyl sulfoxide was proposed as a cryoprotectant in 1960 by Lovelock and Bishop and was rapidly shown to have far more widespread applicability than glycerol (Lovelock and Bishop, 1959). The use of different cryoprotectants, such as glycerol, can also affect the viability of each specific cell type following the freezing and warming process. Even though the oocytes may survive cryopreservation, many fail to fertilize or cleave (Mavrides and Morroll, 2005). Exposure of oocytes to cryoprotectants prior to freezing causes a significant premature exocytosis of cortical granules, which stimulates zona hardening (Fuku, 1995). The premature cortical reaction could explain the poor efficiency of oocyte freezing because the oocyte has already begun its activation events and possible down regulation of cell cycle control proteins (Larman et al., 2006). Zona hardening has been shown to cause a reduction in fertilization rates and a disturbance in the organization of the cytoskeleton and metaphase plate (Fuku, 1995). Since intracytoplasmic sperm injection is largely unsuccessful in the bovine species, zona hardening is detrimental during the cryopreservation of bovine oocytes.

The objective of these experiments was to determine the effects of cryoprotectants on premature zona pellucida hardening using fresh oocytes and also, to determine the effects of cryoprotectants on premature zona pellucida hardening using vitrified oocytes. The specific aims were to compare the effect of dimethyl sulfoxide or glycerol on zona hardening using fresh oocytes and to compare the effect of dimethyl sulfoxide or glycerol on zona hardening using vitrified oocytes. It was hypothesized that glycerol would harden the zona pellucida more than dimethyl sulfoxide and also, that dimethyl sulfoxide would harden the zona pellucida more than the control (Dulbecco's phosphate-buffered saline).

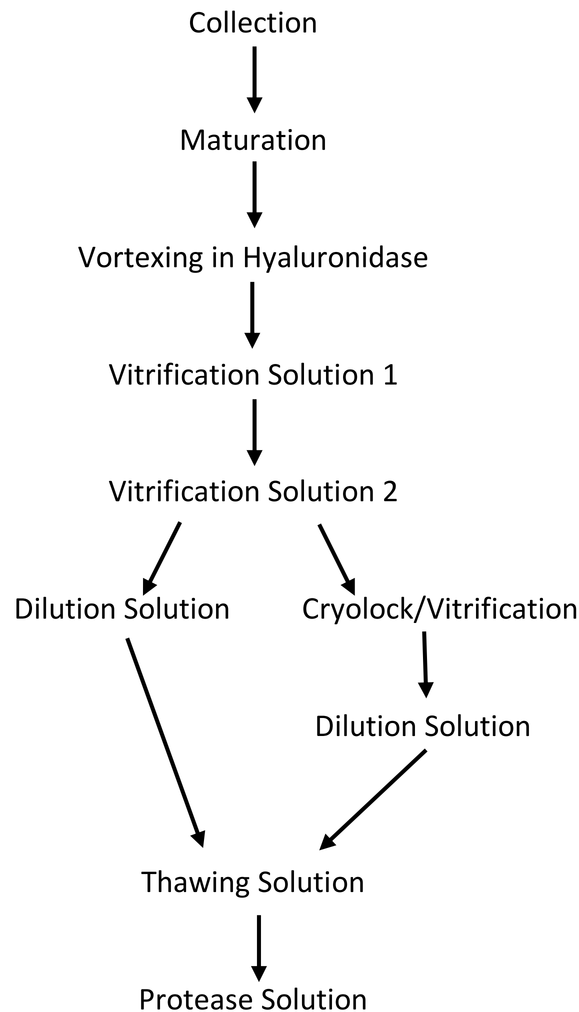
MATERIALS AND METHODS

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

Experimental Design

Oocytes from mixed breed cows were obtained from abattoir derived ovaries through a commercial source and shipped overnight in maturation media consisting of supplemented M199 (Appendix B). After 22 hours of maturation, the oocytes were immediately placed in the vitrification protocol. A total of 117 fresh mature oocytes were used in experiment 1 (n=117) and 113 vitrified mature oocytes were used in experiment 2 (n= 113). Oocytes were randomly assigned to treatment groups: DMSO, glycerol, or the control (DPBS).

