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VITRIFICATION AND DEHYDRATION FOR THE PRESERVATION OF GAMETES

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Medical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program of
Animal and Dairy Sciences

by
Allison E. Moisan
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December, 2006

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ACKNOWLEDGMENTS

I want to thank my graduate advisor Dr. Robert A. Godke and my graduate mentor Dr. Stanley P. Leibo for their support and guidance. I would also like to thank my committee members, Dr. John Lynn and Dr. Kenneth Bondioli for their patience, advice and knowledge throughout my graduate program. I thank Dr. Martha Gómez and Dr. Earl Pope for their help and expertise in establishing the feline IVP system used in my dissertation research.

I wish to express many thanks to the faculty and staff at both the Reproductive Biotechnology Center in Baton Rouge and the Audubon Center for Research of Endangered Species in New Orleans, especially fellow LSU graduate students, for all of their help and advice. Special thanks are expressed to Stella Sullivan, Heather Guttormsen and Karen Smith for their help in the collection of cat semen used in this research project.

I wish to thank Dr. Holly Hale-Danz from the Department of Biological Sciences at LSU for her help with the confocal microscope. I wish to thank the Soccolofski Microscopy Facility in the Department of Biological Sciences, Louisiana State University for the use of their laser scanning confocal and fluorescence microscopes as well as their image analysis system.

I acknowledge the financial support during my graduate program from Dr. Betsy Dresser, Director of the Audubon Center for Research of Endangered Species, the LSU Agricultural Experiment Station and the BEST (Biotechnology Education for Students and Teachers) Program at Louisiana State University Agricultural Center, obtained by Elizabeth Chamberlain an LSU undergraduate student.

I express my most sincere thanks to my loving husband Reynald Jean-Marc Moisan for his support and encouragement.

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ABSTRACT

Of the 36 species of felines in the world, all except the domestic cat are listed as endangered or threatened. To preserve the genetic diversity of felines and other species, genome resource banks have been established. Due to limited availability of germ cells for research, studies must use models to optimize the techniques before they are applied to endangered species. In this study, preservation of oocytes and spermatozoa was examined using the bovine as a model for felines. In the first series of experiments, bovine and feline oocytes were dehydrated, vitrified, warmed and cultured to assess their ability to undergo embryonic development using a choline-based medium (CJ2) for vitrification and warming solution preparation as well as the standard sodium based media. In the second series of experiments, feline spermatozoa were dehydrated using air- and freeze-drying as alternative methods to standard cryopreservation. Assessment was done by examining embryonic development after intracytoplasmic sperm injection (ICSI) and DNA integrity of the dehydrated spermatozoa using the comet assay. In the second series of experiments, bovine and feline oocytes behaved osmotically in response to increasingly concentrated solutions. However, vitrified-warmed bovine oocytes had significantly higher cleavage and blastocyst rates compared with their feline counterparts and development using CJ2 medium was similar to the standard media used for cattle but was detrimental to feline oocytes. In the third experiment, cleavage and blastocyst development of feline oocytes injected with cat spermatozoa preserved using air- and freeze-drying was observed. Also, exposure to the dehydration solution and vitrification did not induce DNA damage but the process of freeze-drying did have significantly higher levels compared with controls. Air-dried sperm did not decondense. In

conclusion, the use of bovine oocytes as a model for feline oocytes was successful. Both bovine and feline oocytes responded similarly to dehydration and vitrification, except when processed using CJ2 medium. Furthermore, feline spermatozoa can be preserved using dehydration as demonstrated by their ability to produce blastocysts. This study has encouraging results for germ cell preservation. However, the efficiency of these procedures must be improved before they can be used as alternative methods of preservation in endangered species.

CHAPTER 1
INTRODUCTION

1.1 General Introduction

1.1.1 Use of ART on Endangered Species

Human activities such as mining, deforestation, agriculture, over-hunting and introduction of foreign species are leading causes of increased extinction rates of numerous species of animals (Sunquist and Sunquist, 2002). The resulting habitat destruction has led to population fragmentation, which results in inbreeding and increased sensitivity to disease. In situ conservation of threatened and endangered species is undoubtedly the best way to overcome such problems. Unfortunately, uncooperative governments, local people and industry make this type of conservation unrealistic in most cases. Therefore, ex situ conservation has become an essential tool in the preservation of endangered species. The use of assisted reproductive techniques (ART) has become a valuable tool to help increase population size, to decrease species inbreeding and to re-introduce lost genetics into the gene pool (Holt and Pickard, 1999). These techniques involve a wide range of relatively noninvasive to extremely invasive methods, such as: artificial insemination (AI), embryo transfer (ET), in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), nuclear transfer (NT) and embryo, oocyte and sperm cryopreservation. Currently, the most widely used technique of assisted reproduction is artificial insemination (Comizzoli et al., 2000).

One factor that severely limits the use of most reproductive strategies is the lack of basic knowledge of the reproductive biology of the species in question. Although there have been innumerable studies of the reproductive biology of domestic species, fewer than 3% of mammalian species have been investigated at all and very little has been done on endangered species (Comizzoli et al., 2000). Species differ in their physiology, their anatomy and their sexual and social behavior. With respect to reproduction, there are differences in ovulation patterns, hormones, estrous cycles, corpus luteum function, pregnancy maintenance, embryonic diapause, site of fertilization, implantation, gestation length, as well as many other facets of reproduction (Holt and Pickard, 1999, Comizzoli et al., 2000).

In particular, the preservation of the germline is essential for the maintenance of a viable population. The standard methods used include sperm, oocyte or embryo cryopreservation. The problem with these methods of preservation is that they must be optimized for each species and access to their genetic material is very limited. The ability to apply these reproductive biotechnologies is hampered due to the large variations in the reproduction of the different species (Comizzoli et al., 2000; Holt et al., 2003).

1.1.2 Methods of Preserving the Germline of Various Species

Even though there is a pressing need for such reproductive techniques, relatively few species have been preserved other than domestic and laboratory animals. Techniques employed for oocyte and embryo preservation are standard equilibrium freezing and nonequilibrium freezing (such as vitrification). Techniques used for sperm preservation include standard cryopreservation or slow cooling, rapid cooling and ultra-rapid cooling or vitrification and most recently, dehydration. Oocytes of all species are sensitive to chilling injury making their cryopreservation very difficult (Vincent and Johnson, 1992; Leibo et al., 1996). Further complicating matters is the small surface-area to volume ratio of oocytes. This reduces the rate of water loss from the cell during dehydration, an important part of standard slow cooling cryopreservation. Dehydration is required to avoid intracellular ice formation (IIF) that may be detrimental to cell survival. Vitrification avoids IIF by rapidly changing the intracellular contents from a liquid to a solid “glass like” state (Rall and Fahy, 1985; for a review see Vajta and Kuwayama, 2006).

Other techniques that have been used to improve the efficiency of oocyte preservation are lipid removal, use of cytoskeleton stabilizers and microinjections of trehalose (Liebermann et al., 2002). Although there have been many studies to improve the efficiency of oocyte preservation, in many nondomestic species, it has not yet been achieved.

Sperm preservation has had significantly more success compared with the oocyte. Compared to the oocyte, sperm have very large surface-area to volume ratio which allows for more rapid dehydration during cryopreservation. The first successful sperm preservation was reported in 1949 (Polge et al., 1949) whereas, oocyte cryopreservation was not successful until 1972 (Whittingham et al., 1972). Semen collection and cryopreservation in cattle is a multimillion dollar industry where preserved sperm are routinely used for artificial insemination. Sperm at various stages of maturation have also been successfully cryopreserved in 50 species (Wirtu, 2004). However, it was not until 1992 that sperm from many strains of inbred mice were successfully cryopreserved using raffinose (Nakagata and Takeshiuma, 1992) and many other strains still do not survive using this technique. Vitrification of human sperm has been recently attempted with and without cryoprotectants to overcome this problem (Nawroth et al., 2002).

Another alternative method used to preserve sperm cells is dehydration. There are three types of dehydration: convective or evaporative drying, freeze-drying and air-drying. All of these types of dehydration render the sperm immotile and therefore unable to fertilize oocytes normally. It was not until the mid 1970s, that Uehera and Yanagimachi (1976) first reported attempting to inject a single sperm directly into hamster oocytes using ICSI. These injected sperm developed into pronuclei, a normal stage in fertilization. Only then was fertilization using dehydrated sperm considered a possibility. Freeze-dried sperm of three species have been used to produce live young of mice (Wakayama and Yanagimachi, 1998), rabbits (Liu et al., 2004) and rats (Hirabayashi et al., 2005). However, no studies have been successfully completed on endangered or exotic species.

1.1.3 Use of Domestic Species as Models for Endangered Species

The limited availability of germ cells from endangered species makes direct research impractical in most instances (Holt and Pickard, 1999). This is a major problem in the advancement of ART in nondomestic species. Due to this limited access, studies using closely related nonendangered species can be done to optimize techniques. Many studies have been completed using domestic species as a model for their endangered counterparts, such as felines, canids, camelids, deer and cattle (for a review see Comizzoli et al., 2000). Models using unrelated species with similar physiological characteristics have also been used. For example, ram sperm cryopreservation protocols have been applied to endangered deer species (Chemineau et al, 1991). Due to limited number of animals available as recipients for embryo transfer, interspecies transfers have been used as a successful tool for endangered species such as the gaur, horse, mouflon and Desert cat (for a review see Comizzoli et al., 2000).

Another limiting factor to the direct study of endangered species is the regulation on animal acquisition and study. To surmount this problem, investigators performing nuclear transfer have used donor cytoplasts to produce cloned offspring of a different species such as the gaur. However, these alternative methods must consider the physiological similarities between species to find the best substitute. One example of such a study is the use of bovine oocytes to test the fertilizability of cryopreserved oryx sperm and other Bos species by IVF (Roth et al., 1998).

As previously mentioned, many mammalian oocytes have large lipid stores, which give them a dark appearance. In mammals, the major lipid storage consists of saturated and monosaturated fatty acids (Gurr and Harwood, 1991 as cited in Kim et al., 2001). In oocytes, lipids are also used as a type of energy source, they are part of the cytoplasmic membrane and organelles and can adjust physiological functions (Kim et al., 2001, Fujihira et al., 2004). Lipid content varies from species to species and also varies with level of maturity. Differences of color of the cytoplasm have been correlated to the amount of lipids within the cytoplasm (dark color and opaqueness of cytoplasm), although other factors such as pigments may also be involved (Fujihira et al., 2004). Analysis of the total level of lipid content in oocytes has been done for various domestic species such as sheep (McEvoy et al., 2000), pigs (Kim et al., 2001) and cattle (Genicot et al., 2005).

Furthermore, the lipid content has been reported to determine chilling sensitivity during cryopreservation (Dobrinsky, 1996; Otoi et al., 1997). The decrease in lipid content observed in frozen-thawed bovine embryos may be a factor in the decreased survival and developmental rates observed although further study of the membranes is required (Kim et al., 2001). Low cryotolerance has been linked to high lipid content and it may also increase the sensitivity of the organelles to low temperatures by altering the composition of their surrounding membranes (Otoi et al., 1997). Lipids are also thought to interact with the oocyte cytoskeleton and these interactions are irreversibly affected during the cryopreservation (Fujihira et al., 2004). In cattle, total lipid content decreases during the maturation process suggesting a vital role in developmental competence of the oocytes (Kim et al., 2001). Decreases in the level of lipids after cryopreservation or vitrification may be due to breaks in the cytoplasmic membranes or damage to the cytoplasm (Fujihira et al., 2004).

1.1.4 Feline Reproduction

In the last few decades, the understanding of feline biology has been greatly improved by the use of captive individuals in zoos and other collections. There are 36 species of cats in the world and almost all are considered either threatened or endangered by the Conservation on the International Trade of Endangered Species of Wild Fauna and Flora (CITES). This global treaty to protect all species of life was established in 1975. Wild felines are found on all continents except Australia and Antartica. Cats come in a large range of body sizes, from the smallest cat weighing only 1.0 kg (Black-Footed cat) to the largest weighing 320 kg (tiger) (Sunquist and Sunquist 2002). Fortunately, they all have very similar anatomy, which makes comparative studies more practical. In general, most studies done in felines have used the domestic cat as the model for their endangered relatives. To date, embryos have been cryopreserved in the domestic cat (Pope et al., 1994; Gómez et al., 2003) as well as in a few endangered felines, such as the tiger (Crichton et al., 2003), the African Wildcat (Pope et al., 2000) and the ocelot (Swanson et al., 2000).

Oocyte cryopreservation is currently limited to the domestic cat. In contrast, sperm cryopreservation has been the most studied compared with oocyte and embryo cryopreservation. Various studies have successfully cryopreserved sperm from domestic felids (Platz et al., 1978, Pope et al., 1991, Hay and Goodrowe, 1993; Lengwinat and Blottner, 1994; Stachecki et al., 1994; Tsutsui et al., 2000; Zambelli et al., 2002) as well as some endangered species (Byers et al., 1989, Donoghue et al, 1992, Swanson et al., 1996a,b; Nelson et al., 1999, Bartels et al., 2000). The big problem associated with sperm preservation in felids is the high incidence of teratospermia, which is highest in the cheetah with 75% abnormal sperm (for a review see Luvoni et al., 2003). Vitrification or dehydration may be valuable alternatives in these species to overcome the problems associated with in vitro fertilization and standard cryopreservation.

1.2 Review of Literature

1.2.1 The Effect of Volumetric Changes of Bovine Oocytes Dehydrated with Two Disaccharides on the Cytoskeleton and Mitotic Spindle

Numerous studies have been done to determine the effect of water movement in living cells exposed to anisotonic solutions. Membrane permeability is a fundamental property of cells, which is characteristic of each cell type (Leibo, 1980). It has been demonstrated that osmotic stress on oocytes is detrimental to their developmental potential (Oda et al, 1992; Agca et al., 2000). The effects of osmotic shock on cells were initially conducted on various somatic cell types such as erythrocytes and on eggs of lower organisms such as sea urchins, amphibians and fish. Since then, the osmotic behavior of different stages of mammalian oocytes and embryos has been examined. Although there has been quite a lot of interest in the permeability of water through the cell membranes of oocytes and embryos of different species, most of the studies have been done in the mouse (Leibo, 1980; Oda et al., 1992; McWilliams et al., 1995; Pedro et al., 1997).

Osmotic behavior of oocytes has also been examined in a few other domestic species including the goat (Le Gal et al., 1994), the human (Hunter et al., 1992) and the cow (Ruffing et al., 1993; Agca et al., 1998, 2000). One study examined the effect of osmotic stress on immature and mature bovine oocytes on fertilization and embryonic development but they used sodium chloride to create the anisotonic solutions (Agca et al., 2000). They reported a significant decrease in developmental rates in all oocytes exposed to hypertonic solutions. However, sodium chloride has been reported to have a detrimental effect on cells in high concentrations due to the increased electrolyte concentration, which causes the destabilization of the plasma membrane (Lovelock, 1954; Stachecki et al., 1998b; Acker and McGann, 2003). Although Myers et al. (1987) examined the osmotic behavior of immature bovine oocytes using sucrose instead of sodium chloride, there has been no report of mature oocyte dehydration using a saccharide.

Unfertilized mouse oocytes and zygotes behave as perfect osmometers (Leibo, 1980). This was determined by placing the cells into increasing hypertonic solutions and then measuring their volume at equilibration. The cell volume relative to the isotonic volume is plotted as a linear function of the reciprocal of the solution's osmolality. This is referred to as a Boyle van't Hoff plot. Extrapolations of the Boyle van't Hoff plots to an infinitely high concentrated solution indicated a nonosmotic volume of 18% for mouse zygotes (Leibo, 1980). This nonosmotic volume was confirmed by other studies, such as 17% for immature bovine oocytes (Myers et al., 1987) and 21% for hamster oocytes (Shabana and McGrath, 1988). This type of plot has also been constructed for various cell types and it has been found that all cells, with the exception of sperm cells and erythrocytes, have a nonosmotic volume of approximately 20%.

Membrane permeability as well as nucleation temperature are used to create thermodynamic models which can be used to optimize equilibrium freezing protocols (Mazur, 1963). Vitrification, a nonequilibrium freezing protocol, employs high concentrations of cryoprotective additives (CPAs). Therefore step-wise addition is used to minimize damage to the cell caused by fluxes in cell volumes (Vajta and Kuwayama, 2006). Cell expansion upon warming and CPA removal is another cause of membrane damage (Hotamisligil et al., 1996). Therefore, ways to minimize the effects of osmotic stress on oocytes must be examined to determine the optimal method of cryopreservation.

The dehydration of oocytes that occurs during cryopreservation is essential to their survival. Due to their low surface to volume ratio, cryopreservation of mammalian oocytes has had low success, with the exception of the mouse (Stachecki and Willadsen, 2000; Parks and Ruffing, 1992). There are two major mechanisms of cryoinjury, intracellular ice formation and solution effects (Mazur et al., 1972). Intracellular ice is formed during rapid cooling when there is not enough time for water to exit the cell. Therefore, large ice crystals form within the intracellular compartments. These crystals will cause breaks in the cell membranes and can also damage cell organelles. If the oocyte is allowed to dehydrate before intracellular nucleation, intracellular ice crystals do form but they are much smaller whereas, the bulk of the ice crystal formation is extracellular and therefore does not cause significant damage to the cell.

During standard cell cryopreservation, CPAs such as glycerol, dimethylsulfoxide (DMSO) and ethylene glycol (EG) are added to the medium. The oocyte can be exposed to the CPAs as a single step or as a multiple step addition. Both permeating and nonpermeating CPAs are used to cause a shift in the isotonic state between the intracellular and extracellular spaces of the cell that causes water to flow out of the cell. Permeating CPAs can produce large cell volume changes during both the freezing and thawing processes. During freezing, the extracellular space will become hypertonic due to the increase in solutes compared with that in the intracellular spaces. Therefore, water leaves the cell resulting in cell shrinkage (Mazur and Schneider, 1986). During thawing, a rapid influx of water into the cell due to the higher intracellular solute concentration, before the permeating CPA can be removed, can result in osmotic shock and cell lysis (Mazur and Schneider, 1986). The water enters the cell faster than the solute can exit therefore increasing the cell volume beyond its lytic volume. Protocols that require minimal cell volume excursions should be used to reduce cellular membrane damage.

In addition to physical damages incurred by volume changes, a toxic solute effect on the cellular membrane and other organelles may be caused by the high concentrations of solutes surrounding the cell as well as the permeating CPAs entering the cell during dehydration. Different CPAs offer different levels of protection during the freezing and thawing process depending on the relative permeability of the particular cell type.

Therefore, cryopreservation protocols have been devised to minimize intracellular ice formation, cell volume excursions, as well as to decrease the time the cells are exposed to high concentrations of solutes in the freezing medium.

Contraction of cell membranes during dehydration around the nucleus causes re-arrangement of organelles including the cytoskeleton and the meiotic spindle. The abruptness and extent of this disruption may be a contributing factor to the decreased survival of cryopreserved oocytes. Most mammalian oocytes are cryopreserved at the metaphase-II (MII) stage. At this stage, the chromosomes are attached to the microtubules at their centrosomes and are pulled into the equatorial region of a barrel-shaped structure before cell division. If the chromosomes are not properly aligned, there is unequal division resulting in aneploidy. During cellular division, the chromatin condenses and distinct chromosomes which then migrate to an equatorial position during prophase. After reaching this equatorial position during metaphase, the chromosomes divide through metaphase-anaphase and then cellular division follows during telophase (cytokinesis) (for a review see Johnson and Everitt, 2000).

Depolymerization of tubulin has been related to the decreased fertilization and developmental rates in many species. Factors known to lead to the disruption of the cytoskeleton and microtubule structure of the metaphase spindle in oocytes are temperature and chemicals used during cryopreservation. The effect of temperature on spindle morphology has been studied in the mouse (Magistrini and Szollosi, 1980; Pickering and Johnson, 1987), human (Pickering et al., 1990) and in cattle (Aman and Parks, 1994). Oocytes of some species, such as the pig, are more sensitive to chilling injury than others. In the pig, removal of some of the cytoplasmic lipids is required for survival post-cryopreservation. In the mouse, after 60 minutes at 0ºC, MII-stage oocytes exhibited complete spindle disassembly (Magistrini and Szollosi, 1980). In the cow, cooling oocytes to 4ºC for 20 minutes resulted in complete disorganization of the meiotic spindle and those maintained at room temperature for 30 minutes had disrupted or abnormal spindle morphologies (Aman and Parks, 1994). In another study, Wu et al. (1999) also observed spindle disassembly and reduced cleavage rates after fertilization in oocytes chilled to 4ºC for 10 minutes but they did not observe any difference in oocytes held at room temperature compared with control oocytes.

The effect on embryonic development of bovine oocytes exposed to room temperature was also examined by Martino et al. (1996) and they observed no difference in cleavage and blastocyst rates compared to control oocytes. Extended exposure of oocytes to room temperature can also cause microtubule disruption. Pickering et al. (1990) observed that human oocytes also exhibited tubulin disassembly if maintained at room temperature for only 30 minutes. Therefore, human oocytes are more sensitive to cooling than bovine and mouse oocytes. This variability in response to different temperatures may explain the need to devise cryopreservation protocols for each species specifically. However, this disruption may or may not be reversible depending on the amount of damage incurred and the species involved. In some species, rewarming of the oocyte, such as in the mouse and to a lesser extent in the human, can result in the reorganization of the meiotic spindle. This ability to reverse the damaging effects of cooling on the cytoskeleton has been reported not to occur in the bovine oocyte (Aman and Parks, 1994). Actin microfilaments are not as sensitive to cooling compared with microtubules, but they can be disrupted by exposure to various CPAs.

Chemicals used during cryopreservation, such as CPAs, have also been shown to have detrimental effects on both the actin and tubulin configurations of oocytes. Propanediol (PrOH) can induce depolymerization in rabbit oocytes and DMSO will do the same in the mouse whereas, DMSO has little detrimental effect on the rabbit cytoskeleton (Vincent et al., 1989). Only a few studies have examined the effects of different CPA exposure to mature bovine oocytes. Saunders and Parks (1999) examined the effects of ethanediol on bovine oocytes. They observed abnormal actin distribution and depolymerization of tubulin in the majority of the treated oocytes. Mixtures of two low concentrations of cryoprotectants, which add up to a high total concentration, have also been shown in some cases to have less of a disruptive effect than the same total concentration using a single CPA (Liebermann et al., 2002). Besides temperature and chemical damage, cryopreservation is also a major contributor to cytoskeletal damage.

1.2.2 Blastocyst Formation from Vitrified Bovine Oocytes, Zygotes and 2-Cell Embryos

One common problem in oocyte cryopreservation is their large, spherical size. Oocytes have a smaller surface area compared with their large internal volume which may contribute to a decrease in their rate of dehydration, an essential step in limiting the formation of intracellular ice. Therefore, either longer exposures to CPAs is required for slow cooling rates to allow for better dehydration or higher concentrations of CPAs compared with embryo protocols are required for rapid cooling protocols to increase the speed of dehydration. Longer exposure of oocytes to CPAs has been thought to be toxic and a major cause of poor survival. The detrimental effects of the increased concentration of CPAs used in vitrification are reduced due to the short time that oocytes are exposed to them during the vitrification.

The degree of oocyte maturation, from the germinal vesicle (GV) stage to the MII stage, has also been shown to have an effect on survival post-vitrification in some species (Parks and Ruffing, 1992). At the MII stage, the DNA of the oocyte is condensed into chromosomes that are aligned along the equatorial region of the metaphase spindle. This stage is thought to be more susceptible to disruption than the GV-stage or the developing embryo in which the DNA is decondensed chromatin of interphase. However, in cattle, GV-stage and MII-stage oocytes behaved similarly to cooling to 10º or 0º for ~30 minutes (Martino et al, 1996). Both stages of oocytes exhibited a decrease in cleavage and blastocyst rates after chilling. Also, during cooling the disruption of the meiotic spindle can cause the chromosomes to separate from the spindle resulting in disjunction and aneuploidy in the fertilized embryo. Only 31% of the frozen-thawed mature bovine oocytes (Saunders and Parks, 1999) and 16% to 19% of human oocytes (Boiso et al., 2002) had normal chromosome arrangements after cooling.

Species differences introduce other factors that can decrease their survival post cryopreservation, such as their cytoplasmic lipid content and chilling sensitivity. Damage to the oocytes can be caused by alterations in the physicochemical properties of the cytoplasmic lipids during the freeze-thaw process (Isachenko et al., 2001a). The exact cause of this damage in unknown but it may be due to the interaction of the lipids with the cytoskeleton. Upon hardening, the lipids may cause disruption in the arrangement of microtubule and microfilaments that make up the cytoskeleton (Isachenko et al., 2001a). Although cytoplasmic lipids are found in oocytes of many species, the type and amount of lipids vary tremendously. Nagashima et al. (1994) found that removal of these cytoplasmic lipids, referred to as delipation, from cleavage-stage porcine embryos increased survival and live young were produced. This process has also been used in porcine GV-stage oocytes with limited success (Park et al., 2005). Although the most extensive research has been done in mice and cattle, oocytes of several other species including human have been successfully preserved using both standard cryopreservation and vitrification.

Vitrification is a type of ultra-rapid cooling first developed by Rall and Fahy in 1985. Vitrification is a quick and simple technique compared with standard slow cooling or equilibrium freezing protocols which take at least 90 minutes to several hours to complete. It also is less expensive because it eliminates the use of programmable freezers (for a review see Liebermann et al., 2002; Shaw and Jones, 2003). Vitrification was first proposed by Luyet (1937) as a method to avoid the damaging crystallization of intracellular water by creating a vitreous state instead. Cooling rates of standard slow cooling are 0.2ºC to 20ºC/minute versus in vitrification, cooling rates exceed 20,000ºC/minute (Vajta et al., 1997). These rates are achieved by using very small volumes of vitrification solution, as little as 1 µl compared with 0.25 ml used in standard cryopreservation in straws (Martino et al, 1996). To achieve these small volumes, various holders or tools have been employed such as straws, open pulled straws (OPS), insemination pipettes, flexipet-denuding pipette (FDP), electron microscope copper grids, microdrops, solid surface metal blocks, nylon coils or mesh, cryoloops and most recently, CryoTops (for a review see Liebermann et al., 2002; Vajta and Kuwayama, 2006).

Besides the use of ultra-rapid cooling rates, high concentrations of CPAs are employed. The process of vitrification uses a 10 fold higher concentration of both permeating (e.g., glycerol, DMSO and EG) and nonpermeating (e.g., sucrose, glucose, trehalose) cryoprotectants. High concentrations of CPAs depress ice crystal formation (Liebermann et al., 2002). Due to the high concentrations of CPAs, besides rapid cooling rates, rapid warming rates are also important to avoid osmotic shock and toxic exposure to the cell. Methods used to minimize the toxic effects of CPAs are the following: Substitution of an amino group for a hydroxyl group to increase the ability of the solution to undergo vitrification, increasing the hydrostatic pressure of the solution to reduce the temperature at which nucleation occurs and reducing the amount of the CPA to its minimal concentration (Liebermann et al., 2002). The latter can be achieved either by using a nonpermeating CPA, such as a saccharide, or by using a combination of two CPAs, each of which will be at a lower concentration but the combined concentration will remain effective.

The first report of successful vitrification was done using mouse embryos (Rall and Fahy, 1985). It was not for another decade that this technique was first successfully used on bovine oocytes (Martino et al., 1996) and early embryos (Vajta et al., 1997). Human oocyte vitrification resulting in a live birth was only recently achieved (Kuleshova et al., 1999). To date, embryos of various species and at various stages of development have been vitrified in the mouse (Rall, 1987; Wood et al., 1993; Rall and Wood, 1994; Nakao et al., 1997; Uechi et al., 1999; Kito et al., 2003), rat (Han et al., 2003; Kono et al., 1988), rabbit (Silvestre et al., 2003; Cai et al., 2005), cat (Crichton et al., 2003), a mustelid (Piltti et al., 2004), pig (Dobrinsky, 1996; Gajda and Smorag, 2000; Berthelot et al., 2000; 2001; Gajda and Smorag, 2002; Esaki et al., 2004), sheep (Naitana et al., 1997; Papadopoulos et al., 2002; Dattena et al., 2004), goat (El-Gayar and Holtz, 2001; Begin et al., 2003), several bovid species (cattle: Massip et al., 1986; Rizos et al., 2003; buffalo: Hufana-Duran et al., 2004), llama (Aller et al., 2002), horse (Hochi et al., 1996; Moussa et al., 2005), a nonhuman primate (Yeoman et al., 2001) and the human (Katayama et al., 2003; Son et al., 2002; 2005).

There has been some success in oocyte vitrification with mammalian species. Oocytes of different maturational stages have successfully been vitrified in various species, such as the mouse (Nakagata, 1989; van der Elst et al., 1992; Wood et al., 1993; Hotamisligil et al., 1996; Isachenko and Nayudu, 1999; Chen et al., 2000; 2001; Lane and Gardner, 2001), hamster (Critser et al., 1986; Lewin et al., 1990; Wood et al., 1993; Lane et al., 1999b), pig (Park et al., 2005) cat (Murakami et al., 2004), sheep (Isachenko et al., 2001b), goat (Begin et al., 2003), a few bovid species (cattle: Vajta et al., 1998; Le Gal et al., 2000; Rho et al., 2002; Men et al., 2003a,b; Chian et al., 2004; Modina et al., 2004; buffalo: Wani et al., 2004), horse (Maclellan et al., 2002) and the human (Kuleshova et al., 1999; Wu et al., 2001; Kuwayama et al., 2005). Although there has been some success in preserving oocytes and embryos of domestic species, very few exotic or endangered species have been preserved using vitrification. This may be due to the limited genetic material available to determine efficient methods of vitrification in these species.

Various chemicals and solutions are used to prepare the vitrification medium. All media begin with a similar isotonic solution containing a buffer, usually either a phosphate-buffered saline or a HEPES-buffered medium (Liebermann et al., 2002). Both of these solutions allow for the manipulations to be done in air without experiencing a pH shift whereas, bicarbonate-buffered medium must be gassed to maintain a proper pH throughout the protocol. The next major component is the CPA used to confer protection during the cooling process. As previously mentioned, high concentrations of CPAs are used to create a viscous solution capable of making the liquid to solid transition without the formation of ice. The most common CPA used for vitrification procedures is EG. It appears to be less toxic to both oocytes and embryos and rapidly diffuses across the cell membranes (Emiliani et al, 2000). Other CPAs used include glycerol, PrOH and DMSO.

In addition, sugars such as sucrose, glucose, fructose, raffinose and most recently trehalose are often an important part of the vitrification solution. Sugars with high molecular weights like disaccharides do not permeate the cell and can therefore reduce the amount of cryoprotectant required for successful vitrification. However, the components and their concentrations used to make up the vitrification solution has varied (for a review see Ali and Shelton, 1993; Pedro et al., 2005). For instance, in some species DMSO is more toxic than EG or sucrose is more effective than trehalose.

Most of the current vitrification solutions contain sodium. Recent studies have demonstrated the detrimental effects of sodium on mouse oocytes (Stachecki et al., 1998a,b). It has been proposed that a sodium ion overload may occur during cooling because the sodium/hydrogen pump is impaired. Stachecki et al. (1998a,b) proposed the use of choline as a substitute cation for sodium and have reported significantly higher blastocyst rates of oocytes cryopreserved in the choline-substituted medium (CJ2) compared with those frozen in standard cryopreservation media. Choline is an organic osmolyte, which is thought to confer protection to cellular membranes during freezing (Stachecki et al, 2002). Unlike sodium, choline is thought not to cross the cell membrane and would therefore not disrupt the intracellular ion load. However, CJ2 medium has only been employed using conventional freezing methods and has not been attempted in conjunction with vitrification to date. Furthermore, mouse oocytes frozen in CJ2 medium are always fertilized after the zona has been artificially breached.

1.2.3 The Effect of Volumetric Changes of Feline Oocytes Dehydrated with Two Saccharides

As previously mentioned, numerous studies have been done to determine the effect of water movement in cells exposed to anisotonic solutions. Membrane permeability is a fundamental property of cells, which is specific for each cell type (Chamberlin and Strange, 1989; Critser et al., 1997). Previous studies have demonstrated that osmotic shock is lethal to cells and must be considered when designing a cryopreservation protocol (Oda et al, 1992; Agca et al., 2000). Osmotic shock occurs when a cell is rapidly diluted out of a hypertonic solution into an isotonic solution. The cell will expand larger than its original isotonic volume and this can lead to cell lysis. The physiological response of cells to anisotonic solutions is cell volume changes.

During cryopreservation, volume changes occur during exposure to the CPA, during freezing and during warming. If these changes are abrupt, cellular membranes may be damaged and cell lysis can occur. Various studies have examined the osmotic behavior of mammalian oocytes and embryos, which include the mouse (Leibo, 1980; Toner et al., 1991; Oda et al., 1992; Pedro et al., 1997), the sheep (Szell et al.1989), the goat (Le Gal et al., 1994), the cow (Ruffing et al., 1993; Agca et al., 2000), the monkey (Songsasen et al., 2002) and the human (Paynter et al., 2001, 2005; McWilliams et al., 1995). In these studies, the effect on fertilization and embryonic development of osmotic stress on immature and mature oocytes was examined. In the mouse, unfertilized mouse oocytes and zygotes were found to behave as ideal osmometers (Leibo, 1980). To determine a cells osmotic behavior, it is placed into increasingly hypertonic solutions and their volumes are measured at equilibrium. The results are plotted as a function of the exposed cell volume relative to the isotonic volume, as a linear function of the reciprocal of the solution's osmolality in a Boyle van't Hoff plot. Extrapolations of the linear regression line for a cell in an infinitely concentrated solution represents the nonosmotic volume of the cell. Nonosmotic volumes range from 18% in the mouse to 32% in cattle (Leibo, 1980 and Ruffing et al., 1993, respectively).

Besides the injury of volume excursions, oocytes must tolerate toxic levels of CPAs generally used in cryopreservation and vitrification protocols. Detrimental effects of CPAs depend on three factors, their concentration, the temperature at which the cells are exposed and the length of time to which the cells are held. Adding to the problem is that CPA toxicity is very speciesspecific. For example, rabbit oocytes exposed to PrOH and DMSO exhibit depolymerization of their microtubules and disruption of the actin microfilaments (PrOH only) (Vincent et al., 1989). In the mouse, PrOH and DMSO cause disruption of actin microfilaments and disorganization in the microtubules at low doses of PrOH (Johnson and Pickering, 1987; Vincent et al., 1990; Joly et al., 1992).

These effects are temperature dependant, as were those during cooling and the detrimental effects on the microtubules may be reversed. Also, exposure of bovine oocytes to EG at room temperature resulted in abnormal spindle morphology greater than with cooling alone (Saunders and Parks, 1999). There has been very little study of the effects of various CPAs on feline oocytes. One study evaluated the effects of PrOH and EG on mature cat oocytes and found that all concentrations used (except 1.5 M PrOH) caused disruption of the meiotic spindle (Comizzoli et al., 2004). The effects of DMSO and that of a combination of CPAs have not yet been examined in the cat. Therefore, the choice of CPAs must be examined for each species before an appropriate protocol can be established.

Dehydration can cause detrimental effects to the cell by altering the structure of the cytoskeleton and displacing the chromosomes. This effect is thought to be more severe in mature, MII-stage oocytes due to the structure of the meiotic spindle. If the spindle is disrupted, normal fertilization cannot occur because the chromosomes are dispersed. This results in disjunction during syngamy and the resulting embryos are aneuploid. As in the case of CPAs, severe dehydration may disrupt the actin microfilaments and the microtubule arrangement of the meiotic spindle. Besides the disruption of cytoskeletal features, cytoplasmic membranes are also affected by severe dehydration. There is a potential loss of some of the cytoplasmic membrane so that when the cell is returned to isotonic conditions, the cell can no longer expand to its original volume and lysis occurs. This loss is thought to result from the fusion of membranes (Wolfe and Bryant, 1999). This theory is, however, disputed as the hydrostatic forces of the lipid bilayers would make this impossible unless all water of hydration was removed (Mazur, 2004). Overall, many factors affect the survival of oocytes during the dehydration process of preservation using cryoprotectants.

1.2.4 Embryo Development of Vitrified Feline Oocytes with Two Different Diluents (CJ2 and M199 Media)

The first report of live offspring in domestic felines produced by ART used cryopreserved sperm in conjunction with artificial insemination (AI) (Platz et al., 1978). Since then, a few nondomestic feline species (cheetah, ocelot and the Leopard cat) have produced offspring after being surgically inseminated with cryopreserved sperm

(Swanson et al, 1996a; Howard et al, 1997). However, oocyte cryopreservation has been much less successful. Only two studies to date have reported in vitro development from either cryopreserved or vitrified-warmed domestic cat oocytes (Luvoni and Pellizzari, 2000; Murakami et al., 2004). There have been no reports of cryopreserved endangered feline oocytes to date.

The relatively low success of oocyte preservation may be due to their high lipid content and the inability of cryoprotectants to pass through the plasma membrane (Luvoni, 2000). However, cryopreservation of cleavage-stage embryos has had greater success in the domestic cat (Gómez et al., 2003) and also in a nondomestic species (tiger) (Crichton et al., 2003). To understand the low success in feline oocyte preservation, Comizzoli et al. (2004) assessed the effect of CPAs on feline oocytes. They observed that exposure to either ethylene glycol EG or PrOH causes a high incidence of abnormal spindle morphologies and decreased blastocyst development, with the exception of 1.5 M PrOH at 25ºC. Luvoni et al. (1997, 2000) also reported detrimental effects of CPAs and cryopreservation on feline oocytes.

Various components make up the vitrification solution used for oocyte preservation. First, there is the base medium which is generally composed of a buffered saline solution, such as TCM-199 or phosphate-buffered saline (PBS). These solutions contain high levels of sodium which is detrimental to embryonic development in mouse oocytes due to the increased cation load during dehydration and cooling process (Stachecki et al., 1998b). To reduce this detrimental effect, Stachecki et al. (1998a) have developed CJ2 in which the sodium is replaced by choline. A 40% increase in blastocyst development was observed compared with a sodium-based medium. This choline-based medium has been also successfully used to preserve human oocytes (Quintans et al., 2002).

Besides the base medium, vitrification solutions contain CPAs such as EG, PrOH, DMSO or propylene glycol (PG). In addition to the permeating CPAs, nonpermeating CPAs such as sucrose, glucose or trehalose have been used in the cryopreservation/ vitrification medium. In the cat, 40% EG and 0.3 M sucrose were used successfully to vitrify oocytes (Murakami et al., 2004). These authors used a two-step dilution beginning with 20% EG only followed by the vitrification solution described above. Two-step dilutions allow for the oocyte to dehydrate slowly avoiding osmotic shock due to the high concentrations of CPAs used. Luvoni et al. (1997) found no significant difference in the resumption of meiosis of cryopreserved feline oocytes using either DMSO or EG.

Various instruments have been used for oocyte cryopreservation. For cryopreservation, standard 0.25 ml straws are used and loaded into a controlled rate freezer. Different instruments have been used for vitrification, such as open pulled straws (OPS) (Vajta et al., 1998), electron microscope grids (Martino et al., 1996), cryoloops (Lane et al., 1999a) and CryoTops (Kuwayama et al., 2005). The last two devices have significantly improved the success of embryonic development after vitrification by decreasing the minimum volume required and subsequently increasing the cooling rates. For example, the OPS method makes use of a 1.5 µl volume whereas, the CryoTop method uses a <0.1 µl volume. Consequently, the cooling and warming rates increases from ~16,000ºC/minute to 23,000ºC/minute for cooling and 14,000ºC/minute to 42,000ºC/minute for warming (Kuwayama et al., 2005). In the cat, only straws have been used to date for oocyte vitrification (Murakami et al., 2004).

1.2.5 Blastocyst Development from Feline Oocytes Injected with Dehydrated Feline Spermatozoa

Natural dehydration of organisms was first described by Antoine van Leeuwenhoek in 1702 when he observed the rehydration of what he called "animalcules" (McGinnis et al., 2005). The technique of dehydration was first attempted by Polge et al. (1949) using fowl sperm. Briefly, they exposed the sperm to a 20% or 30% glycerol solution, cooled it to -79°C, then rewarmed it to -25°C, attached the sample to a freeze-dryer, rehydrated and warmed the sample to 40°C before they assessed motility. The authors reported a maximum of 50% motility but they did not assess fertility of the sperm. There have been a few reports of success in rabbits (Yushchenko, 1957 as stated in Wakayama and Yanagimachi, 1998) and in cattle (Larson and Graham, 1976) after AI with freeze-dried spermatozoa, but these studies could not be verified by others. These reports are considered suspect because the freeze-drying process renders the sperm immotile.

It was not until the advent of intracytoplasmic sperm injection (ICSI) that immotile, dried sperm was re-examined. In brief, a single spermatozoon is selected and injected directly into the cytoplasm of a mature oocyte. This bypasses the normal processes of fertilization, including the acrosome reaction and membrane fusion. These reactions are necessary to initiate pathways responsible for oocyte activation. Activation is required for the oocyte to resume meiosis and allow for the extrusion of the second polar body. Uehera and Yanagimachi (1976) reported the first successful production of embryos by injecting hamster sperm into a mature hamster oocyte. They observed male pronuclear formation within the hamster oocytes and embryos. It was not until decades later that the first accepted report of live young was produced with freeze-dried sperm using ICSI in mice by Wakayama and Yanagimachi (1998). Since this first report, there have been a handful of studies involving the use of freeze-dried or dried sperm. Most of the studies to date have been done in the mouse (e.g., Kusakabe et al., 2001; Pangestu et al., 2000; Bhowmick et al., 2003; Kaneko et al., 2003a,b; Ward et al., 2003). Live young have also been produced using freeze-dried sperm in a few other species, such as the rabbit (Liu et al., 2004) and the rat (Hirabayashi et al., 2005). Embryo production has also been accomplished in the cow (Keskintepe et al., 2002) and the pig (Kwon et al., 2004) using freeze-dried sperm.

There main types of sperm dehydration are convective or evaporative drying, air-drying and freeze-drying. The first employs a very simple procedure of drying a sample at room temperature while freeze-drying involves a three-step process including freezing, sublimation or primary drying and diffusion/desorption or secondary drying (Acker et al, 2004). In convective or evaporative drying, the sample is placed onto a surface across which an inert gas such as nitrogen is passed (forced convection), or it is left out in the open (passive convection). In both cases, as long as the vapor pressure of the surrounding environment is less than that at the surface of the sample, water will evaporate thereby drying the sample (Acker et al., 2004).

Although natural convection is a simple technique, it has a much slower drying rate compared with other drying techniques because it is dependant upon the relative humidity level of the sample's environment. The major disadvantage of this type of drying is that the final moisture level is unknown and there is always humidity left in both open and closed environments. Forced convection can provide more rapid drying rates than natural convection by blowing desiccated air or a gas over the sample. The rate of drying depends on the temperature, velocity of gas and the shape and size of the container holding the sample (for a review see Acker et al., 2004). Because of the continuous flow over the sample, a lower level of dehydration can occur compared with natural convection, as the moisture released from the sample is immediately removed from the environment.

Air-drying is the simplest method of dehydration. It involves the simple evaporation of the sperm sample into the air. The rate of evaporation will vary depending on the temperature and the relative humidity of the environment to which the sample is exposed. To increase the evaporation rates, the temperature of the sample must be raised and the humidity of the room be decreased. With this method of drying, these variables are much more difficult to control compared with convective or freeze-drying.

Freeze-drying involves the freezing of the sample to separate the unbound water from the cells (~80% of water in the original solution) in the form of ice. This is called the primary drying phase whereby the water is removed by sublimation. Usually the sample is kept below 0ºC and at a high vacuum (<100 mbar). This drying occurs until the sample undergoes the glass phase transition at which point secondary drying will occur. The temperature of the container is then raised above the glass transition point to allow for the removal of the bound water (20% water of hydration), during the second phase of drying. This phase allows the remaining water to be removed by desorption (for a review see Acker et al., 2004). This last step is very sensitive to permutations. The temperature must be raised in a controlled manner and not exceed the critical temperature at which the sample looses it ability to rehydrate properly. The resulting product is a lyophilized sample that can be stored at room temperature or refrigerated.

There are several advantages and disadvantages of preserving sperm using the different types of dehydration. Evaporative or convective drying has the advantage of being simple and requires less expensive instruments than does freeze-drying and all steps can be performed at room temperature. The main disadvantage of this method of dehydration is unequal drying rates for the front and end of the container. In order to minimize this effect, the flow rate can be increased and the container size decreased. With freeze-drying, the removal of the water of hydration by desorption enables further dehydration compared with evaporative or convective drying. The rate of drying is dependant on the sample temperature and the vacuum pressure produced by the freeze-drying apparatus. The major problem with this type of drying it that it is very time-consuming as drying may take from several hours to a few days to complete. Although this technique does require the use of liquid nitrogen, it is still more efficient than standard cryopreservation because the dried samples can be stored at room temperature or refrigerated.

Since the first successful report of sperm dehydration, various studies have been reported using different methods of drying. Although simple methods of sperm desiccation like convective (Bhowmick et al., 2003) and evaporative drying (Pangestu et al., 2000) have been studied, the most common method of dehydration remains freeze-drying (Wakayama and Yanagimachi, 1998; Kusakabe et al., 2001; Keskintepe et al., 2002; Bhowmick et al., 2003; Kaneko et al., 2003a,b; Ward et al., 2003; Kusakabe and

Kamiguchi, 2004; Kwon et al., 2004; Liu et al., 2004; Hirabayashi et al., 2005). In general, a 100 μl sperm suspension is transferred to a glass ampule or microcentrifuge tube, which is immediately plunged into LN_2 for 20 to 30 seconds up to 10 minutes. The ampule is then connected to a freeze-dryer for 4 (Kusakabe et al., 2001) to 18 hours (Wakayama and Yanagimachi, 1998; Keskintepe et al., 2002) and then sealed. The inlet pressure reported varied tremendously for each study (Inlet pressure = $32 – 40 \times 10^{-3}$ mbar; 1 to 30 mbar; 190×10^{3} mbar) and may depend solely on the manufacturer's specifications of the freeze-drying apparatus used. In one case, the value reported is much higher than all the other studies (190×10^{-3} mbar). Besides the different methods of drying used, several species have been dried with varied results.

As previously mentioned, production of live young using dehydrated sperm has only been done in three species, the mouse, rabbit and the rat. In other species, various levels of embryonic development have been observed. Blastocyst development has been reported in two species. In the cow, Keskintepe et al. (2002) obtained a 30% blastocyst rate and in the pig, Kwon et al. (2004) had slightly lower blastocyst development (10%). There has been no report of feline sperm dehydration to date.

Dehydration of spermatozoa causes the loss of motility in all species examined. Because of this, in vitro fertilization is not possible and therefore, it was not until the development of ICSI that embryo production was possible (Wakayama and Yanagimachi, 1998). In mice, the sperm heads are removed from the tails before injection because the microtubules needed for pronuclear formation are provided by the oocytes and not the mid-piece as in other species.

In mammals, oocyte activation is critical for fertilization to occur. Oocyte activation is the process by which a metabolically quiescent oocyte is converted into a metabolically active embryo, which can undergo DNA synthesis, RNA translation and cell division (Ozil and Huneau, 2001). Activation can be attained naturally, as in normal fertilization (in vivo or in vitro) or due to oocyte ageing. Alternatively, oocytes can be artificially activated by subjecting them to mechanical, chemical or electrical stimulation. In artificially activated oocytes, the normal sperm-oocyte interactions, such as sperm-zona binding, the acrosome reaction, plasma membrane binding, are bypassed. In this case, oocyte activation must be initiated by some other factor. However, the resulting cascade of events mimics that obtained by natural fertilization (for a review see Williams, 2002). In some species, mechanical damage to the oocyte caused by the injection is sufficient to induce activation. Other species, not properly activated by the mechanical injury of the injection, require chemical or electrical activation protocols.