Table 3.1 Flowchart of Experimental Design



Experiment One Design

Oocytes were shipped in supplemented maturation media M199 and allowed to mature for 22 hours within a 5% CO_2 incubator. After 22 hours, they were vortexed in 1 mg/mL hyaluronidase solution to remove cumulus cells and placed in a warmed holding medium, HEPES-TALP (Appendix B), until entering the vitrification protocol. Oocytes were first exposed to a 50 μL drop of vitrification solution one (Appendix B) under mineral oil and remained there for 5 minutes. Immediately afterwards, the oocytes were transferred to a 50 μL drop of vitrification solution two (Appendix B) under mineral oil and remained there for 45 seconds.

Next, oocytes were placed in the dilution solution (Appendix B) for 5 minutes. Following the dilution solution, oocytes were transferred to a warmed holding medium, HEPES-TALP (Appendix A- Protocol A), until entering the Streptomyces griseus protease solution. Dulbecco's phosphate buffered saline was used as the control, and control treatment oocytes followed the same protocol as the cryoprotectant exposed oocytes. Next, the oocytes were transferred to a 50 µL drop of 0.1% Streptomyces griseus protease solution (Appendix B) under mineral oil where they remained until complete zona digestion was observed. Time of exposure to protease until complete zona pellucida digestion was recorded for each individual oocyte.

Experiment Two Design

Oocytes were shipped in maturation media consisting of supplemented M199 (Appendix B) and allowed to mature for 22 hours within a 5% CO_2 incubator. After 22 hours, they were vortexed in 1 mg/mL hyaluronidase solution to remove cumulus cells and placed in a warmed holding medium, HEPES-TALP (Appendix B), until entering the vitrification protocol. Oocytes were first exposed to a 50 µL drop of vitrification solution one (Appendix B) under mineral oil and remained there for 5 minutes. Immediately afterwards, the oocytes were transferred to a 50 µL drop of vitrification solution two (Appendix B) under mineral oil and remained there for 45 seconds. Within the allotted 45 seconds for vitrification solution 2, oocytes were placed on a cryolock and plunged into liquid nitrogen (Appendix A- Protocol B). All oocytes were warmed within 7 days of vitrifying. To begin the thawing process, oocytes were removed from liquid nitrogen storage and plunged directly into the warmed dilution solution (Appendix B) for 5 minutes. Following the dilution solution, oocytes were transferred to a warmed holding medium, HEPES-TALP (Appendix A- Protocol A), until entering the Streptomyces griseus protease solution. Dulbecco's phosphate buffered saline was used as the control, and the control treatment

oocytes followed the same protocol as the cryoprotectant exposed oocytes. Next, the oocytes were transferred to a 50 µL drop of 0.1% protease solution under mineral oil where they remained until complete zona pellucida digestion was observed. Time of exposure to protease until complete zona pellucida digestion was recorded for each individual oocyte.

Table 3.1 Composition of Cryoprotectant Solutions for Fresh and Vitrified Oocytes

Vitrification Solutions for DMSO Treatment		Vitrification Solutions for Glycerol 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% DMSO	20% DMSO	10% Glycerol	10% Glycerol
0.5 M Sucrose	0.5 M Sucrose	0.5 M Sucrose	0.5 M Sucrose

Preparation of Media

Required media included HEPES-TALP (Appendix B), DMSO vitrification solutions one and two (Table 3.2), glycerol vitrification solution one and two (Table 3.2), dilution solution (Appendix B), and protease solution (Appendix B) were prepared approximately three hours prior to time of use. HEPES-TALP holding media for the treatment and control groups consisted of 20 mL HEPES-TL (Caisson Labs, North Logan, UT, USA) with 60 mg BSA and 200 µL sodium pyruvate and pen/strep (Gibco, No. 15140). Two hours prior to oocyte vitrification, all necessary media dishes were prepared with the premade solutions in petri dishes (35mm, BD

Falcon). All media except HEPES-TALP were covered in mineral oil. HEPES-TALP and the dilution solution were equilibrated for at least two hours on a warming plate at 37°C before being used.