Although much has been learned in the past decade about oocyte activation, the complete signaling pathway is not yet known. Natural activation begins with a trigger such as a spermatozoon binding to an oolema. This initiates a cascade of events involving the release of intracellular calcium stores and an increase in pH, which lead to the exocytosis of cortical granules within the ooplasm and the resumption of meiosis (for a review see Schultz and Kopf, 1995; Tesarik, 1998; Alberio et al., 2001; Williams, 2002). A few candidates as possible triggers are a tyrosine kinase receptor on the sperm membrane or a cytosolic sperm factor within the sperm head. During normal fertilization, a spermatozoon binds to the zona pellucida of an oocyte, undergoes an acrosome reaction and then binds to the oolema. The contents of the sperm head enter the ooplasm and activation occurs (Williams, 2002). Oocytes that have remained unfertilized for a prolonged period of time are referred to as “aged oocytes.” These oocytes can undergo spontaneous activation, or if fertilized, they will be activated and cleave. However, the resulting embryos exhibit fragmentation, apoptosis and poor embryonic development (Fissore et al., 2002).

Activation protocols are used in conjunction with several reproductive techniques, such as nuclear transfer, parthenogenesis and ICSI. The present study deals with the latter. All three types of oocyte activation, mechanical, chemical and electrical have been used in conjunction with ICSI. The most common type of mechanical activation is the damage that results during ICSI, but improper handling can also induce activation. Chemical activation is achieved by use of a variety of chemicals that promote the release of internal calcium stores, such as calcium ionophores, strontium chloride, phorbol esthers, ethanol and the best is ionomycin followed by 6-dimethylaminopurine (DMAP) (Alberio et al., 2001). Finally, an electrical direct current pulse can also induce oocyte activation (Ozil, 1990).

1.2.6 Assessment of DNA Integrity of Dehydrated Feline Spermatozoa with the Comet Assay

Freeze-drying or lyophilization is commonly used in the food and drug industries. It has also been used to preserve bacteria and other microbial organisms, but only recently has this technique been applied to mammalian cells and tissues (for a review see Crowe et al., 2004). Because one first must determine optimal freezing protocols, tissue preservation using this technique has not been as successful as cell drying. However, red blood cells and human fibroblast cells have been successfully freeze-dried (Goodrich et al., 1992; Puhlev et al., 2001). With the success of freeze-dried mouse sperm by

Wakayama and Yanagimachi (1998), dehydration of spermatozoa has been proposed as a cost effective means of preserving the many transgenic strains of mice produced for biomedical research (Kusakabe et al., 2001). This technique may provide an effective method of preserving spermatozoa of many inbred mouse strains, that with conventional freezing, exhibit poor post-thaw recovery.

Desiccation allows sperm to be stored at room temperature compared with cryopreserved samples that must be maintained at ultra-low temperatures. This type of storage would result in a drastic decrease of the cost of maintaining large storage facilities of liquid nitrogen tanks. Furthermore, this simple technique would also be more practical for field work where access to cooling units and liquid nitrogen is unavailable. Transport of dried material is also far less expensive and would require less special handling, allowing for easier movement of specimens between different locations.

Different media have been used to preserve cells during dehydration. In the original publication by Wakayama and Yanagimachi (1998), they used two types: CZB medium without EDTA and DMEM supplemented with 10% fetal bovine serum (FBS). Keskintepe et al. (2002) also used DMEM supplemented with 10% FBS but added glutamine, sodium pyruvate, nonessential amino acids and nucleosides. The most common medium used to protect mouse sperm during desiccation consists of a 10 mM Tris-HCl buffer containing 50 mM EGTA (ethylene glycol-bis [β-animoethyl ether]-N,N,N',N'-tetraacetic acid) in 50 mM NaCl with a high pH (8.0 - 8.4) (Kusakabe et al., 2001). They compared the effects of the CZB medium to the Tris-HCl buffer and found that the latter resulted in a higher percentage of karyologically normal embryos. High concentrations of a calcium-chelating agent, such as EGTA, are used routinely to help maintain chromosome integrity in DNA preparations from eukaryotic cells. This solution has subsequently been used in many studies of different species (Bhowmick et al., 2003; Kaneko et al., 2003a,b; Ward et al., 2003; Liu et al., 2004). Other liquids used for desiccation procedures include distilled water (Hoshi et al., 1994), ethanol, dithiothreitol (DTT), methanol, acetone, or a chloroform-methanol (2:1) mixture (Katayose et al., 1992).

The rehydration of samples reported has been the same for all studies. This was done by simply adding 100 µl (equal volume to that of original sample) of ultra-pure water to the freeze-dried sperm (Wakayama and Yanagimachi, 1998). An increase in the time of injection after rehydration was correlated with a decrease in the rates of activation and fertilization in mice (Wakayama and Yanagimachi, 1998).

The length of storage and the temperature at which the samples are stored has also been evaluated. The longest period reported is for rabbit spermatozoa, which have been stored for more than 2 years at ambient temperature (Liu et al., 2004). Storage time was shown not to have a deleterious effect on the genetic integrity of freeze-dried samples (Ward et al., 2003). However, various storage temperatures that have been studied are: 22 to 25°C, 4°C, -80°C or -196°C. By applying the theory of accelerated degradation kinetics on freeze-dried sperm, it was estimated that samples stored above -80°C for 10 or more years would result in a 0% blastocyst rate in mice (Kawase et al., 2005). However, in the short-term (<1 year), there were no significant differences if freeze-dried sperm were stored at 4°C or -80°C. Until samples have been stored for many years at various temperatures, the true effects on genetic integrity will not be known.

In addition to measuring embryonic development, several studies have examined the effect of sperm dehydration on DNA integrity or cell ploidy. It is well known that freeze-drying causes severe damage to the acrosome and plasma membranes. The deleterious effect of drying on sperm has been done by examination of chromosomal spreads of zygotes (Kaneko et al., 2003a,b; Ward et al., 2003; Kaneko and Nakagata, 2005) and most recently, by use of the comet assay (Kawase et al., 2005).

Briefly, chromosomal spreads are prepared to allow for the counting of the number of chromosomes in the zygotes resulting from oocytes injected with dried sperm. Because oocyte chromosomes seldom show chromosomal aberrations at the zygote-stage, any abnormal chromosomes are considered to be of paternal origin (Kaneko et al., 2003b). In the mouse, the percentage of abnormal sperm was not significantly different from controls (56% to 76%) when samples were stored at 4ºC for up to 5 months (Kaneko et al., 2003b; Kaneko and Nakagata, 2005). However, Ward et al., (2003) found a similar level but it was significantly lower than their controls. The number of chromosomal abnormalities observed in embryos produced by use of freeze-dried sperm was not significantly different from that resulting from ICSI alone (Kusakabe et al., 2001).

The comet assay (single-cell gel electrophoresis or microgel electrophoresis) is an assay used to detect the presence of unbound or fragmented DNA within an individual cell (for a review see Fairbairn et al., 1995; Olive, 2002; Collins, 2004). This technique uses an electrical current to pull the charged DNA from the nucleus. The relaxed or broken DNA strands migrate further resulting in a ‘comet-like’ shape after staining for which it is named. The amount of stain (or fragments) in the tail region is a measure of the amount of DNA damage within the cell. The comet assay was developed by Ostling and Johanson in 1984 to assess DNA damage incurred in somatic cells after irradiation (Fairbairn et al., 1995). Since then, hundreds of investigators have used the comet assay to assess damage in a variety of cell and tissue types (for a review see Fairbairn et al., 1995).

To determine the DNA damage in sperm, few modifications of the comet assay were required. Briefly, the chromosomes must be decondensed after being embedded in an agarose matrix and then the cells are lysed to allow for unbound DNA to migrate away from the sperm head when exposed to a low level electrical current (Kawase et al., 2005). In a study examining freeze-dried sperm, Kawase et al. (2005) observed the presence of comet tail in samples stored at 4ºC for several months but not when samples were fresh or freeze-dried samples were stored at -80ºC. Recently, the comet assay has been used in human fertility clinics as a predictor of male infertility (Morris et al., 2002). The biggest problem associated with this technique is the inability to directly compare results from different studies due to the numerous computer assisted analysis programs and varied protocols. DNA integrity is an important but sometimes forgotten factor that must be examined to ultimately determine the success of a desiccation protocol.

CHAPTER 2
THE EFFECT OF VOLUMETRIC CHANGES OF BOVINE OOCYTES DEHYDRATED WITH TWO DISACCHARIDES ON THE CYTOSKELETON AND MITOTIC SPINDLE

2.1 Introduction

For decades, various methods of mammalian oocyte cryopreservation have been studied but with much less success compared with that of sperm cryopreservation. This is mainly due to the low surface-area to volume ratio of oocytes. In oocytes, insufficient dehydration can result in the formation of ice crystals at subzero temperatures. Vitrification is a form of ultra-rapid cryopreservation that results in a glass-like solidification without ice crystal formation by exposing cells to high concentrations of cryoprotectants (Rall, 1987).

Cooling oocytes below 20°C has been reported to result in decreased fertilization rates and increased polyspermy due to the disorganization or disruption of the meiotic spindle (Magistrini and Szollosi, 1980; Pickering and Johnson, 1987; Pickering et al., 1990; Aman and Parks, 1994; Martino et al., 1996; Wu et al., 1999). After oocyte vitrification, partial or complete disruption of microtubules (Rho et al., 2002) and premature cortical granule exocytosis (Hytell et al., 2000) have been reported. What remains unclear is whether this disruption was caused by the vitrification method or by the exposure to high concentrations of cryoprotectant, in particular, to the high concentration of sucrose used in the process. In mature bovine oocytes, the meiotic spindle is a symmetrical, barrel-shaped structure, located peripherally with anastral poles, to which the microtubules are closely associated (Rho et al., 2002). Microtubules and actin microfilaments are known to be an integral role in the movement of chromosomes and in cell division (Kim et al., 2000). The objective of this study was to examine the effects of increased dehydration of mature bovine oocytes on the cytoskeletal arrangement and the components of the metaphase spindle.

2.2 Literature Review

2.2.1 Studies of Oocyte Dehydration

Numerous studies have been reported on determining the effect of water movement in living cells exposed to anisotonic solutions. Membrane permeability is a fundamental property of cells, which is characteristic of each cell type (Leibo, 1980). It has been demonstrated that osmotic stress on oocytes is detrimental to their developmental potential (Oda et al, 1992; Agca et al., 2000). The effects of osmotic shock on cells were initially conducted on various somatic cell types, such as erythrocytes and on eggs of lower organisms such as sea urchins, amphibians and fish. Since then, the osmotic behavior of several different stages of mammalian oocytes and embryos has been examined.

Although there has been extensive interest in the permeability of water through the cell membranes of oocytes and embryos, most of the studies have been done in the mouse (e.g., Leibo, 1980; Toner et al., 1991; Oda et al., 1992; Pedro et al., 1997). Osmotic behavior has been examined on oocytes of other domestic species such as the goat (Le Gal et al., 1994), the human (Trad et al., 1998) and the cow (Ruffing et al., 1993; Agca et al., 2000). In those studies, the investigators examined the effect on fertilization and embryonic development of osmotic stress on immature and mature bovine oocytes, however, they used sodium chloride to create the anisotonic solutions. They reported a marked decrease in developmental rates of all the oocytes exposed to hypertonic solutions.

Sodium chloride has been reported to have a detrimental effect on cells in high concentrations due to the increased ion load, which causes the destabilization of the plasma membrane (Stachecki et al., 1998b). Although Myers et al. (1987) examined the osmotic behavior of immature bovine oocytes using sucrose instead of sodium chloride, there has been no report of mature oocyte dehydration using a saccharide.

Unfertilized mouse oocytes and zygotes were reported to behave as perfect osmometers (Leibo, 1980). This was determined by placing the cells into increasing hypertonic solutions and then measuring their volume after equilibration. He plotted the cell volume relative to the isotonic volume, as a linear function of the reciprocal of the solution's osmolality (Boyle van't Hoff plot). Extrapolations of the Boyle van't Hoff plots to an infinitely high concentrated solution indicated a nonosmotic volume of 18% for mouse zygotes (Leibo, 1980). This nonosmotic volume agreed well with other studies, such as 21% for hamster oocytes (Shabana and McGrath, 1988) and 17% for immature bovine oocytes (Myers et al., 1987). This type of plot has also been constructed for various cell types, and it has been found that all cells with the exception of sperm cells and erythrocytes, have a nonosmotic volume of approximately 20%.

Membrane permeability as well as nucleation temperature are used to create thermodynamic models, which can be used to optimize equilibrium freezing protocols (Myers et al., 1987). Vitrification is a nonequilibrium freezing protocol which employs high concentrations of CPAs used in step-wise additions to minimize damage to the cell caused by fluxes in cell volumes. Cell expansion during warming and CPA removal are other causes of membrane damage (Hotamisligil et al., 1996). Therefore, ways to minimize the effects of osmotic stress on oocytes needs to be examined to determine the optimal method of cryopreservation.

2.2.2 Cryoinjury Due to Intracellular Ice Formation and Solute Effects

The dehydration of oocytes that occurs during cryopreservation is essential to their survival. Mammalian oocytes have a low surface to volume ratio. Because of this, oocytes will be more difficult to dehydrate, a necessary step in equilibrium freezing. Therefore, oocyte cryopreservation has had relatively low success in most mammalian species, with the exception of the mouse (e.g., Stachecki and Willadsen, 2000; Parks and Ruffing, 1992).

As first hypothesized by Mazur et al. (1972), the major mechanisms of cryoinjury are intracellular ice formation (IIF) and solution effects. During slow-cooling, the cell maintains an equilibrium between its intracellular and extracellular environments through the process of dehydration. Intracellular ice is formed when the equilibrium between these two environments is disrupted (Parks and Ruffing, 1992; Muldrew et al., 2004). When the cooling rate is too rapid to allow for the water to exit the cell, intracellular ice will form resulting in large ice crystals forming within the intracellular compartments. These crystals will cause breaks in the cell membranes and can also damage other cell organelles. If the oocyte is allowed to dehydrate before nucleation, intracellular ice crystals do form but they are smaller and cause less damage to the cell.

During standard cell cryopreservation, cryoprotectant additives (CPAs), such as glycerol, dimethylsulfoxide (DMSO) and ethylene glycol (EG) are added to the media.

The exposure of the oocytes to these CPAs can be done as a single step or a multiple step addition. Both permeating and nonpermeating CPAs are used to cause a shift in the isotonic state between the cell intracellular and extracellular spaces causing water to flow out of the cell. Permeating CPAs can produce large-volume changes during both the freezing and thawing processes. During freezing, the extracellular space will become hypertonic due to the increase in solutes compared with that in the intracellular spaces resulting in water leaving the cell and cell shrinkage (Mazur and Schneider, 1986). During thawing, a rapid influx of water into the cell due to the increased concentrations of solutes within the cell, before the permeating CPA can be removed, can result in osmotic shock and cell lysis (Mazur and Schneider, 1986). The water enters faster than the solute can exit thus, increasing the cell volume beyond its lytic volume. Protocols that require maximal cell volume excursions at a rate that the cells can tolerate should be used to reduce cellular membrane damage.

In addition to physical damages incurred by volume changes, a toxic solute effect on the cellular membrane and other organelles may be caused by the high concentrations of solutes surrounding the cell as well as the permeating CPAs entering the cell during dehydration. Different CPAs confer different levels of protection during the freezing and thawing process depending on the relative permeability of the particular cell type. Therefore, cryopreservation protocols have been devised to minimize intracellular ice formation, cell volume excursions, as well as to decrease the length of time that the cells are exposed to high concentrations of solutes in the freezing medium.

2.2.3 Role of Microtubules During Fertilization and Cellular Division

The contraction of the cell membranes during dehydration around the nucleus causes a re-arrangement of cell organelles including the cytoskeleton and the meiotic spindle. The abruptness and extent of the disruption of the cytoskeleton and meiotic spindle may be a contributing factor to the decreased survival of cryopreserved oocytes. Most mammalian oocytes are cryopreserved at the metaphase-II (MII) stage. At this stage, the chromosomes are attached to the microtubules at their centrosomes and are pulled into the equatorial region of a barrel-shaped structure before cell division. If the chromosomes are not properly aligned, there is unequal division resulting in aneploidy (trisomy). During cellular division, the chromatin condenses and distinct chromosomes form. These chromosomes migrate to an equatorial position during prophase. After reaching this equatorial position during metaphase, the chromosomes divide through metaphase-anaphase and then cellular division follows during telophase, which includes cytokinesis (for a review see Johnson and Everitt, 2000).

Various studies have demonstrated that extended exposure of oocytes to subzero or room temperature and to CPAs can result in depolymerization of the microtubules leading to the dispersal of chromosomes (Magistrini and Szollosi, 1980; Johnson and Pickering, 1987; Pickering and Johnson, 1987; Van der Elst et al., 1988; Pickering et al., 1990; Vincent et al., 1990; Vincent and Johnson, 1992; Aman and Parks, 1994; Fuku et al., 1995b; Martino et al., 1996; Wu et al., 1999).

2.2.4 Disruption of the Cytoskeletal and Microtubule Arrangements

Depolymerization of tubulin has been correlated to a decreased in fertilization and developmental rates in many species. Factors known to lead to the disruption of the cytoskeleton and microtubule structure of the metaphase spindle in oocytes are temperature and chemicals used during cryopreservation. The effect of temperature on spindle morphology has been evaluated in the mouse (Magistrini and Szollosi, 1980; Pickering and Johnson, 1987), human (Pickering et al., 1990) and cattle (Aman and Parks, 1994).

Oocytes of some species, including the pig, are more sensitive to chilling injury than others. In the pig, removal of a small volume of the cytoplasmic lipids is required for survival of post-cryopreservation embryos (Park et al., 2005). In the mouse, after 60 minutes at 0ºC, MII-stage oocytes exhibit complete spindle disassembly (Magistrini and Szollosi, 1980; Pickering and Johnson, 1987). In the cow, cooling oocytes to 4ºC for 20 minutes have resulted in complete disorganization of the meiotic spindle and even holding them at room temperature for 30 minutes, caused disruption or abnormal spindles (Aman and Parks, 1994). Pickering et al. (1990) reported that human oocytes also exhibited tubulin disassembly if maintained at room temperature for only 30 minutes. This indicates that human oocytes are more sensitive to cooling than bovine and mouse oocytes.

This variability to different cooling temperatures may explain the need to devise cryopreservation protocols for specific species. However, this disruption may or may not be reversible depending on the species and the amount of damage incurred. In some species, such as the mouse (Pickering et al., 1990) and to a lesser extent the human (Magistrini and Szollosi,, 1980, Wang et al., 2001), rewarming of oocytes can result in the reorganization of the meiotic spindle. This ability to reverse the damaging effects of cooling on the cytoskeletal arrangement has been reported to not to occur in bovine oocytes (Aman and Parks, 1994). Actin microfilaments are not as sensitive to cooling as the microtubules, but they can be disrupted by exposure to various CPAs (Saunders and Parks, 1999).

CPAs have also been shown to have detrimental effects on both the actin and tubulin configurations of oocytes during cryopreservation. Propanediol (PrOH) can induce depolymerization in rabbit oocytes and DMSO will do the same in the mouse whereas, DMSO has little detrimental effect on the rabbit oocyte cytoskeleton (Vincent et al., 1989). The effects of different CPA exposure have been evaluated on mature bovine oocytes (Fuku et al., 1995b; Agca et al., 1998; Saunders and Parks, 1999). Saunders and Parks (1999) evaluated the effects of EG on bovine oocytes and noted abnormal actin distribution and depolymerization of tubulin in the majority of the treated oocytes. Besides temperature and chemical damage, it has been well documented that cryopreservation is also a major contributor to cytoskeletal damage. Therefore, the objective of this study was to determine the effects of dehydration on the cytoskeleton and meiotic spindle of bovine oocytes.

2.3 Materials and Methods

All compounds unless otherwise stated were purchased from Sigma Chemical Company, St. Louis, MO.

2.3.1 Experimental Design

In the first experiment (Experiment 1), bovine oocytes were allotted to one of six treatments groups for dehydration. For the control groups, MII-stage oocytes (n = 22) exposed to either an isotonic solution of modified TCM-199 alone (Control mTCM) or an isotonic solution of modified M2 alone (Control mM2). Then in the remaining treatment groups MII-stage oocytes were exposed to one of four different saccharide solutions as follows: mTCM + Sucrose (n = 5), mTCM + Trehalose (n = 6), mM2 + Sucrose (n = 12) and mM2 + Trehalose (n = 6).

In the second experiment (Experiment 2), similar bovine oocytes were either dehydrated or not dehydrated and then stained to examine their cytoskeleton and DNA. The six treatment groups were as follows: Control MII-stage oocytes (n = 57) in mTCM or mM2 that were not dehydrated but were stained (Control mTCM and Control mM2) and in the remaining treatment groups, MII-stage oocytes that were dehydrated in one of four different saccharides solutions (as described for Experiment 1) as follows: mTCM + Sucrose (n = 54), mTCM + Trehalose (n = 43), mM2 + Sucrose (n = 49) and mM2 + Trehalose (n = 42).

2.3.2 Oocyte Collection and Processing

Bovine cumulus-oocyte-complexes (COCs) were collected by a private company (Ovagenix, San Angelo, TX) from ovaries obtained from an abattoir in Texas. The COCs were graded and those considered to be of good quality were shipped overnight by express courier in maturation medium, using a portable incubator (temperature 37.8-39.1°C) to the LSU Embryo Biotechnology Laboratory in St. Gabriel, Louisiana. The maturation medium consisted of TCM-199 (12340-030; Gibco, Grand Island, NY) with 0.5 M L-glutamine (G-8540), 1% fetal bovine serum (FBS) (SH30070.02; Hyclone Laboratories Inc., Logan, UT), 10 µg/ml of bovine Luteinizing Hormone (LH) (L-9773), 0.7 IU/ml of Follicle Stimulating Hormone (FSH) (F-2293), 0.01 mg/ml of estradiol (E-2758) and 0.1% penicillin/streptomycin (15140-122; Gibco).

After 22 to 26 hours of maturation, COCs were removed from maturation medium and washed twice in 2 to 3 ml of modified TCM-199 (mTCM). Modified TCM-199 medium was prepared by supplementing TCM-199 with 10% FBS. Cumulus-oocyte-complexes were then transferred to a 15 ml centrifuge tube containing 2.2 mg of hyaluronidase (H-3506) in 4 ml of TCM-199 and were vortexed for ~1 minute to partially remove the cumulus cells surrounding the oocytes. Partially denuded oocytes were washed twice in mTCM and then placed 5 to 6 oocytes per 100 µl droplet of mTCM under mineral oil (M-8410) and incubated (38.5°C) in 5% CO_2 in air for 1 hour prior to starting these experiments.

2.3.3 Dehydration of Oocytes

Solutions used for dehydration consisted of a saccharide (sucrose or trehalose) prepared in either mTCM or mM2. Modified M2 medium was prepared by supplementing M2 medium (M-7167) with 10% FBS. The osmolality of the solutions was determined with a freezing-point osmometer (Model # 3W2; Advanced Instruments Inc., Needham Heights, MA). At least three readings were completed for each solution. The average osmolality of each solution was calculated and was considered to be the osmotic pressure of the solution.

In Experiment 1, oocytes from all treatment groups were partially denuded and transferred in groups of 5 or 6 in a small volume (<5 µl) of mTCM into either mTCM alone or mM2 alone. Both mTCM and mM2 were used in bovine oocyte and embryo manipulations. The volume of the oocytes in these isotonic solutions was recorded as the control volume for the volumetric analyses. The oocytes were allowed to equilibrate for approximately 10 minutes after which digital images were recorded using a personal computer with image capturing software (Scion Image for Windows, Beta 4.0.2; Scion Corporation, Frederick, MD) attached to a Nikon Diaphot inverted microscope. All steps were conducted at room temperature (22°C to 25°C).

Control oocytes remained in mTCM or mM2 while the oocytes for all other treatment groups were then sequentially rinsed through a series of 35 mm petri dishes containing 2 to 3 ml of increasing concentrations of either sucrose (S-1888) or D(+)trehalose (T-5251) dissolved in either mTCM or mM2. For the trehalose treatments, 0.13, 0.25, 0.35, 0.55 and 0.65 M solutions were used and for the sucrose treatments, 0.15, 0.30, 0.50, 0.65 and 0.99 M were used (Table 2.1).

The maximum concentration used for trehalose is lower than that used for sucrose due to its lower solubility. The oocytes were allowed to equilibrate for ~10 minutes in each of the treatment solutions. Digital images were made of the oocytes in each of the saccharide solutions. Oocytes were dehydrated using mM2 (HEPES buffered solution) as the diluent were done in air while those completed in mTCM (bicarbonate-buffered solution) were performed in a modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) that was filled with a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

2.3.4 Volumetric Measurements

The volumes of oocytes exposed to the two isotonic media (Control mTCM and Control mM2) and to each saccharide solution were calculated assuming the oocytes to be spherical. Calibrated digital imaging software (Spot Advanced, Version 3.5.5 for Windows; Diagnostic Instruments Inc., Sterling Heights, MI) was used to measure the diameter of each oocyte. Volume was calculated using the formula for a sphere

Table 2.1 The osmolarities and osmotic pressures of sucrose and trehalose solutions prepared in mTCM and mM2 for Experiment 1

Saccharide in mTCM	Concentration (M)	Osmolarity (mOsm)	Osmotic pressure (1/mOsm)
Sucrose	0.00	0.29	3.44
	0.15	0.46	2.16
	0.30	0.61	1.63
	0.50	0.85	1.18
	0.65	1.01	0.99
	0.99	2.86	0.35
Trehalose	0.00	0.29	3.44
	0.13	0.42	2.36
	0.25	0.57	1.76
	0.35	0.68	1.47
	0.55	0.90	1.11
	0.65	1.02	0.98

Saccharide in mM2	Concentration (M)	Osmolarity (mOsm)	Osmotic pressure (1/mOsm)
Sucrose	0.00	0.28	3.57
	0.15	0.45	2.23
	0.30	0.60	1.66
	0.50	0.85	1.18
	0.65	1.04	0.96
	0.99	2.94	0.34
Trehalose	0.00	0.28	3.57
	0.13	0.42	2.40
	0.25	0.56	1.79
	0.35	0.67	1.50
	0.55	0.89	1.12
	0.65	1.00	1.00

$V = 4/3\pi(d/2)^3$. The mean relative volumes were calculated by taking the average volume of the oocytes exposed to the various saccharide solutions and dividing it by the average isotonic volume for each diluent and expressed as a percentage. This method of determining the relative volumes of oocytes was previously reported by Jackowski et al. (1980), Leibo (1980), Oda et al. (1992) and McWilliams et al. (1995).

2.3.5 Immunostaining of the Cytoskeleton and DNA Staining

Immunostaining was used to identify the presence and location of microfilaments and microtubules and Hoechst staining was used to identify the DNA in Experiment 2. Mature oocytes were obtained and partially denuded and allowed to equilibrate at 38ºC for 1 hour prior to staining as described in section 2.3.2 of this chapter. Approximately 10 oocytes were stained and were used for negative controls.

Briefly, oocytes were transferred from mTCM into either fresh mTCM or mM2 alone and allowed to equilibrate for 10 minutes. They were then sequentially dehydrated using sucrose (0.15, 0.65 and 0.99 M) or trehalose (0.125, 0.35 and 0.65 M) prepared in mTCM or mM2 and allowed to equilibrate at 20ºC to 25ºC for ~10 minutes in each solution. This was under the same conditions as described for the dehydration. The oocytes were fixed in 4% paraformaldehyde (Electron Microscope Science, Fort Washington, PA) containing either 0.99 M sucrose or 0.65 M trehalose and prepared in Dulbecco's phosphate-buffered saline (PBS) (14040-141; Gibco, Grand Island, NY) for 30 minutes and then transferred into ~2 ml of acetone in a glass dish at -20°C for 30 minutes.

The rest of the immunostaining protocol was modified from that reported by Erogulu et al. (1998). Oocytes were rinsed twice in ~2 ml of PBS containing 0.05% BSA

Fraction V (A-7511). Then the oocytes were then incubated for 30 minutes in a ‘blocking solution’ containing 2% goat serum, 2% BSA, 0.01% Triton-X 100 (X-100) prepared in PBS to reduce nonspecific binding. Oocytes were then transferred into a 80 μl droplet of primary antibody, rat anti-α-yeast tubulin (1:20) (provided by Dr. John Lynn, Department of Biological Sciences, Louisiana State University, Baton Rouge, LA), on a slide and incubated for 1 hour at room temperature in the dark. They were then rinsed twice in wash buffer containing 0.05% BSA prepared in PBS and then transferred to a new slide containing a secondary antibody, anti-rat IgG conjugated to fluorescein isothiocyanate (FITC) (1:40) (F-9387) and rhodamine phalloidin (1:40) (R-415; Molecular Probes, Eugene, OR).

The oocytes were incubated as described above for 2 hours. The oocytes bathed in washing solution for 30 minutes prior to being mounted to remove excess stain. The oocytes were mounted using ~100 μl of SlowFade-Antifade (S-2828; Molecular Probes) containing 0.1 μl/ml stock of Hoechst 33342 (B-2261) was added. Oocytes were examined and images recorded with a Nikon 35 mm SLR camera mounted on a Nikon Diaphot fluorescence microscope. Oocytes were also examined using a laser scanning confocal microscope (Model # 3W2; Leica Microsystems, Exton, PA) and imaging software. Laser lines used were 488 nm and 534 nm.

In a preliminary experiment, bovine oocytes (n = 7 per treatment group) were collected and processed as described earlier and fixed as above excluding the addition of either saccharide. All subsequent steps of staining were the same as described above. These oocytes were examined using the same laser scanning confocal microscope and the images were analyzed for the presence and location of actin, tubulin and DNA.

2.3.6 Statistical Analysis

A linear regression was performed on the average relative volumes of oocytes exposed to each saccharide prepared in either mTCM or mM2, over the average osmotic pressure of the solution. Chi-square analysis and Fisher's exact test were performed to test for statistical differences in tubulin and actin among treatment groups ($P \leq 0.05$ statistically different) (Instat Graphpad, Version 3.0, San Diego, CA).

2.4 Results

2.4.1 Volumetric Response to Dehydration

In the first experiment (Experiment 1), the effects on oocyte volumes of various concentrations of each saccharide prepared either in a bicarbonate-buffered (mTCM) or a HEPES-buffered (mM2) solution were examined. For both diluents, bovine oocytes behaved osmotically when transferred from an isotonic solution into increasingly hypertonic solutions. For each treatment, the number of oocytes used was as follows. A total of 22 mature bovine oocytes were used as controls (Control mTCM and Control mM2), for sucrose in mTCM ($n = 4$ or 5), trehalose in mTCM ($n = 5$ or 6), sucrose prepared in mM2 ($n = 5 - 12$) and trehalose in mM2 ($n = 5$ or 6). The effect of increased dehydration due to increased osmotic pressure found in the bovine oocytes is shown in Figure 2.1.

The resulting data obtained from oocyte dehydration demonstrate that bovine oocytes exhibit a linear decrease in volume as a function of the reciprocal of osmolality. The osmotic pressures of the isotonic solutions of mTCM and mM2 alone were 344

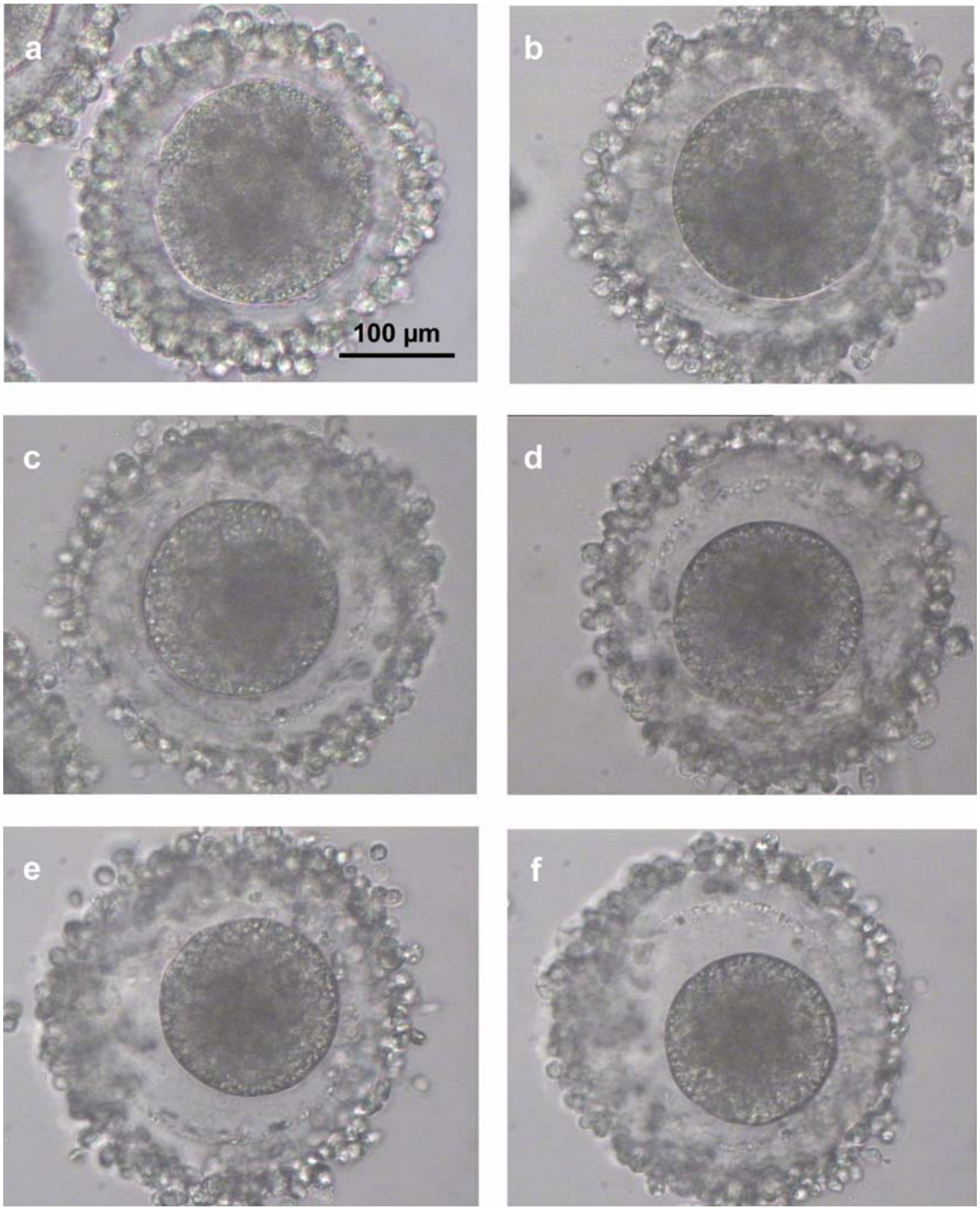

Figure 2.1 Photomicrographs of bovine oocytes suspended in various concentrations of trehalose prepared in mM2. (a) Oocyte exposed to mM2 alone for ~10 minutes at room temperature in air, (b) 0.13 M trehalose, (c) 0.25 M, (d) 0.35 M, (e) 0.55 M and (f) 0.65M. Note the increasing perivitelline space between the oocyte and the zona pellucida. Images were obtained using Scion Imaging Software attached to a Nikon Diaphot microscope with 20X objective. Images were adjusted for brightness and contrast using Adobe Photoshop, Version 6.0. (Scale bar = 100 µm, set for all images).

mOsm and 357 mOsm, respectively. Oocytes exposed to 0.99 M sucrose in mTCM contracted to 39% of their isotonic volume, while those exposed to 0.65 M trehalose in the same diluent contracted to 35%. Oocytes exposed to the same osmolalities of sucrose and trehalose but prepared in mM2, were dehydrated to 32% and 39%, respectively. The results in Figures 2.2 and 2.3 are a Boyle van't Hoff plots for bovine oocytes.

2.4.2 Immunostaining Results

In the second experiment (Experiment 2) bovine oocytes were exposed to the highest concentrations of sucrose and trehalose in Experiment 1 to assess the effect of dehydration on the cytoskeleton and DNA spindle. After being stained, oocytes were mounted and examined both by fluorescence microscopy and by confocal microscopy. Immunostained oocytes were examined for the location of the actin microfilaments and the alignment of the microtubules and chromosomes. All negative controls exhibited no evidence of autofluorescence (Figure 2.4).

In a preliminary Experiment in which oocytes were fixed without being exposed to a saccharide, all of the control oocytes (n = 7) had intact meiotic spindles with co-localization of actin and tubulin. The location of DNA was not analyzed in this experiment. The results listing the percentage of oocytes with intact or disrupted tubulin and actin are presented in Table 2.2. In all saccharide-treated oocytes, there were significantly fewer intact meiotic spindles compared with controls. Of the oocytes treated with trehalose prepared in either mTCM (n = 7) or mM2 (n = 7), 57% had intact microtubules and the majority exhibited co-localization of the actin and tubulin.

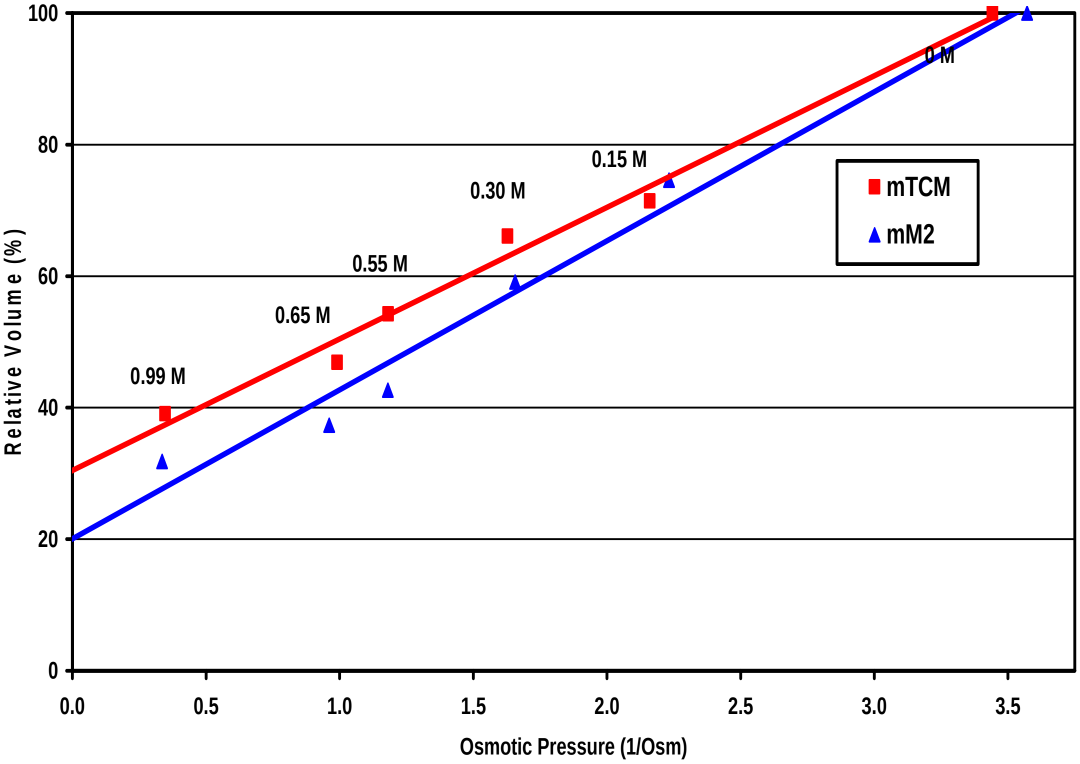

Figure 2.2 Boyle van't Hoff plots of bovine oocytes exposed to various solutions of sucrose prepared in mTCM or mM2. The points represent the mean relative volumes of oocytes. The correlation coefficients (R^2) for sucrose prepared in mTCM and mM2 were 0.99 and 0.98, respectively.

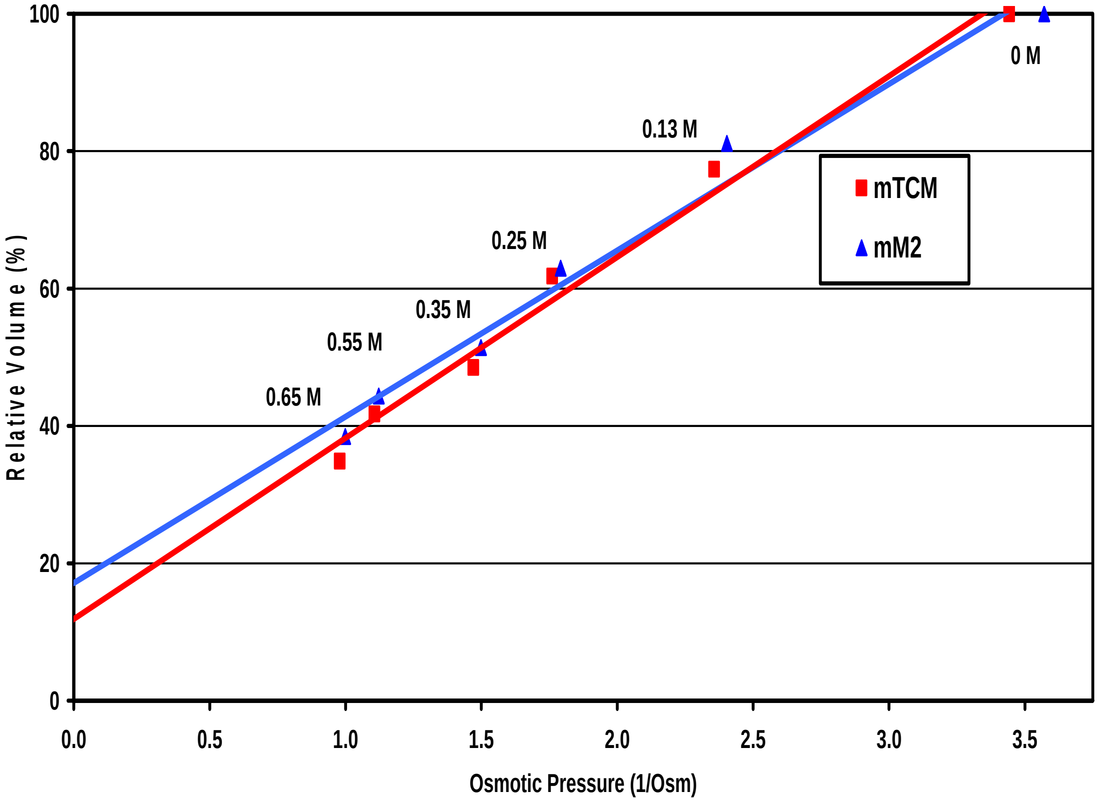

Figure 2.3 Boyle van't Hoff plots of bovine oocytes exposed to various solutions of trehalose prepared in mTCM or mM2. The points represent the mean relative volumes of oocytes. The correlation coefficients (R^2) for trehalose prepared in mTCM and mM2 were both 0.98.

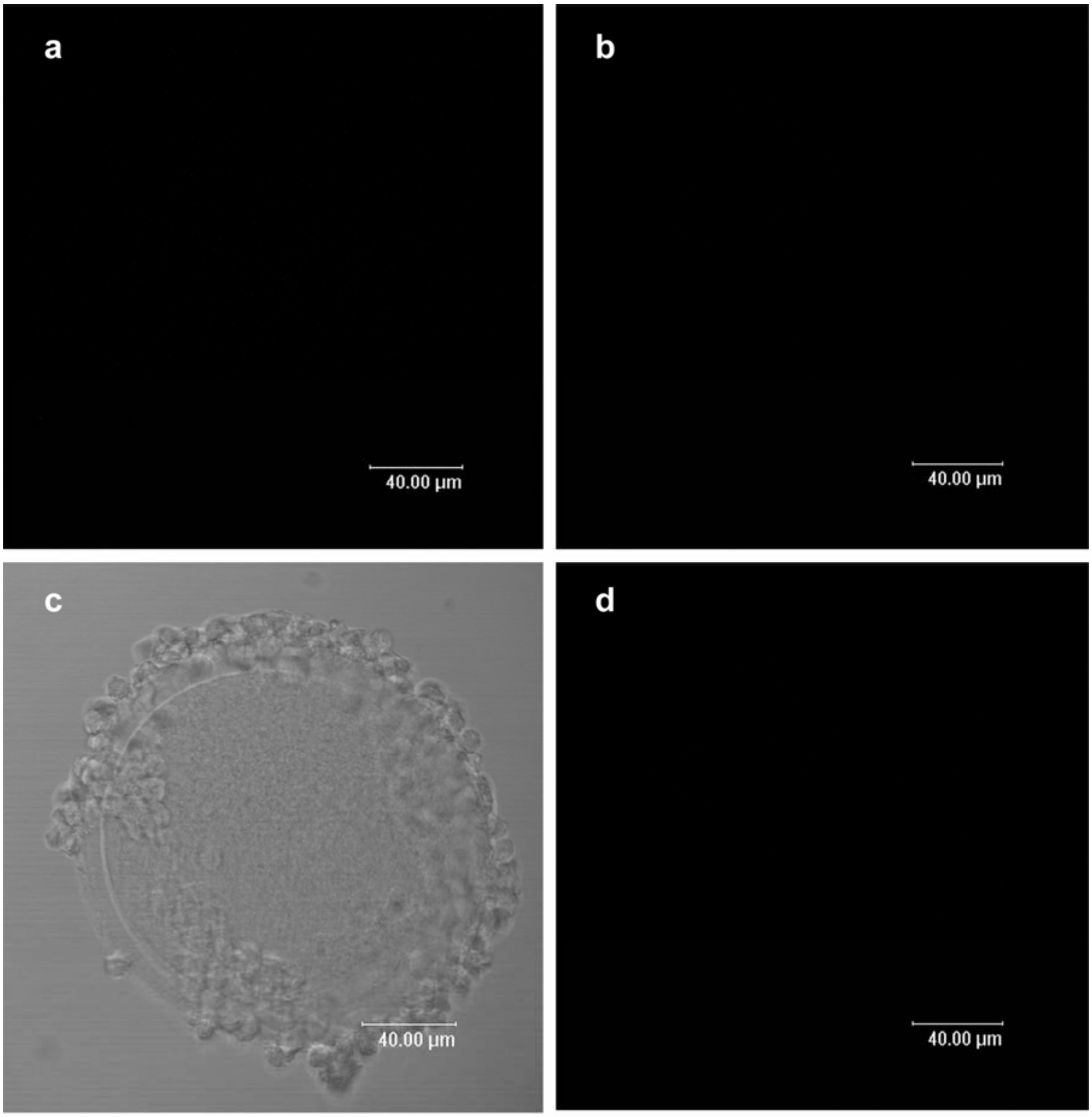

Figure 2.4 Photomicrographs of negative control for the primary antibody (rat-anti-α-tubulin) and rhodamine phallodin. Bovine oocytes were immunolabeled as previously described with the omission of primary antibody. Oocytes were incubated in mTCM or mM2 alone for the same period of time used for staining. (a) and (b) No presence of non-specific binding of secondary antibody or autofluorescence, (c) DIC image of oocyte, (d) overlay of tubulin and actin, no staining observed. Images were captured using a laser scanning confocal microscope and imaging software, with a 63X oil immersion objective. All oocytes were fixed in 4% paraformaldehyde, post-fixed in acetone at –20ºC, immunolabeled and mounted in 50% (v/v) glycerol.

This was, however, significantly higher than the results observed for oocytes exposed to sucrose prepared in either media. Only 2 (29%) of the sucrose-treated oocytes had intact spindles and co-localization was also reduced.