Vitrification and Warming Procedures

Oocytes were exposed to dimethyl sulfoxide, glycerol and control treatment groups in both experiments. In each replication, the oocytes were randomly assigned to a treatment group. Groups of two to five oocytes would be placed into the designated vitrification solution for 5 minutes. They were then transferred to the higher concentration vitrification solution and removed within 45 seconds. In the first experiment using fresh oocytes, after the higher concentration vitrification solution, they were immediately plunged into the dilution solution where they remained for 5 minutes. After being removed from the dilution solution (Appendix B), oocytes were briefly washed in HEPES-TALP and transferred to the protease solution (Appendix B). In the second experiment using vitrified, after the higher concentration vitrification solution, the oocytes were placed on the cryolock device, excess media removed and plunged into liquid nitrogen within 45 seconds. The caps of the cryolocks were pre-cooled in liquid nitrogen and were put on with pre-cooled forceps. The cryolocks were stored in liquid nitrogen for no more than 7 days. Upon thawing, the cryolocks were plunged into dilution solution where they remained for 5 minutes. Oocytes were then briefly washed in HEPES-TALP and transferred to the protease solution for complete zona pellucida digestion.

Zona Pellucida Digestion

All oocytes in both experiments were placed in 50 µL drops of a 0.1% Streptomyces griseus protease solution under mineral oil at room temperature. They remained in the protease solution until the zona pellucida was completely digested.

Statistical Analysis

The first experiment consisted of 5 replicates with 41 oocytes each in the DMSO and glycerol treatment groups and 35 oocytes in the control group. The second experiment consisted of 3 replicates with 37 oocytes in the DMSO treatment group, 41 in the glycerol treatment group, and 36 in the control group. Assessment of zona hardening was based on time the oocytes time of expose to protease until the zona pellucida had been completely digested. The data was analyzed with Sas 9.4, using Least Squares Means and contrasts statements. Values with a $P \leq 0.05$ were considered statistically significant.

RESULTS

Experiment One

Results from the protease digestion of the zona pellucida using fresh mature oocytes showed a statistically significant difference between DMSO, glycerol and PBS treatments with the P-value of $P= 0.0142$. When comparing DMSO digestion to glycerol digestion, there was statistical significance with $P=0.0238$. However, there was no statistical significance when comparing DMSO and PBS ($P=0.3325$). Glycerol compared to PBS showed a statistical significant difference with $P=0.0058$. Lastly, when comparing DMSO and glycerol together to PBS, results showed it was statistically significant with $P=0.0262$ (Table 3.5). The treatments DMSO, glycerol, and PBS had an average digestion time of 16.79 minutes, 20.94 minutes, and 15.2 minutes, respectively. Figure 3.1 shows oocytes at different stages throughout zona pellucida digestion: A) after removal from maturation media consisting of supplemented M199, 3 oocytes are surrounded by cumulus cells; B) a denuded oocyte with a thick, fully intact zona pellucida; C) oocyte is approximately half way through protease digestion with thinning of the

zona pellucida; D) zona pellucida has completely digested and only the vitelline membrane surrounds the nuclear contents.

Table 3.2 Zona Pellucida Mean Protease Digestion Time using Fresh Oocytes

Treatment	N	Digestion Time
DMSO	41	16.79 ± 2.6272
Glycerol	41	20.94 ± 2.6272
DPBS	35	15.2 ± 2.6552