The majority of the 57 control oocytes 54 (79%) contained intact spindles, as indicated by their microtubule arrangement, while only 12 (21%) had disrupted microtubules (Figure 2.5). Representative micrographs of immunostaining of oocytes exposed to sucrose or trehalose prepared in mTCM and mM2 are in Figures 2.6 and 2.7. Of the oocytes exposed to 0.65 M trehalose prepared in mTCM (n = 43) and mM2 (n = 42), 12 and 13 oocytes exhibited disrupted microtubule arrangements and 31 and 29 oocytes, no organized structure was observed, respectively. Of the oocytes exposed to 0.99 M sucrose in mTCM (n = 54) and mM2 (n = 49), 14 and 16 had disrupted spindles and in 39 and 33 oocytes, no organized tubulin structure was noted, respectively. Only one intact spindle was observed in all the saccharide-treated oocytes (Table 2.3).

Co-localization of actin filament and microtubules was observed in 93% of the control oocytes (Control mTCM and Control mM2) and in 1% to 30% of the sucrose and trehalose treated bovine oocytes. The difference between the number of oocytes with actin present and those exhibiting co-localization with the microtubules was due to a diffuse staining throughout the oocytes exposed to sucrose prepared in mTCM. In some cases, when the actin extending from one side of the oocyte to the other, in close proximity to the spindle (Figures 2.6 and 2.7, panel d). Bovine oocytes stained for DNA with Hoechst were used to confirm the location of the chromosomes within the

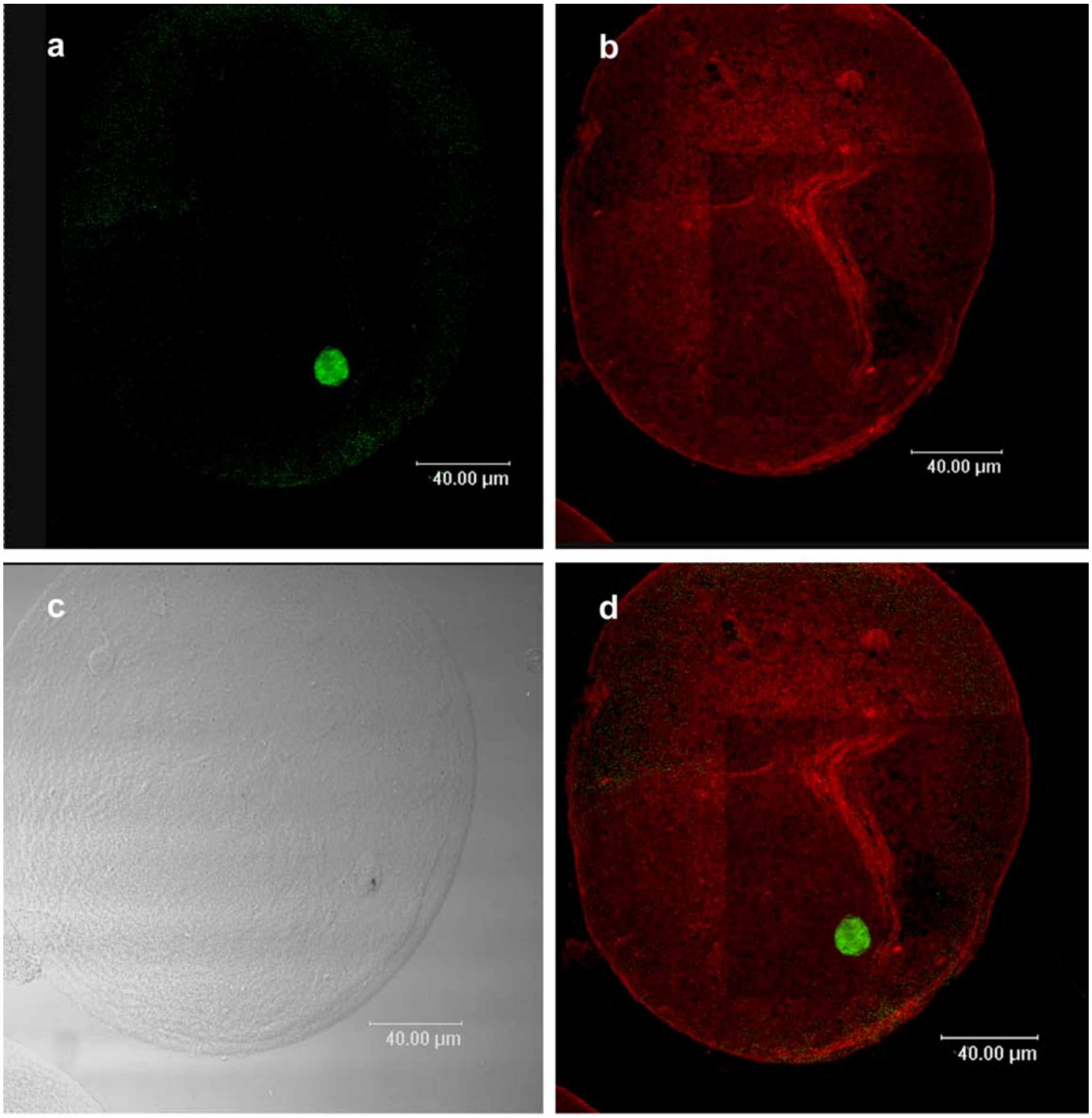

Figure 2.5 Photomicrographs of mature bovine oocytes immunolabeled with rat-anti-α-tubulin (green) and stained with rhodamine phallodin (red). (a) Image of an intact metaphase plate, (b) Image of a large bundle of actin microfilaments, (c) DIC image of oocyte, (d) overlay of tubulin and actin labeling demonstrating any regions of co-localization (orange). Images were captured using a laser scanning confocal microscope and imaging software, with a 63X oil immersion objective. All oocytes were fixed in 4% paraformaldehyde, post-fixed in acetone at –20ºC, immunolabeled and mounted in 50% (v/v) glycerol.

Table 2.2 Summary of cytoskeletal integrity and co-localization of actin and tubulin in bovine oocytes exposed to 0.65 M trehalose and 0.99 M sucrose prepared in both mTCM and mM2 and allowed to recover before fixation (Preliminary Experiment)

		Meiotic spindle			Actin
Treatments	No. of oocytes	Intact	Disrupted	Absent	Co-localized
Control	7	7 (100)	0 (0)	0 (0)	7 (100)
mM2 + Trehalose	7	4 (57)	2 (29)	1 (14)	6 (86)
mTCM + Trehalose	7	4 (57)	1 (14)	2 (29)	5 (71)
mM2 + Sucrose	7	2 (29)	2 (29)	3 (43)	18 (33)
mTCM + Sucrose	7	2 (29)	2 (29)	3 (43)	28 (52)

Numbers in parentheses represent percentage of the total number of oocytes stained for each treatment. No statistical significances in this table ($P \leq 0.05$).

Table 2.3 Summary of cytoskeletal integrity and co-localization of actin and tubulin in bovine oocytes exposed to 0.65 M trehalose and 0.99 M sucrose prepared in both mTCM and mM2 (Experiment 2)

		Meiotic spindle			Actin	
Treatments	No. of oocytes	Intact	Disrupted	Absent	Present	Co-localized
Control	57	45 (79)^a	12 (21)^a	0 (0)^a	57 (100)^a	53 (93)^a
mM2 + Trehalose	42	0 (0)^b	13 (24)^a	29 (62)^b	13 (24)^b	13 (24)^a
mTCM + Trehalose	43	0 (0)^b	12 (28)^a	31 (72)^b	13 (24)^b	13 (24)^a
mM2 + Sucrose	49	0 (0)^b	16 (30)^a	33 (61)^b	18 (33)^b	16 (30)^a
mTCM + Sucrose	54	1 (2)^b	14 (26)^a	39 (72)^b	28 (52)^b	6 (11)^a

Numbers in parentheses represent percentage of the total number of oocytes stained for each treatment. ^{a,b}Values with different superscripts within columns are significantly different ($P \leq 0.05$).

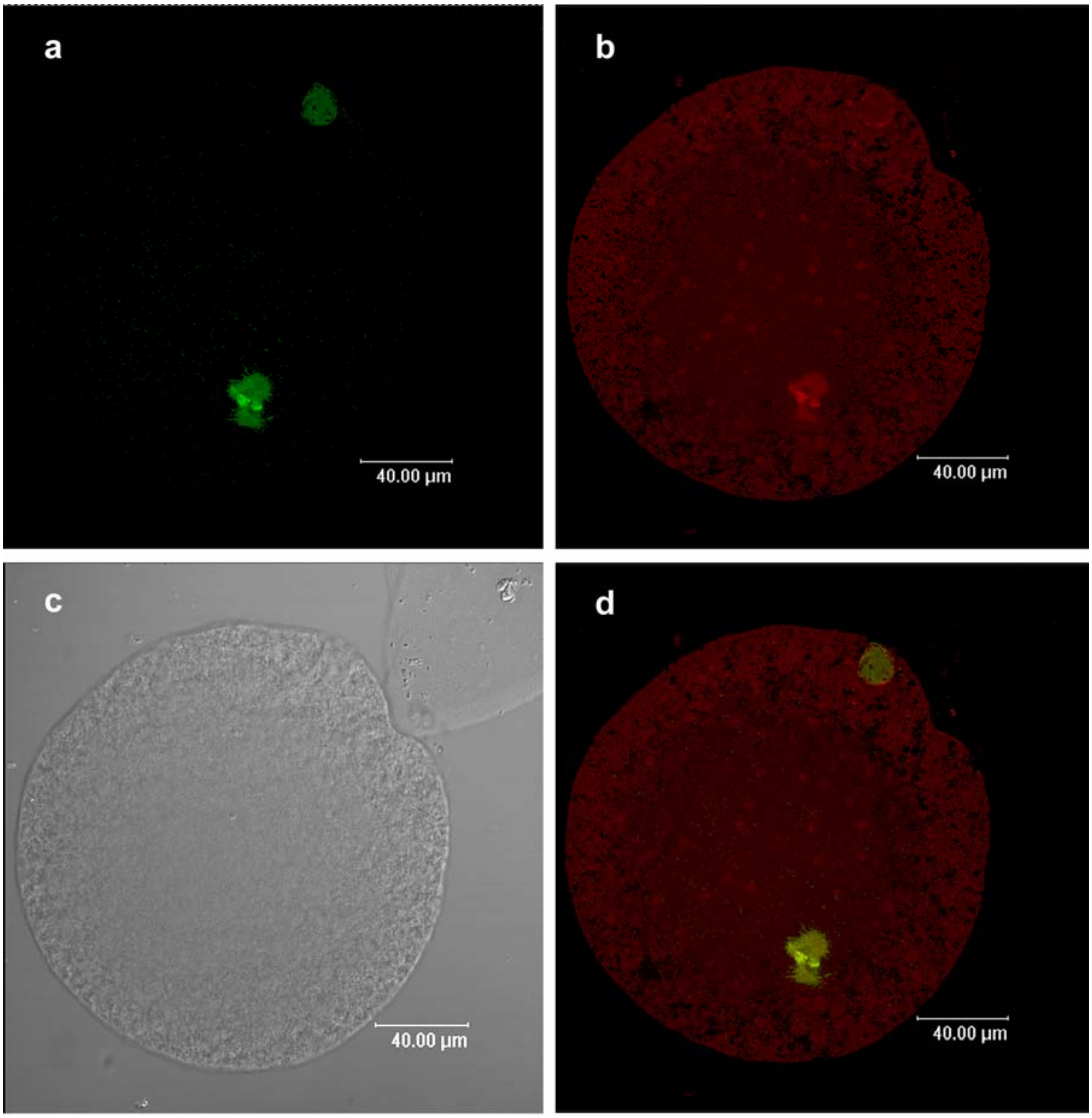

Figure 2.6 Photomicrographs of mature bovine oocytes exposed to 0.65 M trehalose prepared in mTCM, fixed and immunolabeled with rat-anti-α-tubulin (green) and rhodamine phallodin (red). (a) Image of a disrupted metaphase spindle as well as a second region of tubulin with no distinct organization, (b) Image of actin microfilaments, (c) DIC image of oocyte and (d) overlay of tubulin and actin labeling, demonstrating a high degree of co-localization (orange). Images were captured using a laser scanning confocal microscope and imaging software, with a 63X oil immersion objective. All oocytes were fixed in 4% paraformaldehyde, post-fixed in acetone at –20ºC, immunolabeled and mounted in 50% (v/v) glycerol.

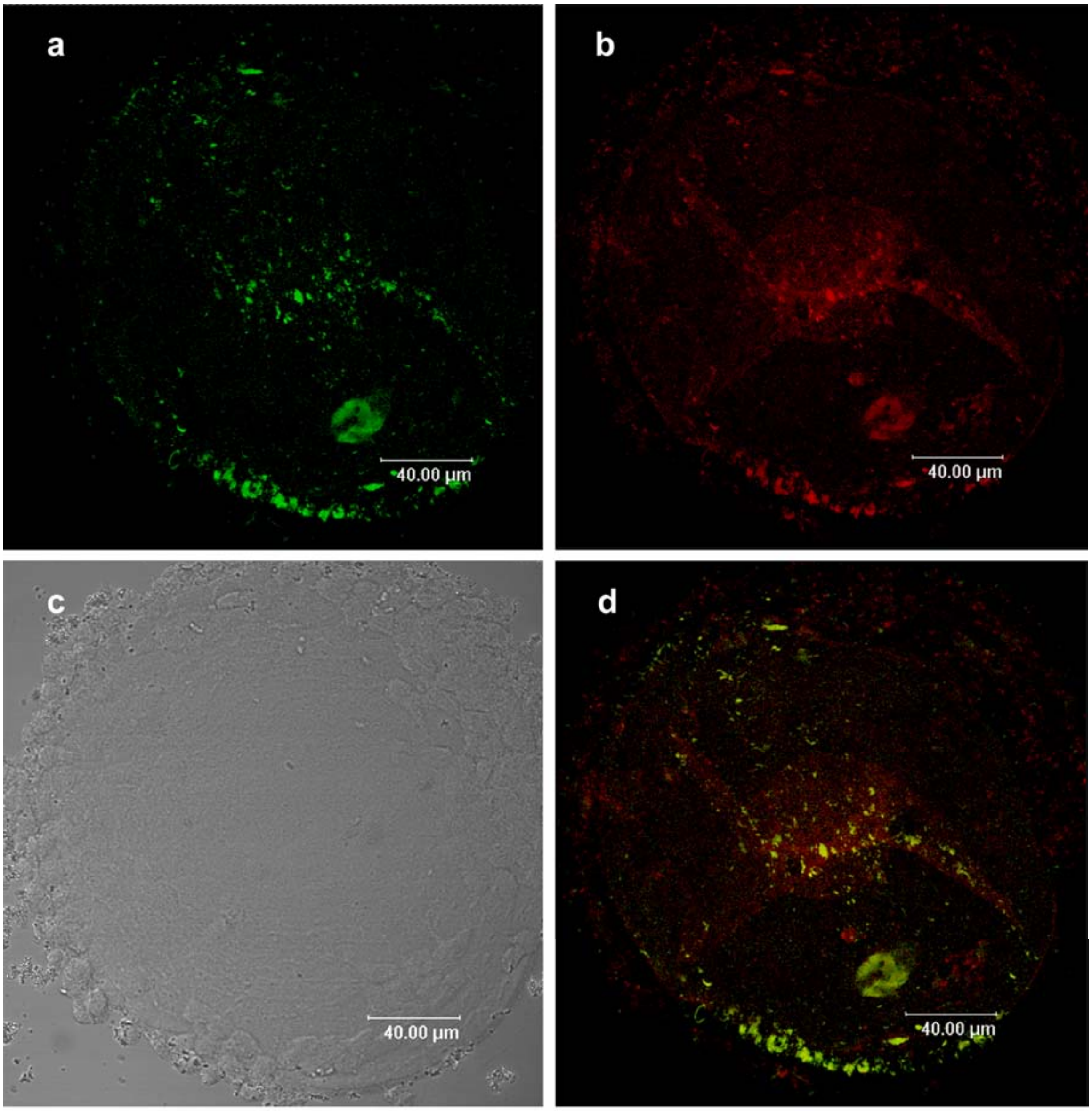

Figure 2.7 Photomicrographs of mature bovine oocytes exposed to 0.99 M sucrose in mM2, fixed and immunolabeled with rat-anti-α-tubulin (green) and rhodamine phallodin (red). (a) Image of a slightly disrupted metaphase spindle, (b) Image of actin microfilaments aggregated in a similar region as the tubulin, (c) DIC image of oocyte and (d) overlay of tubulin and actin labeling, demonstrating co-localization (orange). Images were captured using a laser scanning confocal microscope and imaging software, with a 63X oil immersion objective. All oocytes were fixed in 4% paraformaldehyde, post-fixed in acetone at –20ºC, immunolabeled and mounted in 50% (v/v) glycerol.

Metaphase spindle. Representative micrographs are included in Figures 2.8 and 2.9. In all cases in which the microtubules were intact, the chromosomes were intimately associated with the spindle structure, as revealed by Hoechst staining. Simultaneous visualization of all 3 dyes was only possible using standard fluorescence microscopy and not by laser-scanning confocal microscopy because the microscope that was used was not equipped with an ultraviolet laser. Two distinct regions of bright Hoechst staining were observed in all mature oocytes used in this study, one for the metaphase plate and the other representing the location of the first polar body.

2.5 Discussion

When suspended in increasingly concentrated solutions of an impermeant solute, such as a saccharide, the relative volume of a cell decreases in relation to the reciprocal of the osmolality to which it is exposed (Ruffing et al., 1993). Mature bovine oocytes have higher permeability coefficients of water and cryoprotectant compared with GV-stage oocytes (Vincent and Johnson, 1992; Ruffing et al., 1993). This may account for the differences observed in cryopreservation protocols for MII-stage oocytes compared with the GV-stage or other stages of oocytes. Therefore, mature bovine oocytes were chosen to study the effects of dehydration on the cytoskeletal arrangement and meiotic spindle prior to vitrification. Reports of cytoskeleton and spindle disruption microfilaments were not confined to the meiotic spindle, long fibers of actin were observed from cryopreserved mouse and bovine oocytes that have been previously been reported (Vincent et al., 1989; Fuku et al., 1995a; Eroglu et al., 1998). However, there have not been any reports on the effects of dehydration due to high concentrations of saccharides as used prior to vitrification of mature bovine oocyte.

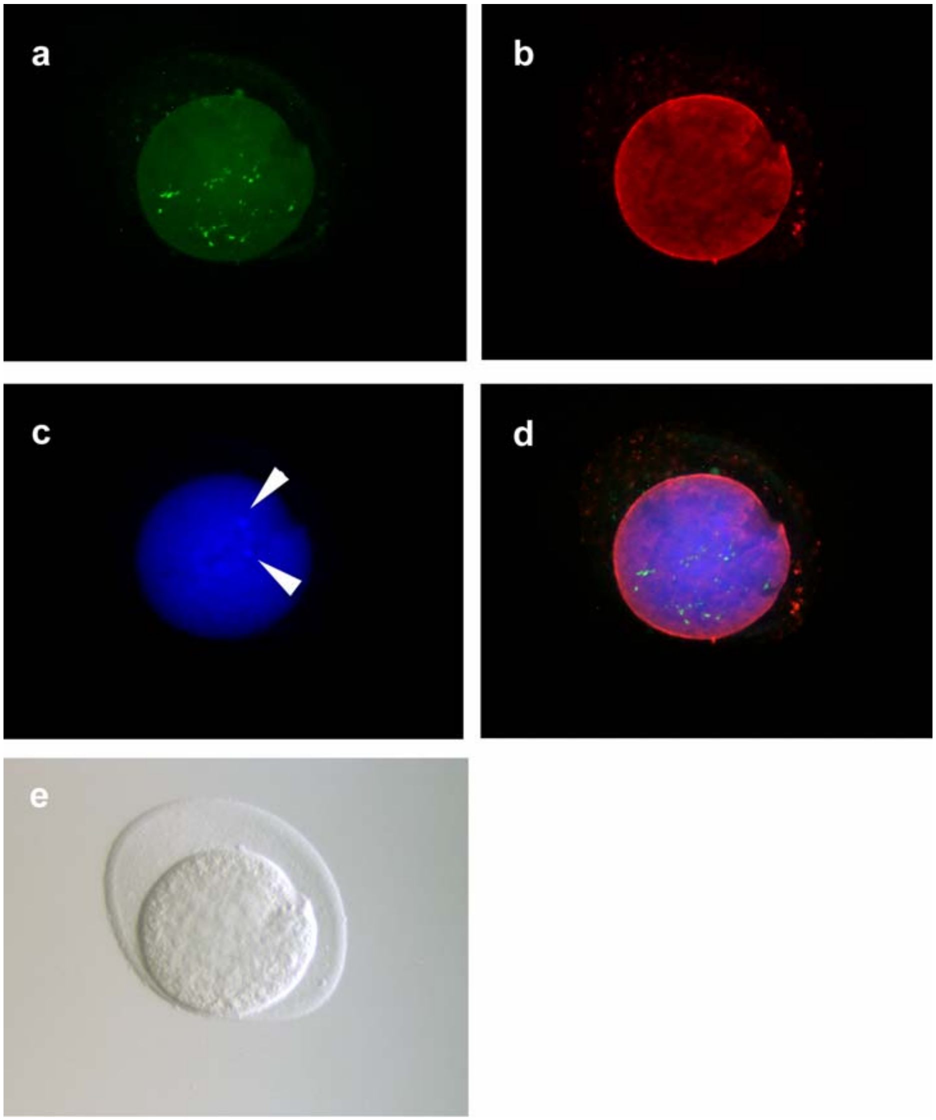

Figure 2.8 Photomicrographs of a mature bovine oocyte exposed to 0.99 M sucrose prepared in mM2 and then triple stained for tubulin, actin and DNA to allow for simultaneous visualization. (a) Stained oocyte visualized using a FITC filter to visualize tubulin (green) staining using a 40X objective on a Nikon Diaphot fluoresce microscope, (b) The same oocyte visualized using a TRITC filter for actin (red) staining, (c) Oocyte visualized using UV filter with arrows denoting the location of the DNA (blue), (d) Overlay of all three stains using a tri-filter block and (e) Brightfield image of the same oocyte. The oocyte was fixed in 4% paraformaldehyde, post-fixed in acetone at –20ºC, immunolabeled and mounted in 50% (v/v) glycerol. Brightness and color adjusted using Adobe Photoshop software 6.0.

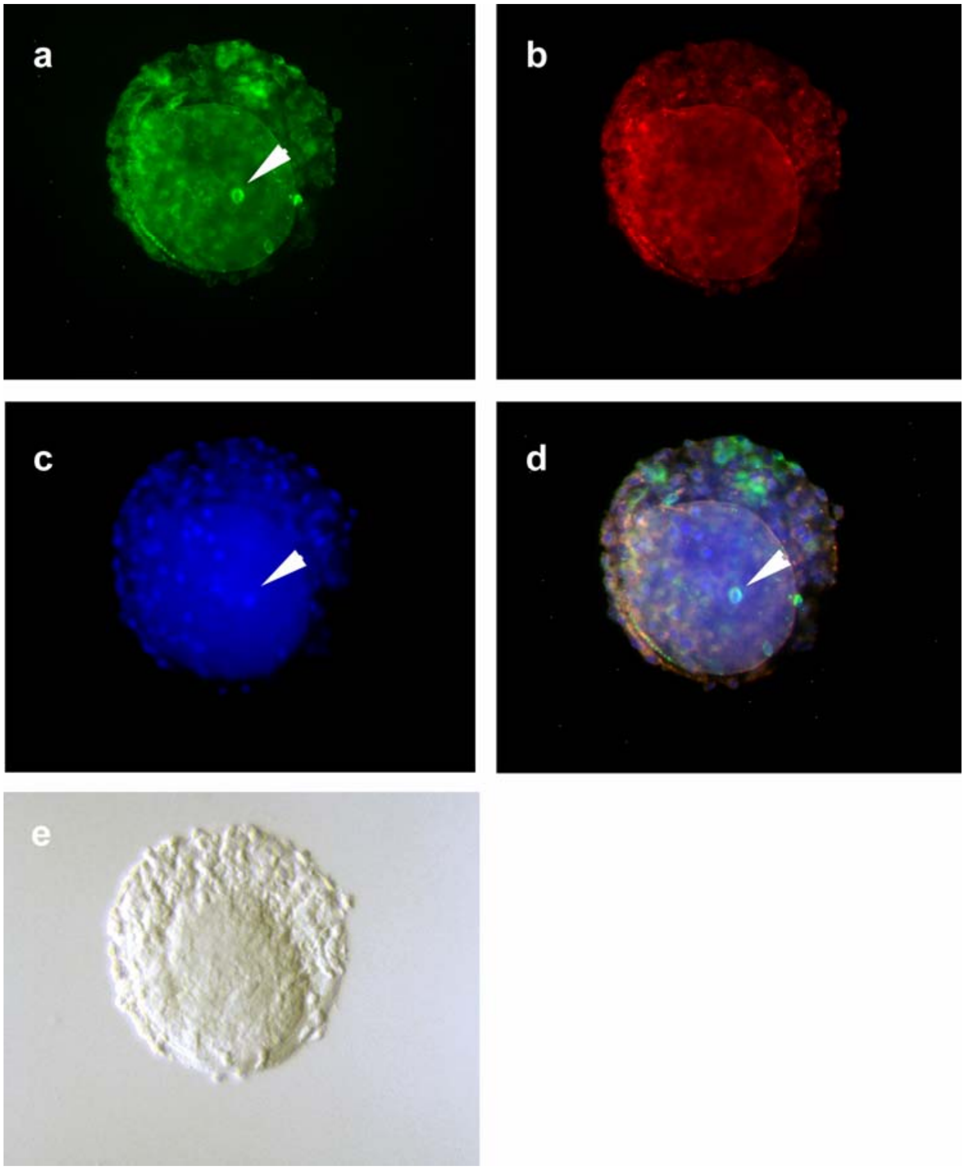

Figure 2.9 Photomicrographs of a mature bovine oocyte exposed to 0.65 M trehalose prepared in mM2 and triple stained for tubulin, actin and DNA to allow for simultaneous visualization. (a) Stained oocyte visualized using a FITC filter to visualize tubulin (green) staining using a 40X objective on a Nikon Diaphot fluorescence microscope, (b) The same oocyte visualized using a TRITC filter for actin (red) staining, (c) Oocyte visualized using UV filter with arrows denoting the location of the DNA (blue), (d) Overlay of all three stains using a tri-filter block and (e) Brightfield image of the same oocyte. The oocyte was fixed in 4% paraformaldehyde, post-fixed in acetone at –20ºC, immunolabeled and mounted in 50% (v/v) glycerol. Brightness and color adjusted using Adobe Photoshop software 6.0.

In the first experiment, volumetric measurements of bovine oocytes at equilibrium in hypertonic solutions were made. At high concentrations of saccharides, oocytes undergo dehydration to restore a balance of water activity between their milieu and their intracytoplasmic environment, to a minimum of 13% to 23% of their isotonic volume as reported for mouse and human oocytes (McWilliams et al., 1995). This maximal level of dehydration represents the nonosmotic volume of an oocyte. In this study, mature bovine oocytes exhibited a decrease in relative volume with increasing osmotic pressure to a minimum of ~32% of their isotonic volume in 0.99 M sucrose in mM2 ($R^2 = 0.98$). Bovine oocytes behaved similarly when exposed to increasing concentrations of sucrose or trehalose in both isotonic solutions. Lysis occurred in a few oocytes when they were exposed to a high concentration of sucrose prepared in either diluent. Under normal conditions, sucrose will not diffuse across the cell membrane. However, at high concentrations, there may be leakage due to the effects of severe dehydration on the plasma membrane (Oda et al., 1992) at elevated temperatures. Lyis occurs once cells are returned to the isotonic solution.

In the preliminary immunostaining study, bovine oocytes were fixed without the addition of sucrose to the fixation medium. This allowed time for partial recovery of the microtubule structure after the initial dehydration. Reversal of this microtubule disruption due to cooling has been found in mouse oocytes. In humans, only a partial re-organization of the meiotic spindle was observed after the oocytes were cooled (Pickering et al., 1990; Wang et al., 2001). However, in bovine oocytes, little to no reversal of the de-polymerization of tubulin was observed after simple cooling and rewarming or when oocytes were exposed to room temperature (Aman and Parks, 1994). This difference is thought to be attributed to the significantly higher amount of pericentriolar material available in mouse oocytes that allow the re-polymerization of microtubules compared with other species (Aman and Parks, 1994). Microtubules in control oocytes in our study were intimately associated with the chromosomes forming the meiotic spindle, which appeared as a symmetrical, barrel-shaped structure with anastral poles. This observation agrees with previous studies on the cytoskeletal arrangement in bovine oocytes during maturation and cooling (Kim et al., 2000; Rho et al, 2002). The few disrupted spindles may represent immature oocytes that have not properly extruded their first polar body. This is in strong contrast to the results obtained with the dehydrated oocytes. Therefore, in Experiment 2, sucrose or trehalose was added to the fixation medium to avoid any recovery of the cytoskeleton or meiotic spindle.

In the second experiment, the highest concentrations of saccharides (0.99 M sucrose and 0.65 M trehalose) used in Experiment 1 were used to determine the effect of dehydration on cytoskeletal structures and the meiotic spindle. Microtubules and microfilaments are integral cytoskeletal components, required for proper cell division and the maintenance of the meiotic spindle (Kim et al., 2000). Immunohistochemistry was used to visualize microtubules and actin microfilaments while Hoechst staining was used to identify the positioning of the chromosomes.

Only one intact meiotic spindle was observed out of the 188 oocytes that were exposed to saccharides. Furthermore, only 24% to 30% of these oocytes had any visible tubulin structure. This level of disruption is thought to be a major factor in the relatively low developmental rates obtained with vitrified-warmed oocytes after they are fertilized. Without an intact meiotic spindle, normal fertilization and development will not occur. A region rich in microfilaments has been reported to co-localize with the meiotic spindle (Kim et al., 2000). In our study, co-localization of actin with tubulin was observed in most of the control oocytes (93%), but was observed in only a few of the oocytes that had been subjected to dehydration. There was a similar degree of microtubule disruption regardless of the saccharide used or the medium in which it was prepared.

Staining with Hoechst dye revealed the close proximity of the chromosomes to the microtubules in all treatments with observable meiotic spindles and in all of the controls. The major cause of the microtubule disruption observed in dehydrated oocytes may be due to the severe reduction in cell volume. This may cause the displacement of cytoskeletal structures responsible for maintaining the meiotic spindle in position. Rupture of coronal extensions to the oocyte during severe dehydration may also contribute to the disruption of the cytoskeleton found in this study.

2.6 Conclusions

Bovine MII-stage oocytes behave osmotically in response to increased osmotic pressure. Also, oocytes exposed to high concentrations of saccharides exhibited a high degree of meiotic spindle disruption, which may result in the inability of these oocytes to be fertilized and to develop. The use of sucrose as the saccharide for dehydration resulted in a higher percentage of spindle disruption in both media used compared with the use of trehalose in both media. Further study is needed to determine whether the spindle can be re-organized after vitrification and before fertilization by sequential rehydration. Protective properties of trehalose as well as the use of microtubule stabilizers may also be beneficial for its use in vitrification.

CHAPTER 3
BLASTOCYST FORMATION FROM VITRIFIED BOVINE OOCYTES, ZYGOTES AND 2-CELL EMBRYOS

3.1 Introduction

Gamete and embryo preservation is proposed as being essential for conserving the genome of all species (Karow and Critser, 1997; Watson and Holt, 2001; Holt et al., 2003). Although sperm and embryos of many species are readily cryopreserved, oocyte preservation has been less successful. Many characteristics of oocytes including their low surface to volume ratio, stage of maturation and species-specific differences, such as sensitivity to chilling and osmotic shock, have contributed to their poor cryopreservation success (Parks and Ruffing, 1992). Although oocyte vitrification was developed in 1985 by Rall and Fahy, it was not for another decade that progress in oocyte preservation increased compared with traditional slow cooling protocols for oocytes.

Vitrification avoids the deleterious effects of ice formation by solidifying the intracellular water into a metastable glass-like substance by using ultra-rapid cooling rates (Taylor et al., 2004). Although there has been an increase in the success using this method, oocyte vitrification results in relatively low blastocyst development (~20%) compared with vitrified cleavage-stage embryos with >50% blastocyst development in cattle (Vajta et al., 1998). Further impeding the efficiency of oocyte vitrification is the multitude of different approaches used including the use of different mixtures of cryoprotective additives (CPAs) and their concentrations, the number of steps, length of exposure and even the holders used (Vajta, 2000). New techniques to improve its efficiency have concentrated on minimizing the volume of solution vitrified, micromanipulation of the oocyte as well as the addition of chemicals to confer stability to the cytoskeletal arrangements. However, until efficient, standardized vitrification protocols can be established for domestic species, the use of oocyte vitrification will remain a research technique instead of a commercially applied method of preserving the female genome. The objective of this study was to devise a protocol to preserve bovine oocytes and early cleavage-stage embryos with vitrification by comparing their subsequent embryonic development after in vitro fertilization (IVF).

3.2 Literature Review

3.2.1 Problems Associated with Preserving Oocytes and Embryos

One common problem in oocyte cryopreservation is their large, spherical size. Their relatively small surface-area compared with their large internal volume decreases their rate of dehydration. Dehydration is important to limit the formation of intracellular ice during crypreservation. Furthermore, during slow cooling, oocytes are exposed to CPAs for a long period of time. For rapid cooling, high concentrations of CPAs are required for rapid dehydration. Longer exposure of oocytes to CPAs has been thought to be toxic and a major cause of poor survival. The detrimental effects of the increased concentration of CPAs used in vitrification are limited due to their short exposure during the vitrification and rapid dilution during warming.

The stage of maturation, from the germinal vesicle (GV) stage to the metaphase-II (MII) stage, has been shown to influence oocyte survival post-vitrification in some species (Parks and Ruffing, 1992). At the MII stage, the DNA of the oocyte is condensed into chromosomes that are aligned along the equatorial region of the metaphase spindle. This stage of development is more susceptible to disruption than the embryo in which the DNA is in the form of decondensed chromatin. During cooling, the metaphase spindle can be disrupted and the chromosomes separated from the spindle resulting in dysjunction and aneuploidy in the developing embryo. For example, in two of many on studies of oocyte cryopreservation, only 31% of the frozen-thawed bovine oocytes (Saunders and Parks, 1999) and 16% to 19% of human oocytes (Boiso et al., 2002) had normal chromosome arrangements.

Species differences introduce other factors to cryopreservation, such as cytoplasmic lipid content and chilling sensitivity. The presence of cytoplasmic lipids in the oocytes can cause damage during the freeze-thaw process due to alterations in their physicochemical properties (Isachenko et al., 2001a). The exact cause of this damage in unknown but it may be due to the interaction of the lipids with the cytoskeleton. The lipids may cause disruption in the arrangement of microtubules and microfilaments that make up the cytoskeleton upon their premature release (Isachenko et al., 2001a). Although cytoplasmic lipids are found in many species oocytes, there is marked variation in the type and amount of lipids. Removal of cytoplasmic lipids through a process referred to as delipation has been found to increase the tolerance to chilling of cleavage-stage porcine embryos (Nagashima et al., 1994; Ushijima et al, 2004). This process has also been used in porcine GV-stage oocytes with relative success (Park et al., 2005). Although the most extensive research has been done in mice and cattle, oocytes of several other species including human have been successfully preserved using both standard cryopreservation and vitrification (e.g., Wood et al., 1993, Vajta et al., 1998, Kuleshova et al., 1999, LeGal et al., 2000, Chen et al., 2001, Chian et al., 2004, Kuwayama et al., 2005).

3.2.2 Vitrification as an Alternative Method of Preservation

Vitrification is a quick and simple technique compared with standard slow cooling or equilibrium freezing protocols that take at least 40 minutes to several hours to complete. It also is less expensive by not requiring the use of programmable freezers. Vitrification was first proposed by Luyet in 1937 as a method to avoid the damaging crystallization of intracellular water. During the standard slow cooling process, cooling rates are around 1ºC/minute whereas, during vitrification, cooling rates can exceed 20,000ºC/minute (Vajta et al., 1997). The high cooling rates obtained during vitrification are achieved by the use of very small volumes of vitrification solution, as little as 1 µl compared with 0.25 ml used in standard oocyte and embryo cryopreservation in straws. To handle such small volumes, various holders or tools have been employed such as straws, open pulled straws (OPS), insemination pipettes, flexipet-denuding pipette (FDP), electron microscope copper grids, microdrops, solid surface metal blocks, nylon coils or mesh, cryoloops and recently, Katayama et al. (2003) has recently introduced a new device, the CryoTop (for a review see Liebermann et al., 2002).

Besides the use of ultra-rapid cooling rates, the vitrification process employs high concentrations of CPAs. Vitrification uses a 10 fold higher concentration of both permeating (e.g., glycerol, dimethylsulfoxide and ethylene glycol) and nonpermeating (e.g., sucrose, glucose, trehalose) cryoprotectants. High CPAs depress ice crystal formation (Luyet and Gehenio, 1952). Because of the high concentrations of CPAs used in the vitrification procedures, rapid warming rates as well as rapid cooling rates are also important to avoid osmotic shock to the cells.

Methods used to minimize the toxic effects of CPAs include substitution of an amino group for a hydroxyl group in the CPAs to increase the ability of the solution to undergo vitrification, increasing the hydrostatic pressure of the solution to reduce the temperature at which nucleation occurs and reducing the amount of the CPA to its lowest effective concentration (Liebermann et al., 2002). The latter can be achieved either by using a nonpermeating CPA, such as a saccharide, or by using a combination of two CPAs, each of which will be at a lower concentration with the combined concentration will remain effective.

3.2.3 Current Status of Oocyte and Embryo Vitrification

The first report of successful vitrification of mammalian gametes was done using mouse embryos (Rall and Fahy, 1985). It was not for another decade that this technique was first successfully used on bovine oocytes (Martino et al., 1996) and early stage-embryos (Vajta et al., 1997). Human oocyte vitrification resulting in a live birth was achieved shortly after that (Kuleshova et al., 1999). To date, embryos at various stages of development have been vitrified in the mouse (Rall, 1987; Nakao et al., 1997; Uechi et al., 1999; Kito et al., 2003; for a review see Shaw and Jones, 2003), rat (Han et al., 2003; Kono et al., 1988), rabbit (Silvestre et al., 2003; Cai et al., 2005), cat (Crichton et al., 2003), a mustelid (Piltti et al., 2004), pig (Dobrinsky et al., 2000; Gajda and Smorag, 2000; Berthelot et al., 2000; 2001; Gajda and Smorag, 2002; Esaki et al., 2004), sheep (Naitana et al., 1997; Papadopoulos et al., 2002; Dattena et al., 2004), goat (El-Gayar and Holtz, 2001; Begin et al., 2003), several bovid species (cattle: Massip et al., 1986; Rizos et al., 2003; buffalo: Hufana-Duran et al., 2004), llama (Aller et al., 2002), horse (Oberstein et al., 2001; Moussa et al., 2005), a nonhuman primate (Yeoman et al., 2001)

and the human (Son et al., 2002; 2005). Although this list does not contain all of the publications for each species, it demonstrates the large number of studies involving embryo vitrification.

Oocyte vitrification has, however, been for less successful. Oocytes at different maturational stages (GV, MI or MII) have been successfully vitrified in the mouse (van der Elst et al., 1992; Wood et al., 1993; Hotamisligil et al., 1996; Isachenko and Nayudu, 1999; Chen et al., 2000; 2001b; Lane and Gardner, 2001), hamster (Lewin et al., 1990; Wood et al., 1993; Lane et al., 1999b), pig (Park et al., 2005), cat (Murakami et al., 2004), sheep (Isachenko et al., 2001b), goat (Begin et al., 2003), a few bovid species (cattle: Vajta et al., 1998; Le Gal et al., 2000; Rho et al., 2002; Men et al., 2003a,b; Chian et al., 2004; Modina et al., 2004; buffalo: Dhali et al., 2000; Wani et al., 2004), horse (Maclellan et al., 2002) and the human (Kuleshova et al., 1999; Wu et al., 2001a; Kuwayama et al., 2005). Although there has been some success in preserving oocytes and embryos of domestic species, very few exotic or endangered species have been preserved using vitrification. This is likely due to the limited genetic material available to determine efficient methods of vitrification in these species.

3.2.4 Components of the Vitrification Medium

Various chemicals and solutions are used to prepare the vitrification medium. All media begin with a similar basic solution with buffering capability, usually either a phosphate-buffered saline or a HEPES-buffered medium (Liebermann et al., 2002). Both of these allow the manipulations to be done at the bench without experiencing a pH shift whereas, a bicarbonate-buffered medium must be gassed to maintain a proper pH throughout the protocol.

The next major component is the CPA used to confer protection during the cooling process. High concentrations of CPAs are used to allow the intracellular and extracellular environments to solidify into a vitreous state when cooled to subzero temperatures. One common CPAs used in vitrification is ethylene glycol (EG). It appears to be less toxic to both oocytes and embryos and rapidly diffuses across the cell membranes (Emiliani et al, 2000). Other commonly used CPAs are 1,2-propanediol (PrOH), glycerol and dimethylsulfoxide (DMSO).

In addition, sugars such as sucrose, glucose, fructose, raffinose and most recently trehalose, are often an important part of the vitrification solution. Sugars with high molecular weights, like disaccharides, do not permeate the cell and can, therefore, help in the dehydration of cells during freezing by increasing the extracellular solute concentration (Muldrew et al., 2004). However, the components and their concentrations used to make up the vitrification solution vary from species to species.

Most current vitrification solutions contain sodium. Recent studies have demonstrated the detrimental effects of sodium on mouse oocytes during freezing (Stachecki et al., 1998a,b). It has been proposed that a high electrolyte concentration may occur during cooling (Lovelock, 1954). During freezing, the sodium/hydrogen exchange system is impaired and salt loading can occur. This creates a hypertonic intracellular environment resulting in cell lysis (Acker and McGann, 2003). Stachecki et al. (1998a,b) proposed the use of choline as a substitute cation for sodium and have reported that significantly higher blastocyst rates resulted from oocytes frozen in the choline-substituted medium (CJ2) compared with those frozen in standard cryopreservation media. Choline is an organic osmolyte that is thought to confer protection to cellular membranes during freezing (Stachecki et al, 2002). Unlike sodium, choline is thought not to cross the cell membrane and would therefore not disrupt the intracellular ion load. However, CJ2 medium has only been employed using conventional freezing methods and has not been used in conjunction with vitrification to date.

3.3 Materials and Methods

All compounds unless otherwise stated were purchased from Sigma Chemical Company, St. Louis, MO.

3.3.1 Experimental Design

In this experiment, MII-stage bovine oocytes were allotteded to one of the following nine treatment groups: Control oocytes (n = 251) subjected to standard in vitro production (IVP) (Control IVP), MII-stage oocytes (n = 116) vitrified using mTCM (mTCM-MII), MII-stage oocytes (n = 114) vitrified using mCJ2 (mCJ2-MII), IVF derived presumptive zygotes (n = 131) vitrified after 13 to 15 hours of IVF (Zygote), IVF derived 2-cell embryos (n = 122) vitrified after IVF and cultured for 28 to 29 hours (2-Cell) and with each treatment listed oocytes and embryos were used as controls by subjecting them to these same solutions without vitrification: mTCM-MII CPA (n = 116), mCJ2-MII CPA (n = 107), Zygote CPA (n = 89) and 2-cell CPA (n = 70). These MII-stage bovine oocytes were used as controls to assess the effect of the vitrification solutions alone on embryonic development. They were subjected to the vitrification and warming process omitting the loading onto the CryoTop and plunging into liquid nitrogen. There were four treatment replications in this experiment.

3.3.2 Oocyte Collection, Fertilization and Culture

Bovine cumulus-oocyte-complexes (COCs) were collected by a commercial company (BoMed; Madison, WI) from ovaries harvested from mature cows at an abbatoir. The COCs were then shipped overnight by express courier in maturation medium, in a portable incubator (temperature 37°C - 39°C) to the laboratory at the Audubon Research Center in New Orleans, Louisiana. Maturation medium consisted of TCM-199 (12340-030; Gibco, Grand Island, NY) with 0.5 M L-glutamine (G-8540), 1% fetal bovine serum (FBS) (SH30070.02; Hyclone Laboratories Inc., Logan, UT), 10 μg/ml of bovine LH (Luteinizing Hormone) (L-9773), 0.7 IU/ml of FSH (Follicle Stimulating Hormone) (F-2293), 0.01 mg/ml of estradiol (E-2758) and 0.1% penicillin/ streptomycin (15140-122; Gibco). In four replicate runs, COCs were randomly allocated to one of nine treatment groups. COCs in the control in vitro production group (Control IVP; n = 251), were subjected to standard in vitro fertilization (IVF) and then cultured in CR1aa medium (Rosenkrans and First, 1994) in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 38°C for up to 9 days.

The vitrification treatment groups consisted of COCs vitrified at the presumptive MII stage (in two different media), or COCs inseminated and then vitrified either as presumptive zygotes or as 2-cell embryos. MII-stage oocytes were subjected to vitrification in mTCM or mCJ2 medium (n = 116 and n = 114, respectively) after 20 to 21 hours of IVM and then fertilized by IVF. TCM-199 was modified by adding 10% FBS (mTCM) and CJ2 medium was also modified by the addition of 10% FBS (mCJ2). These modified solutions were used to prepare both the vitrification and warming media for each of theses groups of oocytes, as described below. Presumptive zygotes (n = 131) were vitrified after 13 to 15 hours of insemination. The 2-cell embryos (n = 122) resulting from IVF that were cultured for 28 to 29 hours before vitrification.

For in vitro embryo production, COCs were removed from in vitro maturation (IVM) at 22 hours of maturation, washed twice in Brackett-Oliphant medium (BO medium) (Brackett and Oliphant, 1975) supplemented with 3% bovine serum albumin Fraction V (BSA) (A-4503) (BSA-BO, medium used in the insemination droplet) and then transferred to insemination droplets. Insemination droplets were prepared using a 0.5 ml straw of cryopreserved Holstein bull (7H5188; GeneX, Baton Rouge, LA) sperm in all replicates. One straw was thawed in air for 30 seconds, then placed in a water bath (38ºC to 40ºC) for 1 minute. The spermatozoa were mixed with 10 ml of BO medium supplemented with 1 mM caffeine sodium benzoate (BO-Caff) (C-4144) and centrifuged for 6 minutes at 500 x g in a 15 ml microcentrifuge tube. The supernatant discarded and the pellet re-suspended in another 10 ml of medium and re-centrifuged. The final pellet was re-suspended in an equal volume of BO-caffeine and 0.6% BSA-BO media. The volume of medium to resuspend the sperm was adjusted so that the insemination droplets contained ~1 x 10^6 sperm/ml.

Groups of 25 to 30 COCs were placed into each 100 µl droplet of the sperm suspension in a 35 mm dish and covered with 2 to 3 ml of mineral oil (M-5310) and incubated for 12 hours at 38°C in 5% CO_2 in humidified air. After insemination, the presumptive zygotes were stripped of their cumulus cells by vortexing them for 3 minutes in TCM-199 supplemented with 2.2% hyaluronidase (H-3506). Zygotes were washed in two 35 mm dishes each containing 2 to 3 ml CR1aa day 0 to 3 medium (CR1aa day 0-3) and then placed into 80 µl droplets of CR1aa day 0-3 (10 to 15 zygotes/droplet) in a 35 mm petri dish, covered with mineral oil and incubated. CR1aa day 0-3 medium consisted of CR1aa stock solution (Rosenkrans and First, 1994) supplemented with 2% BME amino acids (B-6766), 1% MEM nonessential amino acids (11140-050; Gibco - Invitrogen), 50 µg/ml of gentamicin solution (15750-060; Gibco - Invitrogen), 0.01 M L-glutamine (G-5763) and 3% BSA Fraction V (A-7511). The dish was then placed into a sealed bag (0181216; Kapak sealpak pouches - Fisher Scientific, Pittsburg, PA) and filled with 5% CO_2, 5% O_2 and 90% N_2 gas and incubated at 38°C.