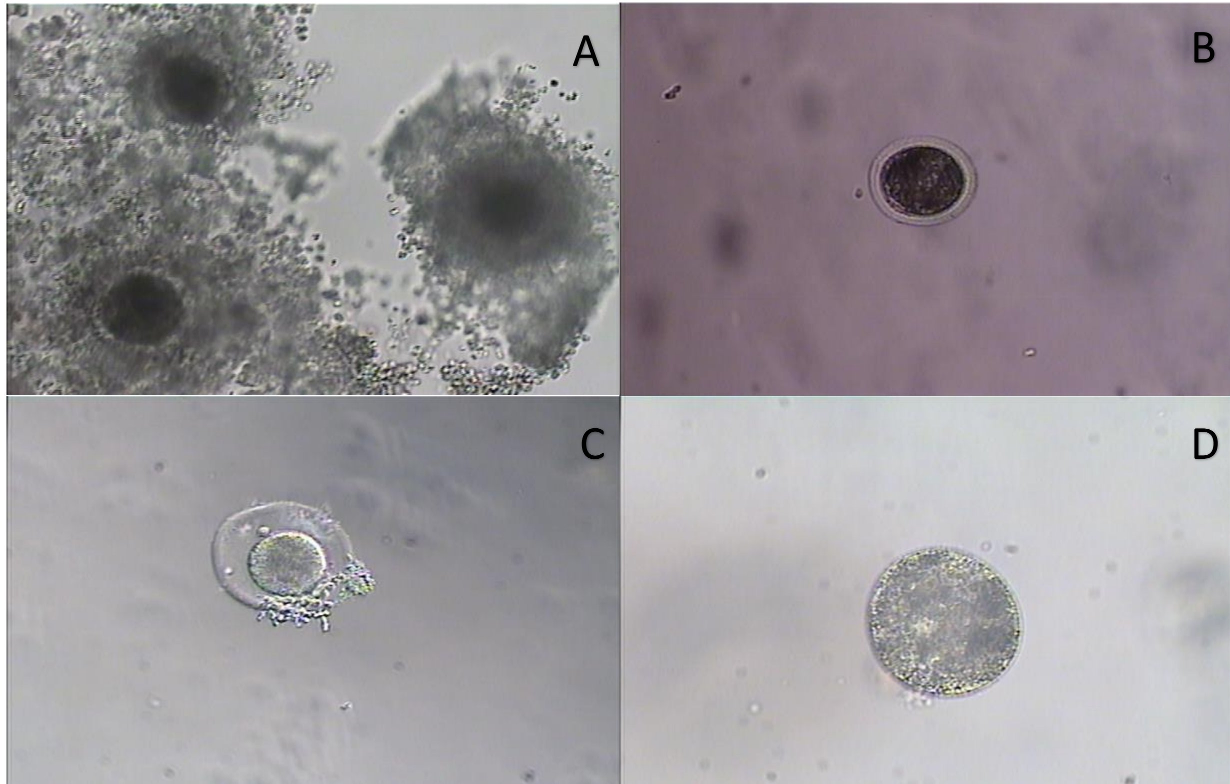
± Standard Deviation

Table 3.3 Comparison of Zona Pellucida Protease Digestion Time between Treatments using Fresh Oocytes

Cryoprotectant	P value
DMSO vs. Glycerol	0.0238
DMSO vs. Control (PBS)	0.3325
Glycerol vs. Control (PBS)	0.0058
DMSO & Glycerol vs. Control (PBS)	0.0262

* Contrasts with a probability value of $P \leq 0.05$ were considered significantly significant

Figure 3.2 Stages of Zona Pellucida Digestion by Protease



A- Cumulus-oocyte-complex
B- Denuded oocyte
C- Partial zona pellucida digestion
D- Full zona pellucida digestion

Experiment Two

Results from the protease digestion of the zona pellucida using vitrified mature oocytes showed no statistical significant difference between DMSO, glycerol and PBS treatments (P=0.3437). When comparing DMSO digestion to glycerol digestion there was no statistical significance (P=0.5301). DMSO versus the control and glycerol versus the control both showed no statistical significance with P-values of P=0.1699 and 0.3813, respectively. Lastly, when comparing DMSO and glycerol together to PBS, results showed it was also not statistically significant with P=0.2004 (Table 3.6). The treatments DMSO, glycerol, and PBS had an average

digestion time of 22.78 minutes, 19.56 minutes, and 14.94 minutes, respectively. Figure 3.1 shows oocytes at different stages throughout zona pellucida digestion: A) after removal from maturation media consisting of supplemented M199, 3 oocytes are surrounded by cumulus cells; B) a denuded oocyte with a thick, fully intact zona pellucida; C) oocyte is approximately half way through protease digestion with thinning of the zona pellucida; D) zona pellucida has completely digested and only the vitelline membrane surrounds the nuclear contents.

Table 3.4 Zona Pellucida Mean Protease Digestion Time using Vitrified Oocytes

Treatment	**N**	**Digestion Time**
DMSO	37	22.28 ± 4.9906
Glycerol	41	19.56 ± 5.0011
DPBS	36	14.94 ± 4.9951

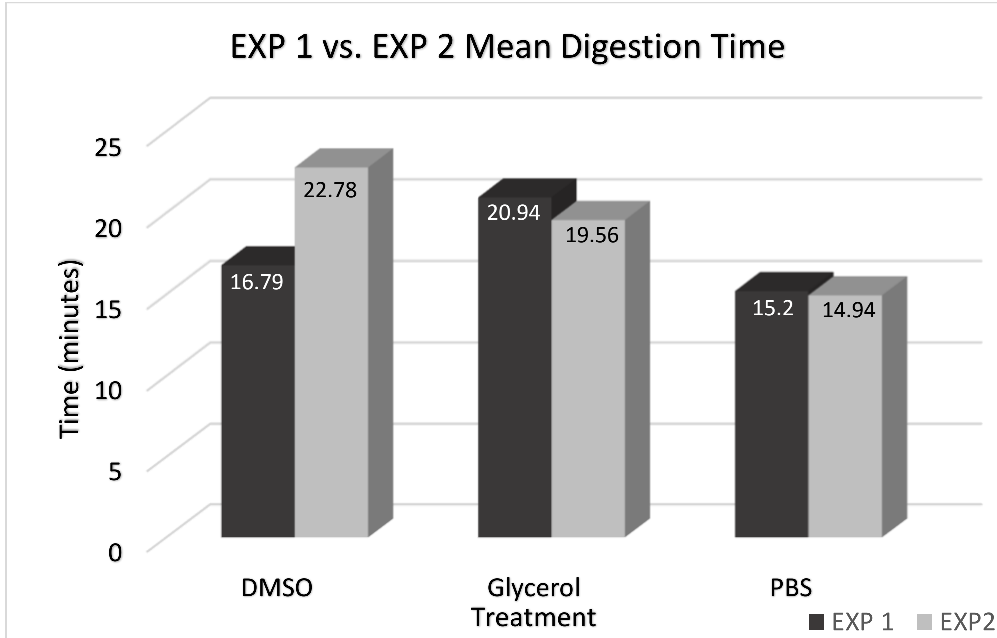
± Standard Deviation

Table 3.5 Comparison of Zona Pellucida Protease Digestion Time between Treatments using Vitrified Oocytes

Cryoprotectant	**P value**
DMSO vs. Glycerol	0.5301
DMSO vs. Control (PBS)	0.1699
Glycerol vs. Control (PBS)	0.3818
DMSO & Glycerol vs. Control (PBS)	0.2004

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

Figure 3.3 Protease Digestion of the Zona Pellucida using Fresh and Vitrified Oocytes



DISCUSSION

The aim of these experiments was to determine if zona pellucida hardening is affected by choice of cryoprotectant in the cryopreservation of mature oocytes via vitrification. Determining which cryoprotectants have the greatest effect on zona pellucida hardening in the vitrification of bovine oocytes would be beneficial because it would potentially increase fertilization rates and have positive implications regarding the largely unsuccessful efforts of bovine intracytoplasmic sperm injection. Without zona pellucida hardening during cryoprotectant exposure, activation of the oocyte after ICSI would enable the cortical granule reaction to have the greatest effect on the zona pellucida. Within these experiments, progress was made towards determining which cryoprotectants would minimize the amount of zona pellucida hardening in bovine vitrification.

In the first experiment, significance between all three treatment groups was observed (P=0.0142). In the first comparison, zona hardening was statistically different (P=0.0238) when

DMSO and glycerol were compared to each other. However, significance was not observed when DMSO was compared to the control (P=0.3325), suggesting that DMSO did not harden the zona pellucida compared to the control (PBS). Comparing zona hardening in glycerol to the control showed the greatest significance (P=0.0058) of all the comparisons. When DMSO and glycerol zona digestion observations were combined and compared to the control, zona hardening showed to statistically different (P=0.062). All of these data support our hypothesis that glycerol hardens the zona pellucida more than DMSO, suggesting that DMSO is the superior cryoprotectant in the cryopreservation of bovine oocytes.