On day 3, dividing embryos were transferred to 80 µl droplets of CR1aa day 3 to 7 medium (CR1aa day 3-7) in a 35 mm dish covered in oil, bagged and incubated further as previously described. CR1aa day 3-7 medium consisted of CR1aa stock solution supplemented with 2% BME amino acids, 1% MEM nonessential amino acids, 50 µg/ml of gentamicin solution, 0.01 M L-glutamine, 0.5% FBS and 3% BSA Fraction V.

3.3.3 Oocyte and Embryo Vitrification

The vitrification solution for all treatment groups, with the exception of those oocytes that were to be vitrified in the CJ2 solution, consisted of mTCM containing 20% EG (E-9129) + 20% DMSO (D-4540) + 0.65 M trehalose (T-5251). Only in the mCJ2 MII-stage oocyte treatment group, was the TCM-199 replaced by a choline-based solution (CJ2) (Stachecki et al., 1998a).

Vitrification of oocytes and embryos was completed using the same protocol. Briefly, groups of 6 to 12 oocytes or embryos were rinsed in mTCM or mCJ2, transferred into a 35 mm dish containing 5% EG + 5% DMSO prepared in mTCM or mCJ2 for 45 seconds, then transferred into a 35 mm dish containing 10% EG + 10% DMSO in the appropriate diluent for 45 seconds and then transferred into the vitrification solution.

Once in the vitrification solution, the oocytes or embryos were pipetted in <0.1 µl volume of vitrification medium onto the surface of a thin polypropylene strip attached to a plastic handle, called a CryoTop (Katayama et al., 2003). The entire CryoTop was immediately plunged directly into liquid nitrogen (LN_2). Protective sheaths were affixed to the CryoTops to protect the oocytes and embryos and then they were loaded onto canes and stored in LN_2 for ~2 hours (Figure 3.1).

3.3.4 Oocyte and Embryo Warming

Before being warmed, the protective sheath was removed. Then, the vitrified samples were warmed and liquefied by rapidly transferring one CryoTop at a time directly from LN_2 into a 35 mm dish containing ~2 to 3 ml of 0.25 M trehalose prepared in mTCM or mCJ2 at 37°C. Oocytes or embryos were then sequentially transferred at 1-minute intervals into 0.19 M and 0.13 M trehalose also prepared in either diluent. They were washed in mTCM medium and then transferred to the appropriate culture medium and incubated at 38°C. This process was repeated until all oocytes or embryos were recovered. All embryos were cultured as previously described for control IVP embryos. Cleavage was evaluated on day 3 post-insemination and blastocyst development was assessed on days 7 and 9 post-insemination.

3.3.5 Controls for the Effect of the Media on Embryonic Development

To determine the effect on embryonic development of the media alone, COCs and embryos were processed as described previously for each treatment, but they were not placed onto the CryoTop or plunged into LN_2 (nonvitrified). For all CPA controls, COCs were sequentially moved through the vitrification CPAs and then were immediately placed directly into the warming solutions in decreasing concentrations.

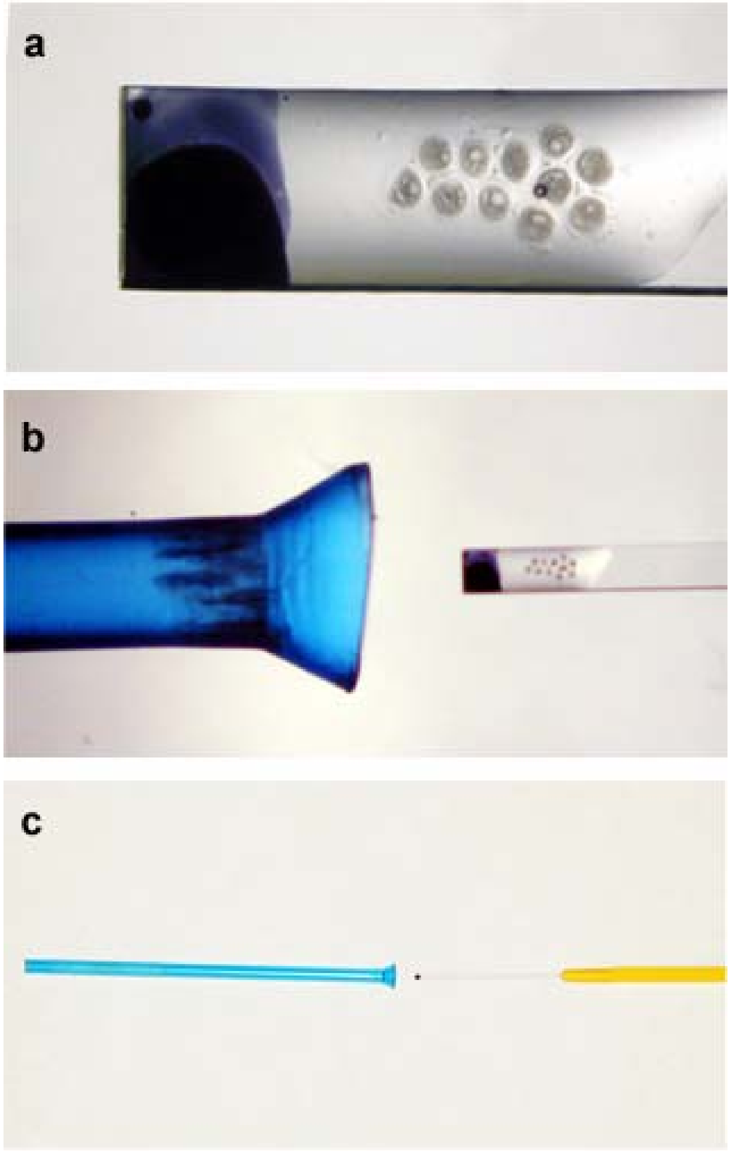

Figure 3.1 Photographs of the CryoTop used for oocyte and embryo vitrification. (a) Bovine oocytes loaded onto the tip of a CryoTop in minimal volume, (b) CryoTop with oocytes being placed into its protective sheath and (c) Lower magnification of CryoTop showing holder (yellow) and protective sheath with plug (blue).

After being rinsed in the last warming solution, the oocytes or embryos were placed into mTCM medium until all oocytes were processed. In the case of the CPA control oocytes, this step was followed by insemination and then culture. In the case of the presumptive zygotes and 2-cell embryos, they were placed into culture and assessed as previously described.

3.3.6 Statistical Analysis

Cleavage and blastocyst rates across treatment groups and culture intervals were analyzed for statistical significance by use of Chi-square tests ($P \leq 0.05$ statistically different) (Instat Graphpad Version 3.0, San Diego, CA).

3.4 Results

3.4.1 Controls for Vitrification Media and Procedures

To determine the effect of the cryoprotectants alone on the embryonic development oocytes, presumptive zygotes and 2-cell embryos were exposed to the vitrification and warming solutions without vitrification. Oocytes exposed to vitrification and warming solutions prepared in either mTCM (mTCM-MII; n = 116) or mCJ2 medium (mCJ2-MII; n = 107) and the presumptive zygotes (n = 89) had similar cleavage (65%, 62% and 74%, respectively) and blastocyst rates (13%, 8% and 15%, respectively). Cleavage rates of oocytes or presumptive zygotes exposed to vitrification and warming solutions were not significantly different from those of the Control IVP oocytes (71%). In contrast, blastocyst development on day 9 post-insemination of oocytes (13% and 8%) and of presumptive zygotes (15%) exposed to vitrification and warming solutions was significantly lower than the Control IVP oocytes (29%). Although 2-cell embryos (n = 70) had a significantly higher cleavage rate (84%) (≥4-cell development) compared with all other treatment groups (mTCM-MII, mCJ2-MII and Zygotes), they exhibited blastocyst rates on day 9 (33%) similar to those of the Control IVP oocytes (Figure 3.2).

3.4.2 Oocyte and Embryo Vitrification

In this study, the development of vitrified oocytes and embryos was examined. All treatments were compared with oocytes subjected to standard IVF procedures. Two vitrification solutions were used for oocytes, mTCM (mTCM-MII) and mCJ2 medium (mCJ2 MII). Photographs of cleavage-stage and blastocyst-stage embryos produced from control oocyte groups and vitrified oocytes and embryo groups are shown in Figures 3.3 and 3.4. Of the vitrified-warmed oocytes, 54% of those vitrified using mTCM (n = 116) and 48% of those vitrified using mCJ2 (n = 114) cleaved by day 3 post-insemination. These cleavage rates were significantly lower than the 71% cleavage rate of the control IVP oocytes (n = 251). The vitrified oocytes also exhibited a decrease in blastocyst rates (4% each, respectively) and none hatched by day 9 post-insemination compared with 29% blastocysts and with 3% that hatched in the control IVP oocytes.

Vitrified-warmed presumptive zygotes (n = 131) responded like the vitrified-warmed oocytes, with 53% cleavage and only 7% of them forming blastocysts. There was no statistical difference between the cleavage and blastocyst rates of vitrified oocytes and those of presumptive zygotes. However, the vitrified-warmed 2-cell embryos had significantly higher cleavage and blastocyst rates than the vitrified-warmed oocytes and the presumptive zygotes. Of the vitrified-warmed 2-cell embryos (n = 122), 69% cleaved (≥4-cell development) and 28% developed into blastocysts by day 9 post-insemination,

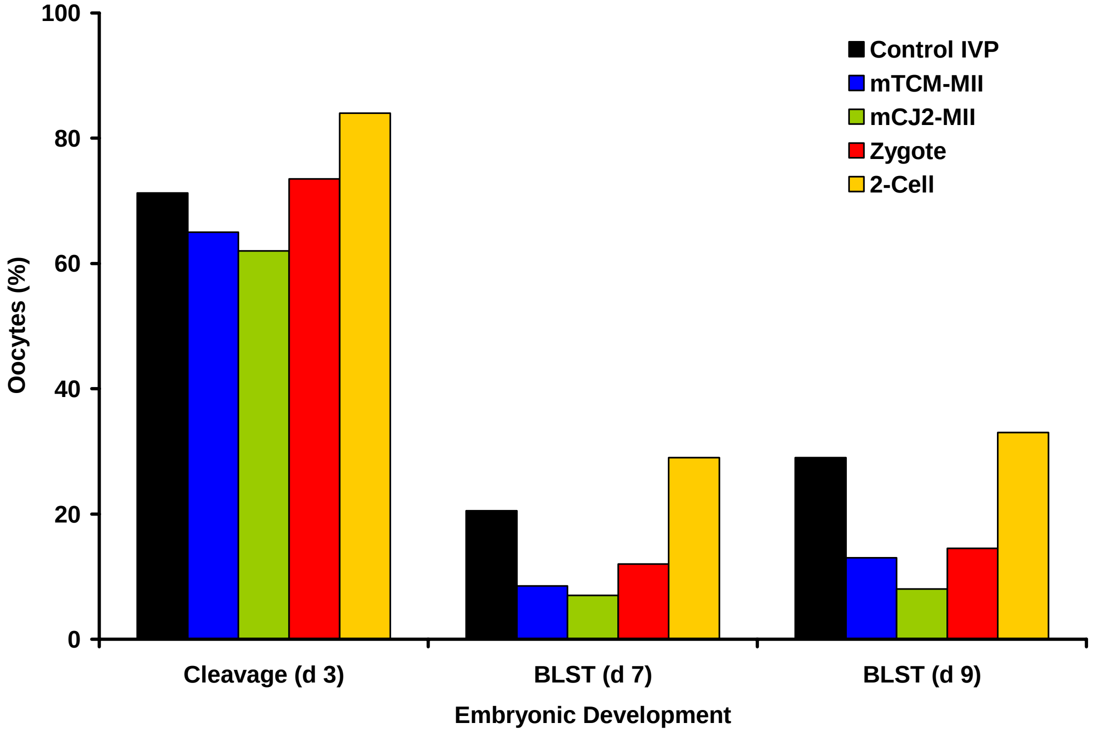

Figure 3.2 Average cleavage (day 3) and blastocyst rates (day 7) of control in vitro produced (IVP) and oocytes and embryos exposed to vitrification and warming solutions alone (CPA controls). BLST = Blastocyst.

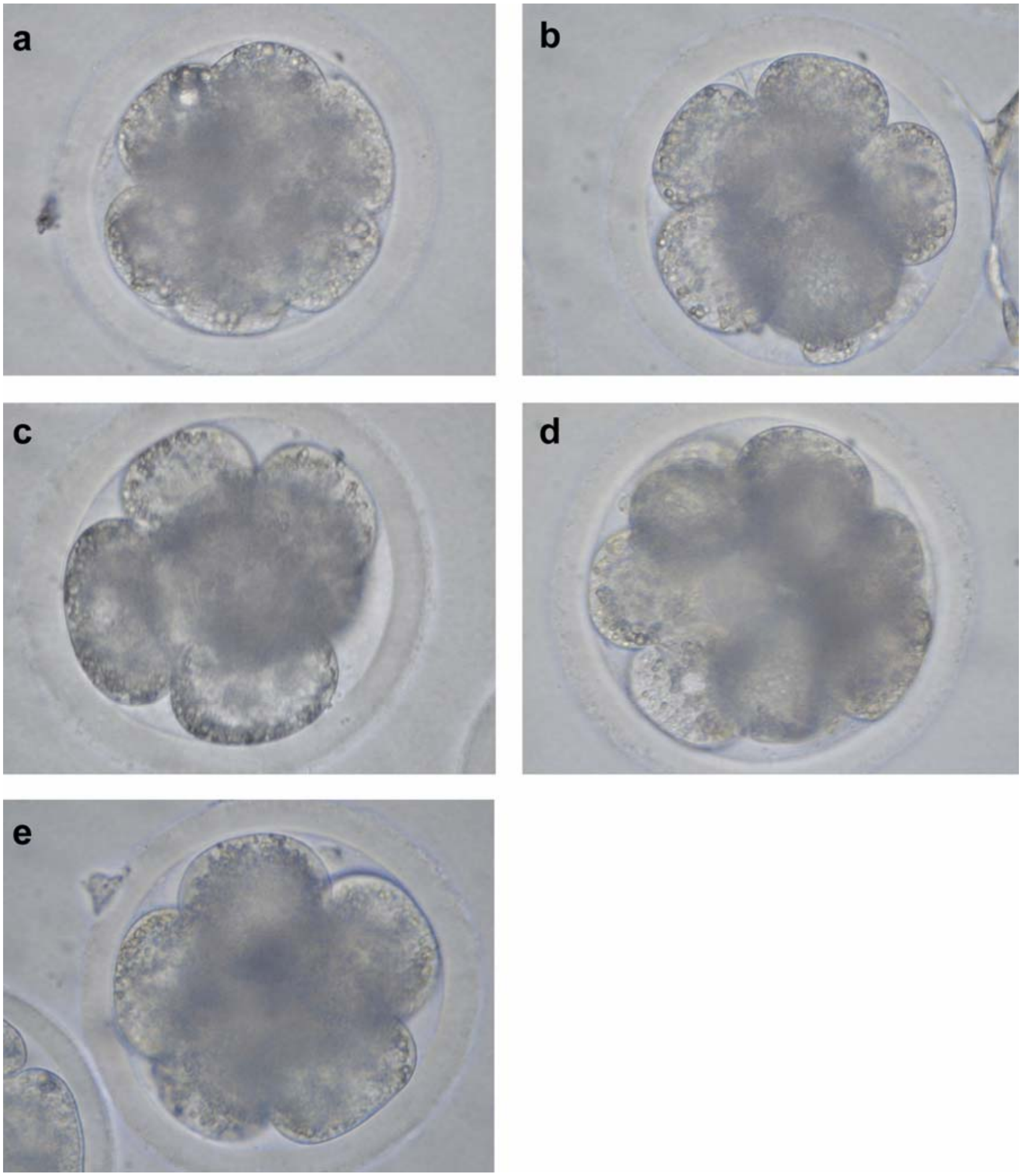

Figure 3.3 Photographs of cleavage-stage embryos produced from vitrified oocytes and embryos and control IVP embryos on day 3 post-insemination. (a) Control IVP embryos, (b) Vitrified-warmed MII-stage oocytes prepared in mTCM and (c) mCJ2, (d) Vitrified-warmed presumptive zygote and (e) Vitrified-warmed 2-cell embryo. Magnification photographed with a 40X objective.

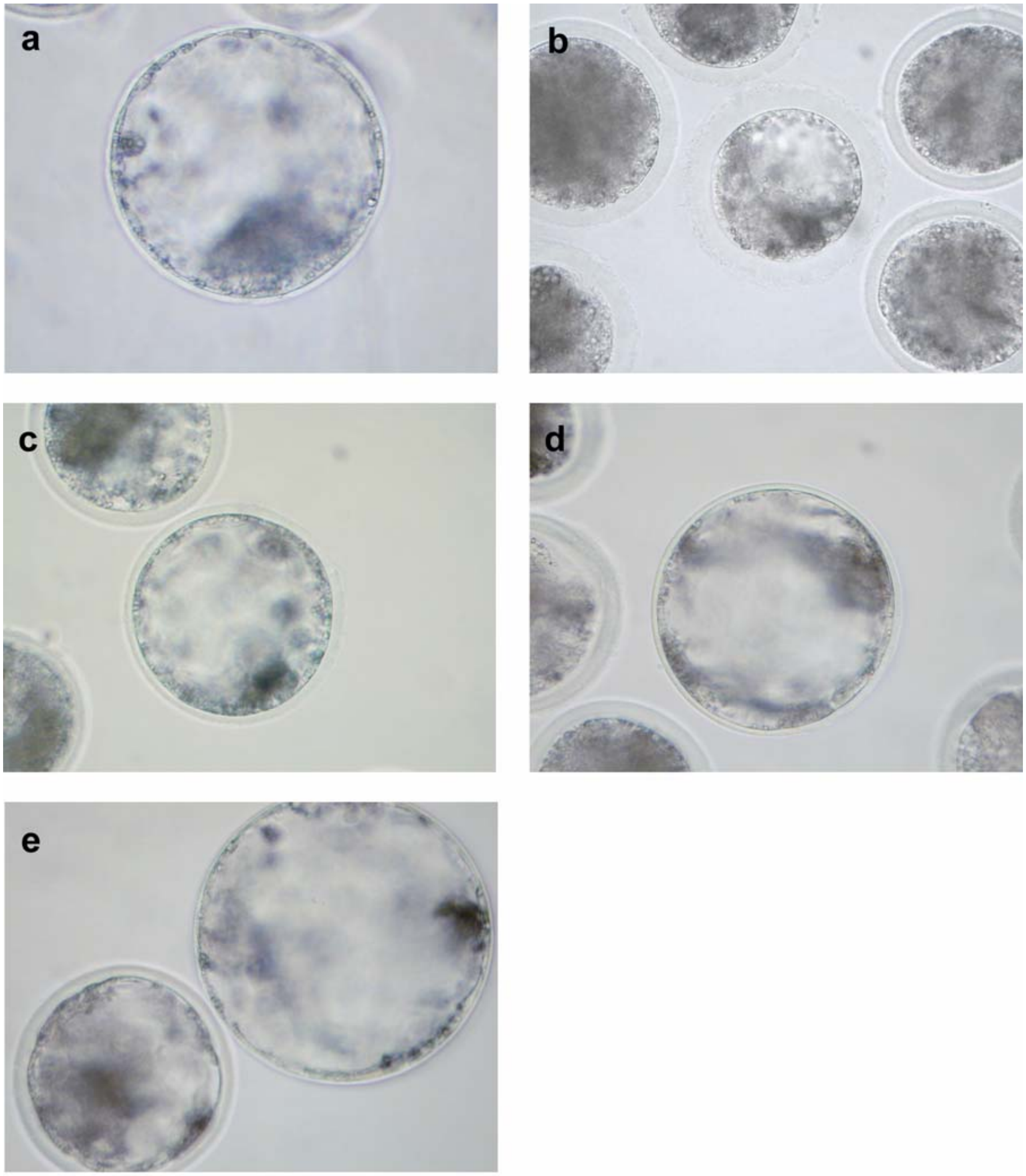

Figure 3.4 Photographs of blastocyst-stage embryos produced from vitrified oocytes and embryos and control IVP oocytes on day 9 post-insemination. (a) Control IVP embryo, (b) Vitrified-warmed MII-stage oocytes prepared in mTCM and (c) mCJ2, (d) Vitrified-warmed presumptive zygote and (e) Vitrified-warmed 2-cell embryo. Magnification photographed with a 20X objective.

and 2% of them hatched. These results were not significantly different from the development rates obtained for the control IVP oocytes (Figure 3.5 and Table 3.1).

3.5 Discussion

Although there have been numerous studies of vitrification of bovine embryos, the majority of these have been with late-stage embryos. Only one study to date used OPS vitrification to cryopreserve zygotes and early cleavage-stage bovine embryos (Vajta et al., 1998). Recently, a new tool called the CryoTop was developed to allow for an even smaller volume of vitrification medium to be used and therefore yields quicker cooling and warming rates for embryos (Katayama et al., 2003). The OPS methods of vitrification uses a 1.5 µl volume compared with a <0.1 µl volume using the CryoTop. Cooling and warming rates are also increased from ~16,000ºC/minute to 23,000ºC/minute for cooling and 14,000ºC/minute to 42,000ºC/minute for warming (Kuwayama et al., 2005). This new instrument has been used to improve the efficiency of successful vitrification of human oocytes (Kuwayama et al., 2005). Those investigators obtained a maximum of 50% blastocyst development using this technique on embryos compared with 0% using standard equilibrium freezing.

The objective of the experiment described herein was to use the CryoTop as a device for the vitrification of early cleavage-stage bovine embryos, as assessed by their embryonic development in vitro. The development rate of presumptive zygotes after vitrification was not significantly different from that of vitrified oocytes. This is not surprising as the zygotes are still unicellular and have a similar surface-area to volume

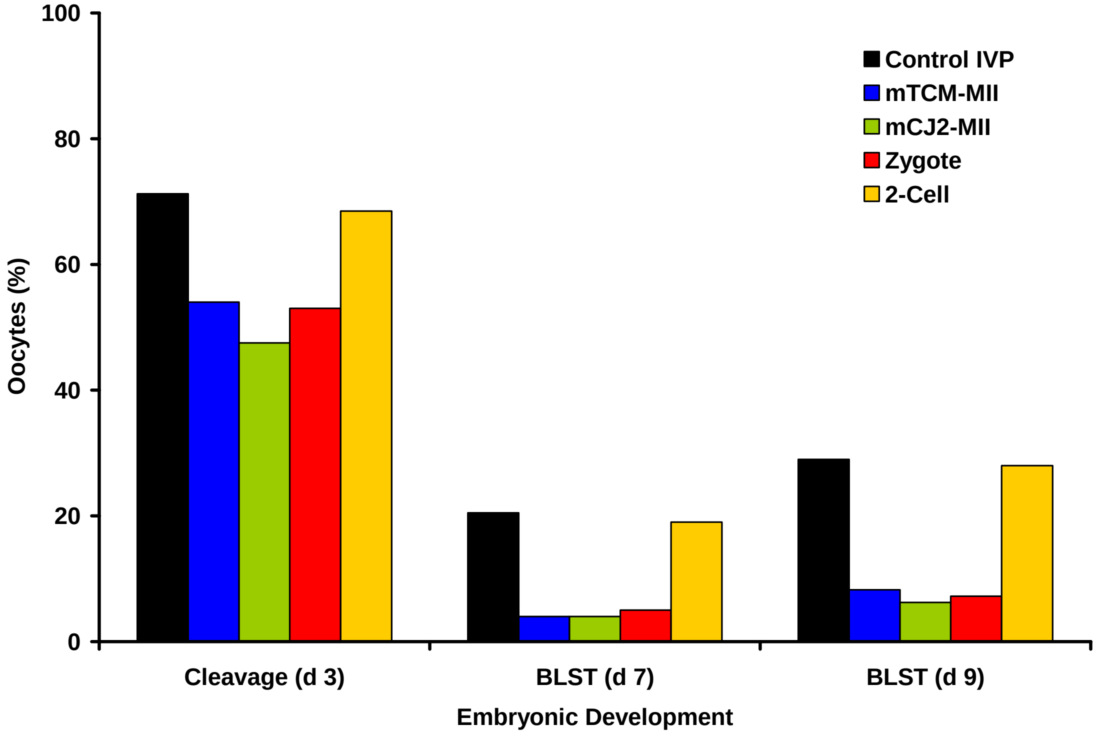

Figure 3.5 Average cleavage and blastocyst rates of control and vitrified oocytes and embryos. BLST = Blastocyst.