It has been documented that when mouse and human oocytes are exposed to cryoprotectants during cryopreservation there is a rise in intracellular calcium, prematurely inducing the cortical granule reaction, subsequently hardening the zona pellucida. Morley and Whitfield reported that DMSO caused an intracellular calcium rise in various cell lines (Morley and Whitflield, 1993). Premature intracellular calcium rise has not been studied in bovine oocytes and should be an important area of interest in research going forward. Utilizing fluorescence microscopy to evaluate calcium fluctuations as a result of oocyte exposure to cryoprotectants will provide insightful information as to which cryoprotectants have the most significant impact on zona pellucida hardening. In a study conducted by Hardin and colleagues, deleterious trends from cryoprotectants used in vitrification protocols had negative effects on the oocytes ability to repair the metaphase spindles and other organelle disruptions after cryopreservation (Hardin et al., 2016). In 1977, Fahy and Karow showed that damage after freezing and thawing in certain cases is actually correlated with the concentration of cryoprotectants (Fahy and Karow, 1977). Since there is not a universal cryopreservation protocol

for vitrifying bovine oocytes, using an alternate protocol with different concentrations of cryoprotectants could produce different results.

In experiment two, we did not find significance between the three treatment groups (P=0.3437). There was no statistical difference in any of the comparisons made: DMSO versus glycerol (P=0.5301); DMSO versus control (P=0.1699); glycerol versus control (P=0.3818); or DMSO and glycerol versus control (P=0.2004). These results suggest that the cryoinjury due to vitrification caused too great of a variability in the zona pellucida digestion time in order for the effect of cryoprotectant to be efficiently measured. Our hypotheses could neither be accepted nor rejected upon analysis of this data from this experiment. Although we did not find significance, there is a trend that both DMSO and glycerol cause an increase in zona pellucida digestion time compared to PBS.

Damage to oocytes during vitrification has been well documented in many studies, which can include premature cortical granule release, zona pellucida hardening, depolymerization of the microtubules, and misalignment of the chromosomes. Previous experiments have shown that vitrification has negative effects on spindle morphology and other organelle morphology in mature oocytes (Purohit, 2012) and could possibly be affecting the mature oocytes after vitrification in this experiment. Most importantly, the mechanism of meiotic spindle regeneration after oocyte cryopreservation is essentially unknown (Ma, 2010). Al-Hasani and Gook demonstrated that cryopreservation induced zona pellucida hardening and premature release of the cortical granule content (Al-Hasani, 1987; Gook, 1993). Any and all of these cryoinjuries could have had an effect on the high variability of zona digestion encountered in experiment two.

In both experiments, the dilution solution, or warming solution, consisted of PBS, bovine calf serum, and sucrose. Warming can be defined as the conversion of a glass to a highly viscous

supercooled liquid. Studies conducted by several research teams showed that large macromolecules such as calf serum are responsible for excessive lipid accumulation through increased lipid uptake from the medium and/or through disturbance of mitochondria metabolism, which increases oocyte cryosensitivity. (Leroy et al., 2005; Abe et al., 2002). Physical changes of lipids submitted to freezing temperatures are among the major causes of cellular cryodamage (Pereira and Marques, 2008). However, Shinsuke and Mazur propose that the degree of cryoinjury or survival of the oocyte is almost entirely dependent on the warming rate: the faster the warming rate, the higher the survival (Shinsuke and Mazur, 2009). If any ice crystallization occurs during freezing, oocytes will possibly have already been irreversibly injured, and could render the thawing process ineffective. This also could be a potential explanation as to why we encountered such high variability in zona pellucida digestion times among the cryoprotectants.

The structure of DMSO could also have affected the zona pellucida digestion time after vitrification. Dimethyl sulfoxide's structure is quite different that glycerol in that it contains a sulfoxide group. Zona proteins 3 of the zona pellucida contains disulfide bridges (Mitchell, 2014). In theory, the sulfoxide group of DMSO could form disulfide links with ZP3 producing intermolecular bonds. If this is happening during vitrification, this could be affecting zona pellucida hardening, possibly changing zona pellucida permeability or structure. This also offers an explanation as to why there was an increase in zona pellucida digestion time after vitrification in DMSO.

When comparing the mean zona pellucida digestion times of each treatment between experiment 1 and experiment 2 (Figure 3.2), we observed a significant increase in DMSO. In experiment 1, the mean digestion time was 16.79 minutes and in experiment 2 was 22.78 minutes. DMSO was the only treatment group that showed an increase in digestion times,

suggesting that vitrification caused the cortical granule reaction, subsequently, hardening of the zona pellucida. In glycerol and PBS, a slight decrease was observed in mean digestion time: 20.94 minutes to 19.56 minutes and 15.2 minutes to 14.94 minutes, respectively. The decrease in mean digestion time after vitrification suggests that there was no cortical granule reaction, and therefore, the oocytes were not viable after vitrification. The high variability encountered in experiment 2 is likely due to the variability of survival. Some oocytes had the cortical granule and some did not.

Overall, from the first experiment, we can conclude that glycerol should not be used as a cryoprotectant for vitrification in bovine oocytes. Although we did not find significance, we observed a trend that DMSO possibly causes zona pellucida hardening. Even though cryoinjury caused high variability in our attempts to measure zona pellucida hardening after vitrification, we believe our results show that the cryoprotectants used in vitrification bears further investigation regarding zona pellucida hardening.

CHAPTER IV
SUMMARY AND CONCLUSION

SUMMARY

Oocyte cryopreservation is an attractive option to a number of industries including preservation of valuable cell lines, human infertility, livestock production and endangered species. All of these areas depend on the successful storage and subsequent viability of cryopreserved oocytes. To ensure high levels of cell viability post thaw, cryoprotectants are used to lower freezing temperature, allowing for greater dehydration of cells to prevent intracellular ice formation (Watson, 1995). Especially in vitrification, the ultra-fast freezing method that transforms liquid into a glass-like structure instead of forming ice crystals, high concentrations of cryoprotectants are needed to circumvent the rapid decrease in cooling temperatures, which can be toxic to oocytes. Therefore, cryoprotectant toxicity can be seen as the most limiting factor for the success of cryopreservation by vitrification (Fahy, 2009). The most pertinent cryoinjury in bovine oocytes from the cryoprotectants in vitrification is zona hardening. Zona hardening is brought about by the fusion of cortical granules to the plasma membrane and the release of their contents into the zona pellucida layers (exocytosis) (Sun, 2003). The membrane fusion event is calcium-dependent and is normally triggered by the increase in intracellular calcium initiated by sperm-egg fusion (Kline and Kline, 1992). However, it has been documented that oocyte and/or embryo exposure to cryoprotectants during cryopreservation causes a rise in intracellular calcium, prematurely inducing the cortical granule reaction. 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). Since intracytoplasmic sperm injection is largely unsuccessful in the bovine species, evaluating zona

pellucida hardening in mature bovine oocytes could further our insight on determining an optimal cryoprotectant and concentration for the vitrification of bovine oocytes.

CONCLUSION

The data in the first experiment shows that glycerol hardens the zona pellucida significantly more than DMSO or the control. While we could not definitively determine if DMSO hardens the zona pellucida more than the control, we observed trends that with further research and increase in sample size, could support the hypothesis that DMSO hardens the zona pellucida more than the control. Even though our efforts were unsuccessful in further showing that cryoprotectants effects on zona pellucida hardening in experiment two, the results were useful in suggesting that the cryoinjury caused by vitrification could have more negative effects on oocytes than we fully comprehend. Many aspects of cryopreservation are still not fully understood, which prompts further imperative research into cryoprotectants, optimal protocols, and other factors affecting the complete restoration of structure and function in the mature bovine oocyte after thawing.

Future research is needed on the varying cryoprotectants and their effects on zona pellucida hardening in mature bovine oocytes. More specifically, on the cryoprotectant induced premature intracellular calcium rise. In human and mouse oocytes, exposure of oocytes to cryoprotectants prior to freezing causes a rise in intracellular calcium, therefore activating significant premature exocytosis of cortical granules, in turn stimulating zona hardening (Fuku, 1995). The premature cortical reaction could explain the poor efficiency of oocyte freezing because the oocyte has already begun its activation events and possible down regulation of cell cycle control proteins (Larman et al., 2006). In bovine oocytes, very minute amounts of research have been conducted regarding zona pellucida hardening and essentially none on intracellular

calcium rise. Because intracytoplasmic sperm injection is immensely problematic in bovine oocytes, preventing zona pellucida hardening would undoubtedly increase the efficacy of this technique.

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APPENDIX A
PROTOCOLS

Vitrification of Bovine Oocytes- Protocol A

1. Prepare equilibration and vitrification solutions before beginning (Appendix B).
2. Place 3-5 oocytes in 50 µL drop of equilibration solution (solution one) under mineral oil for 5 minutes, pipette oocytes up, down, and around the dish for continued time during the 5 minute interval.
3. After 5 minutes in the first solution, move oocytes to second solution (50 µL drop under mineral oil) for 30 seconds, pipetting them around the dish.
4. In the last 15 seconds of a total of 45 seconds in the second solution, remove oocytes from the second solution and place on the tip of a cryolock with minimal media.
5. Continue immediately to Warming Protocol A.

Vitrification of Bovine Oocytes- Protocol B

1. Prepare equilibration and vitrification solutions before beginning (Appendix B).
2. Place 3-5 oocytes in 50 µL drop equilibration solution (solution one) under mineral oil for 5 minutes, pipette oocytes up, down, and around the dish for continued time during the 5 minute interval.
3. After 5 minutes in the first solution, move oocytes to second solution (50 µL drop under mineral oil) for 30 seconds, pipetting them around the dish.
4. In the last 15 seconds of a total of 45 seconds in the second solution, remove oocytes from the second solution and place on the tip of a cryolock with minimal media.
5. After oocytes are placed on the tip of the cryolock and excess media removed, plunge cryolock into liquid nitrogen.

6. With previously cooled forceps, gently secure the cap, that is also previously cooled, onto the cryolock device under the liquid nitrogen.
7. Move all cryolock with caps into canister for storage.
8. Repeat until all oocytes have been vitrified.
9. Continue immediately to Warming Protocol B.

Warming of Bovine Oocytes- Protocol A

1. Plunge cryolock into dilution solution with oocytes facing upwards, gently moving cryolock to remove oocytes.
2. After 5 minutes in dilution solution, transfer oocytes to warmed HEPES-TALP wash medium.
3. Briefly pipette oocytes up and down in HEPES-TALP and transfer oocytes to protease solution.

Warming of Bovine Oocytes- Protocol B

1. Remove cap from cryolock under liquid nitrogen.
2. Immediately plunge cryolock into dilution solution with oocytes facing upwards, gently moving cryolock to remove oocytes.
3. After 5 minutes in dilution solution, transfer oocytes to warmed HEPES-TALP wash medium.
4. Briefly pipette oocytes up and down in HEPES-TALP and transfer oocytes to protease solution.

Protease Digestion of Zona Pellucida

1. After the HEPES-TALP wash, move oocytes to 50 μL drops of protease solution under mineral oil.
2. Oocytes remain in drop until complete zona digestion is observed.

APPENDIX B
MEDIA FORMULATIONS AND STOCK SOLUTIONS

Media Formulations:

HEPES-TALP Medium

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

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 µL
Na Pyruvate	Stock Solution	P-4562	100 µL
Glutamine	100x stock solution	G-8540	100 µL
EGF	1000x stock solution	E9644	1 µL
FSH (Folltropin)	1000x stock solution	Bioniche	10 µL

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	0.856 g

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	0.856 g

Vitrification Solution One for Glycerol

Vitrification Solution Two 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	0.856 g

PBS	2 mL
20% Calf Serum	1.0 mL
20% Ethylene Glycol	1.0 mL
20% Glycerol	1.0 mL
0.5 M Sucrose	0.856 g

Dilution Solution for Warming

PBS	8 mL
20% Calf Serum	2 mL
0.25 M Sucrose	0.856 g

Protease Solution for Zona Digestion

PBS	20 mL
0.1% Protease	0.02 g

Stock Solutions:

1. **Pen/ Strep**: Gibco 15140. Aliquot ~ 500 µL of new pen/strep solution into sterile centrifuge tubes. Store at -20°C until use.
2. **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.
3. **Hyaluronidase**: Sigma H-3506. Prepare a 1mg/mL stock 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.
4. **Protease**: Sigma P8811-1G. Dissolve 0.02 g of protease in 20 mL of PBS. 1 mL aliquot of new protease solution into sterile 1.5 mL centrifuge tubes. Store at -20°C until use.

VITA

Kaci Denise Rogers was born to William and Dawn Rogers in February, 1994. She has one sibling, Kali Dawn Rogers, who is a candidate for the degree of Elementary Education from Louisiana State University. Kaci attended Winnfield Senior High School in Winnfield, Louisiana, where she graduated in 2012.

Kaci attended Louisiana State University from 2012 to 2016. In May of 2016, she graduated from Louisiana State University with a Bachelor of Science in Animal, Dairy, and Poultry Science.

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