Table 3.1 Summary of embryonic development of control and vitrified bovine oocytes, presumptive zygotes and 2-cell embryos

		Oocytes/embryos developing			
Treatment	No. of oocytes	CLVG (d 3)	BLST (d 7)	BLST (d 9)	HBLST
Control IVP	251	71	21	29	3
mTCM-MII	116	54*	4*	8*	0
mCJ2-MII	114	48*	4*	6*	0
Presumptive Zygotes	131	53*	5*	7*	0
2-Cell Embryos	122	69	19	28	2

mTCM-MII = mature oocytes vitrified in mTCM and then fertilized by IVF, mCJ2-MII = mature oocytes vitrified in mCJ2 and then fertilized by IVF, CLVG = Cleavage, BLST = Blastocysts, HBLST = Hatched blastocysts.

*Statistical difference $P<0.05$; Chi-square analysis of each treatment was compared with IVP controls (Control IVP) within columns.

ratio as the unfertilized oocyte. However, the presence of the meiotic spindle in oocytes is usually thought to make them more susceptible to damage compared with that of zygotes. Also, the percentage of oocytes that were not mature before IVF and thus, could not have been fertilized is similar to that in the vitrified oocyte group (~20%).

In contrast, the 2-cell embryos are only selected after their first cleavage and therefore should have higher developmental rates, since they have been selected. These embryos had a similar or higher developmental rates compared with the IVP controls. Higher survival of 2-cell embryos may also result from their ability to dehydrate quicker due to their higher surface-area to volume ratio of the cell membranes compared with the unicellular zygote. Having been fertilized and having cleaved, the 2-cell embryos were much more resistant to the deleterious effects of cryoprotectants and vitrification. A similar result has also been reported with early and later stages of cleaved bovine embryos that were vitrified with the OPS method (Vajta et al., 1998). However, these investigators found a higher blastocyst rate for zygotes (30%) compared with the blastocyst rate found for presumptive zygotes in this study (7%).

The increased cooling and warming rates obtained using the CryoTop yields significantly higher survival and developmental rates of mature bovine oocytes compared with other vitrification methods, such as the OPS (Kuwayama et al., 2005). The results for cleavage of vitrified oocytes in mTCM vs. mM2 (60% vs. 54%) noted by other investigators (Kuwayama et al., 2005) were similar to those obtained in this study, however, blastocyst development rate was lower (23% vs. 8%). This decrease may be due to the quality of the oocytes used or the season in which the experiments were conducted. The cleavage (range: 22% to 76%) and blastocyst (range: 3% to 15%) rates observed in this experiment were not different from previous reports using different instruments for oocyte and embryo vitrification (Martino et al., 1996; LeGal et al., 2000; Rho et al., 2002; Modina et al., 2004).

Currently, most cell handling and cryopreservation media contain sodium salts (Stachecki et al., 1998b). However, several studies have mentioned that sodium toxicity is the major factor in cell damage during cryopreservation. This toxicity has been attributed to alteration of cellular function and disruption of the cell membrane. Thus a suitable replacement for sodium was examined and choline was chosen. Using a choline-based medium, Stachecki et al. (1998a,b) compared the embryonic development of mouse oocytes post-vitrification, using two different diluents including the standard carbonate-buffered medium, TCM-199 containing sodium and this choline-substituted medium, CJ2.

With the sodium-free medium, Stachecki and colleagues obtained a blastocyst rate greater than 50% (Stachecki et al., 1998b; Stachecki and Willadsen, 2000) compared with 9% using a sodium-based medium. However, in the present study, no significant difference was found in the cleavage and blastocyst rates of bovine oocytes that were vitrified in either mTCM or mCJ2. There was also no difference in the effect of either medium on development of oocytes exposed to the media alone (CPA Controls). Thus, this study demonstrates that CJ2 is a suitable alternative medium for bovine oocyte vitrification. Although there was no significant increase in development, embryos derived from vitrified oocytes using a choline-substitute medium seemed to develop faster and they were of better quality, as judged by blastomere size and lack of fragmentation.

Due to the high concentrations of CPAs used in vitrification protocols, the effect of the vitrification and warming solutions must be examined separately to determine whether the decreased survival is due to the vitrification process or to the toxicity of the medium used. In this study, each treatment group was exposed to the vitrification and warming solutions but without being vitrified and were then placed into culture. There was no effect of the solutions on the cleavage rates, but there was a decrease in blastocyst development of the vitrified oocytes and zygotes compared with control IVP oocytes. These results suggest that there was no block to fertilization due to changes in the zona pellucida or plasma membrane as a result of the media. However, the decrease at the blastocyst rate may be due to detrimental effects on the chromosomes that are activated at the 8- to 16-cell stage in cattle. Other organelles and cytoskeletal components may also be affected by the high concentrations of cryoprotectants used during the vitrification process.

In summary, more than 50% of vitrified oocytes and presumptive zygotes cleaved after vitrification, but this rate was significantly lower when compared with control IVP oocytes. Vitrified 2-cell embryos had greater cleavage and blastocyst rates compared with vitrified oocytes and zygotes and were not different from the control IVP oocytes. Exposure to vitrification and warming solutions had little effect on cleavage rates compared with control IVP oocytes but did have an effect on blastocyst development. In this study, 2-cell embryos were more resistant to the deleterious effects of cryoprotectants and vitrification.

3.6 Conclusions

Bovine oocytes and early-cleavage stage embryos can be vitrified using the CryoTop device. Using this technique, 2-cell embryos are more resistant to damage caused by the vitrification and warming media as well as to the vitrification process. Unfertilized oocytes behaved similarly to presumptive zygotes in both their ability to survive vitrification and their susceptibility to these high concentrations of cryoprotectants. It may be beneficial to decrease the concentration of CPAs used in their vitrification so as to increase their blastocyst rates. In this study, use of a choline-substituted medium was as efficient for the use in bovine oocyte vitrification as TCM-199, the standard bovine embryo handling medium. Although all stages of oocytes and embryos evaluated in this study cleaved after subsequent culture post-vitrification, further study is needed to improve the efficiency of this procedure before it can be made commercially available to the livestock industry.

CHAPTER 4
THE EFFECT OF VOLUMETRIC CHANGES OF FELINE OOCYTES DEHYDRATED WITH TWO SACCHARIDES

4.1 Introduction

Cryopreservation of feline oocytes and embryos would allow assisted reproductive techniques (ART) to be applied to domestic as well as to endangered feline species. All of the feline species, with the exception of the domestic cat, are listed as either endangered or threatened by the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). Although embryo cryopreservation has been successfully applied to both domestic and a few nondomestic species, oocyte cryopreservation has been more problematic. To date, successful oocyte cryopreservation resulting in blastocyst formation has only been reported in one study (Murakami et al, 2004), and no live young were born.

4.2 Literature Review

4.2.1 Oocyte Dehydration in Other Species

Numerous studies have been completed to determine the effect of water movement in various cell types exposed to anisotonic solutions. Membrane permeability is a fundamental property of cells, which is specific for each cell type (Leibo, 1980). Previous studies have demonstrated that osmotic shock is lethal to cells and needs to be considered when preparing a cryopreservation protocol (Oda et al, 1992; Agca et al., 2000). Osmotic shock may occur when a cell is rapidly transferred from a hypertonic solution into an isotonic solution. The cell will expand beyond its original isotonic volume as water enters rapidly and this can cause cell lysis. The physiological response of cells to anisotonic solutions is to undergo volume changes. During cryopreservation, volume changes occur during the exposure to the cryoprotectant additives (CPAs), during freezing, during warming and during CPA removal. If these changes are large and abrupt, cell membranes are damaged causing cell lysis.

Numerous studies have examined the osmotic behavior of mammalian oocytes and embryos. To date, most reports have been on either the mouse (e.g., Leibo, 1980; Toner et al., 1991; Oda et al., 1992; Pedro et al., 1997) or the cow (e.g., Ruffing et al., 1993; Agca et al., 2000). In these studies, the effect of osmotic stress on fertilization and embryonic development was examined on immature and mature oocytes. In the mouse, unfertilized mouse oocytes and zygotes were reported to behave as ideal osmometers (Leibo, 1980). To determine the osmotic behavior of a cell, it is placed into increasingly hypertonic solutions and its volume is measured at equilibrium. The results are plotted as the cell volume relative to the isotonic volume as a function of the reciprocal of the solution's osmolality in a Boyle van't Hoff plot. Extrapolation of the linear regression line for a cell in an infinitely concentrated solution represents the solids volume of the cell. Nonosmotic volumes of oocytes range from 18% in the mouse to 32% in the cow, respectively (Leibo, 1980; Ruffing et al., 1993).

4.2.2 Effects of Cryoprotectants on Embryonic Development

Besides the injury of volume excursions, oocytes and ova must tolerate exposure to high concentrations of CPAs generally used in cryopreservation. Detrimental effects of CPAs depend on three factors: their concentration, the temperature at which the cells are exposed and the duration of CPA exposure. Adding to the problem is the fact that there are differences in sensitivity of oocytes among species to various CPAs. For example, rabbit oocytes exposed to propanediol (PrOH) and dimethylsulfoxide (DMSO) exhibit depolymerization of their microtubules and disruption of the actin microfilaments (only PrOH) (Vincent et al., 1989). In the mouse, PrOH and DMSO cause disruption of actin microfilaments and at low concentrations of PrOH caused disorganization of the microtubules (Johnson and Pickering, 1987; Vincent et al., 1990a; Joly et al., 1992).

These effects are temperature dependant as during cooling. Also, exposure of bovine oocytes to ethylene glycol (EG) at room temperature resulted in abnormal spindle morphology greater than with cooling alone (Saunders and Parks, 1999). There have been few studies of the effects of various CPAs on feline oocytes. One study examined the effects of PrOH and EG on mature cat oocytes and found that all concentrations used (except 1.5 M PrOH) caused disruption of the meiotic spindle (Comizzoli et al., 2004). The effects of DMSO and that of a combination of CPAs have not yet been reported to date in the cat. Therefore, the choice of CPAs needs to be examined for each species before an appropriate protocol can be established.

4.2.3 Effect of Dehydration on the Oocyte

Dehydration can cause detrimental effects to the cell by altering the structure of the cytoskeleton and displacing the chromosomes. This effect is thought to be more severe in metaphase-II (MII) oocytes because of the presence of the meiotic spindle. If the spindle is disrupted, normal fertilization does not occur because the chromosomes are dispersed. This results in disjunction during syngamy and the resulting embryos are most often aneuploid. As in the case of CPAs, severe dehydration can disrupt the actin microfilaments and the microtubule arrangement of the meiotic spindle. Besides the disruption of cytoskeleton, the cell membranes may also be affected by severe dehydration. There is potential loss of membrane so that when the cell is returned to isotonic conditions, the cell can no longer expand to its original volume and lysis occurs. This loss of membrane is thought to result from the fusion of membranes in plant cells (Wolfe and Bryant, 1999). This theory has been disputed on the grounds that the hydrostatic forces of the lipid bilayers would make this impossible unless all water of hydration was removed (Mazur, 2004). Overall, many factors affect the survival of oocytes during the dehydration process of preservation using cryoprotectants. The objective of this experiment was to determine the effect of volumetric changes on the subsequent embryonic development after in vitro fertilization of dehydrated feline oocytes.

4.3 Materials and Methods

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise stated.

4.3.1 Experimental Design

In the first experiment (Experiment 1), feline oocytes were allotted to one of three treatments for dehydration as follows: MII-stage oocytes (n = 10) were exposed to mM199 alone (0 M saccharide) (Control mM199), MII-stage oocytes (n = 17) exposed to increasing concentrations of sucrose (0.15, 0.30, 0.50, 0.65 and 0.99 M) prepared in mM199 and MII-stage oocytes (n = 18) exposed to trehalose prepared in mM199 (0.13, 0.25, 0.35, 0.55 and 0.65 M). This mM199 medium was prepared using M199 Earle's salt solution supplemented with 10% fetal bovine serum.

In the second experiment (Experiment 2), MII-stage feline oocytes were allotted to three treatment groups as follows: Control MII-stage oocytes (n = 38) exposed to mM199 alone, fertilized in vitro, and then cultured to assess embryonic development, MII-stage oocytes (n = 15) exposed to sucrose prepared in mM199 and MII-stage oocytes (n = 14) exposed to trehalose prepared in mM199, as in the first experiment, and then fertilized in vitro to assess the effects of dehydration on their embryonic development.

4.3.2 Oocyte Collection and Maturation

Ovaries were collected by local veterinary clinics during standard ovary/hysterectomies of queens at various stages of their reproductive cycle and transported in a 100 ml plastic sample vial (9853Q47; Thomas Scientific, Swedesboro, NJ) containing 50 ml of TL HEPES (04-616F; Cambrex Bio Science, Walkersville, MD) supplemented with 50 μg/ml of gentamicin (G-1397) in a cooler at room temperature to the laboratory at the Audubon Center for Research on Endangered Species in New Orleans, Louisiana. Fresh ovaries were processed within 6 hours post-collection as previously described by Gómez et al. (2000), with only minor modifications. Briefly, ovaries were separated from their connective tissue and tract remnants using scissors, washed in two 35 mm petri dishes (351029; Falcon - Becton Dickinson, Franklin Lakes, NJ) containing 2 to 3 ml of oocyte holding medium (He199) and then held in the same medium until processed.

The holding medium consisted of Medium 199 (M-3769) supplemented with 71 mM sodium bicarbonate (S-5761), HEPES buffer (H-6147), 0.4% BSA (A-8412) and 1 mM L-glutamine (G-8540), 0.36 mM pyruvic acid (P-4562), 2.22 mM L(+)lactic acid (L-4388) and 50 μg/ml of gentamicin sulfate (G-1264) (pH = 7.7-7.9; osmolality = 285 - 295 mOsm). A few ovaries at a time were transferred into a 60 mm petri dish containing 2 to 3 ml of He199. Each ovary was held with a pair of serrated, curved forceps in a 35 mm petri dish (351008; Falcon - Becton Dickinson) containing He199, the ovary was bisected along its longitudinal axis using a scalpel blade (# 10). Each half was then scrapped with the blade to puncture all follicles. This caused cumulus-oocyte-complexes (COCs) to be released into the medium.

COCs were collected and transferred into another 35 mm dish containing He199. The ovaries were minced a second time and again COCs were collected. Once the COCs had been collected, they were washed in another 35 mm dishes containing He199 and then washed through two successive dishes containing in vitro maturation (IVM) medium. The IVM medium consisted of M199 Earle's salts solution (9102; Irvine Scientific, Santa Ana, CA) supplemented with 100 IU/ml of human chorionic gonadotropin (hCG) (CG-10), 200 IU/ml of equine chorionic gonadotropin (eCG) (G-4877), 100 IU/ml of Epidermal Growth Factor (EGF) (E-9644), 0.3% BSA Fraction V (81-068-2; Serological Proton, Kankakee, IL), 2 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM lactic acid, 1.12 mM L-cysteine (C-6852) and 50 µg/ml of gentamicin (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm). All solutions were filtered using a 0.2 µm pore acrodisk.

Oocytes were graded based on the number of cumulus cell layers. Oocytes with two or more layers of cumulus cells and an even dark cytoplasm were assigned quality grade scores of Grade A & B. Oocytes with fewer than one layer of cumulus cells and/or an uneven lipid distribution or light cytoplasm were assigned a score of Grade C. Groups of 20 to 30 oocytes were transferred to 800 µl of IVM medium in a 4-well culture dish (176740; Nunclon - VWR International, West Chester, PA). These dishes were placed into a humidified, gassed (5% O_2, 5% CO_2 and 90% N_2) sealed in a plastic bag (0 181216; Kapak sealpak pouches - Fisher Scientific, Pittsburg, PA) in an incubator at 38°C for 24 hours.

4.3.3 Dehydration of Oocytes

Solutions used for dehydration consisted of a saccharide (sucrose or trehalose) prepared in mM199. This mM199 medium was prepared using M199 Earle's salt solution supplemented with 10% fetal bovine serum (FBS) (SH30070.02; Hyclone Laboratories Inc., Logan, UT). The osmolality of the solutions was determined in triplicate with a freezing-point osmometer (Model # 3W2; Advanced Instruments Inc., Needham Heights, MA). The average osmolality of each solution was calculated and was considered to be the osmotic pressure of the solution. Cumulus cells were removed from mature oocytes. Briefly, oocytes were transferred into a 1.5 ml microcentrifuge tube containing 0.05% hyaluronidase (H-4272) in IVM medium and then vortexed for 75 seconds. The contents of tube were transferred into a 35 mm dish and quickly rinsed with mM199 to recover denuded oocytes. Denuding was completed by mechanical stripping with a 200 μm-diameter pulled micropipette. The denuded oocytes were transferred into a fresh 35mm dish containing mM199 and incubated at 38°C in 5% CO_2 in air for 30 minutes before being assigned to treatment groups.

All steps were executed in a warm room (28°C to 30°C). Oocytes dehydrated in mM199 medium, a bicarbonate-buffered solution, were placed in an incubator (38ºC) in a humidified atmosphere of 5% CO_2 in air. For the volumetric analysis, the denuded oocytes were transferred into mM199 alone and allowed to equilibrate. These oocytes in mM199 were considered to be controls (Control mM199). In each solution, the oocytes were allowed to equilibrate for ~10 minutes after which images were recorded with a digital camera attached to a Nikon inverted microscope.

The oocytes were then sequentially rinsed in 2 to 3 ml of medium with increasing concentrations of either sucrose (S-1888) or D(+)trehalose (T-5251) diluted in M199 medium. For the sucrose treatments, 0.15, 0.30, 0.50, 0.65 and 0.99 M solutions (Sucrose) were used and for the trehalose treatments, 0.13, 0.25, 0.35, 0.55 and 0.65 M (Trehalose) were used (Table 4.1). The oocytes were allowed to equilibrate for ~10 minutes in each saccharide solution and digital images were recorded. After being suspended in the highest concentration of saccharide, dehydrated oocytes were transferred back through the saccharide solution in order of decreasing concentrations, allowing them to equilibrate for 10 minutes at each step. The rehydrated oocytes were then transferred to mM199 medium and placed into an incubator for ~30 minutes until they were exposed to in vitro fertilization (IVF) (Experiment 2).

4.3.4 Volumetric Measurements

In Experiment 1, the volumes of oocytes exposed to each saccharide solution and the diluents were calculated assuming the oocytes to be spherical. Calibrated digital imaging software (Spot Advanced, Version 3.5.5 for Windows; Diagnostic Instruments Inc., Sterling Heights, MI) was used to measure the diameter of each oocyte. Volume was calculated using the formula for a sphere $V = 4/3\pi(d/2)^3$. The mean relative volumes were calculated by taking the average volume of an oocyte for a particular diluent and dividing it by the average volume for oocytes exposed to the diluent alone and expressed

Table 4.1 The osmolarities and osmotic pressures of sucrose and trehalose solutions prepared in mM199 medium

Saccharide	Concentration (M)	Osmolarity (mOsm)	Osmotic pressure (1/mOsm)
Sucrose	0.00	0.30	3.36
	0.15	0.46	2.17
	0.30	0.63	1.60
	0.50	0.86	1.16
	0.65	1.05	0.95
	0.99	1.47	0.68
Trehalose	0.00	0.30	3.36
	0.13	0.42	2.37
	0.25	0.56	1.78
	0.35	0.68	1.47
	0.55	0.90	1.11
	0.65	1.01	0.99

as a percentage. This basic method of determining the relative volumes of oocytes was previously reported with only minor modifications (Jackowski et al., 1980; Leibo, 1980; Oda et al., 1992; McWilliams et al., 1995).

4.3.5 In Vitro Fertilization and Culture

In Experiment 2, ejaculated sperm was collected from toms housed at the Audubon Center for Research of Endangered Species using a teaser female and an artificial vagina (AV) as approved by the Audubon's Institutional Animal Care and Use Committee. The AV consisted of a small glass bottle filled with ~40 ml of warm water (42°C to 45°C), with a balloon with the tip cut out in which was inserted a small plastic cup (Figure 4.1). A small amount of K-Y lubricant was applied to the balloon edge to ease penetration. Using a teaser female in estrus, the tom was allowed to mount her and his penis was guided into the AV, holding the bottle at a 45°angle. Once the ejaculate was produced, the AV was gently removed and the sperm sample was transferred to a 1.5 ml microcentrifuge tube and held in a 500 ml water bottle at ~30°C until processed.

The sperm sample was then mixed (1:1) with refrigeration medium (90129; Irvine Scientific) and placed into a 500 ml bottle filled with water and refrigerated for 24 hours before use. Insemination droplets were prepared by adding 2 µl of the refrigerated pellet into 400 µl of pre-warmed HEPES-Tyrode's solution (HeTY) medium. This HeTY medium consisted of a Tyrode's salt solution and contained 1.5% M199 HEPES, 7.5% BSA (pH = 7.3 - 7.4; osmolality = 285 - 295 mOsm). Sperm progressive motility and concentration were assessed using a hemocytometer to determine the amount of sperm to be added to the IVF droplets (50 x 10^4 sperm/ml).

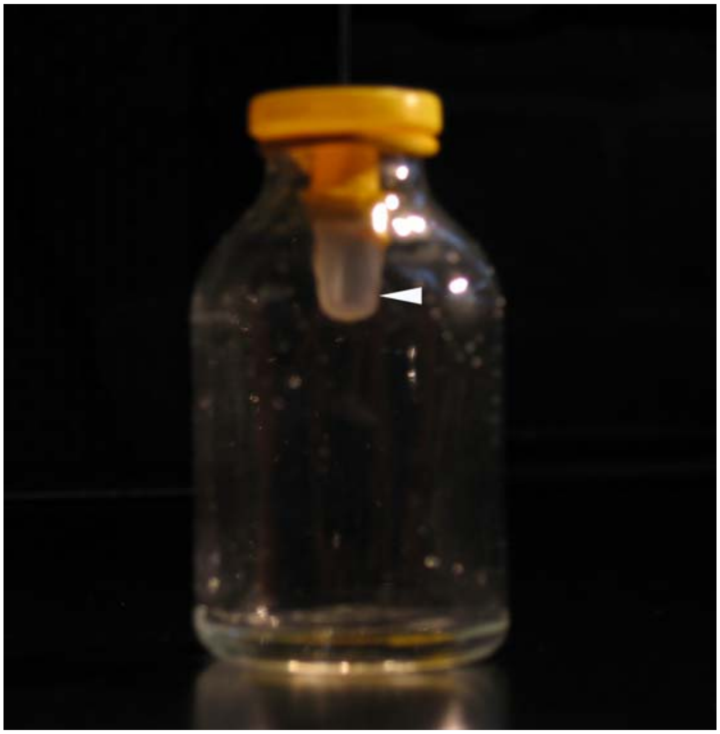

Figure 4.1 The artificial vagina (AV) used for semen collection from domestic toms. The AV consisted of a small glass bottle filled with 40 ml of water (42°C to 45°C), and a balloon (yellow) was placed into the bottle and the top was stretched over the neck of bottle. The tip of the balloon was cut out and a small plastic cup (white arrow) was inserted. The plastic cup was the bottom of a 1.5 microcentrifuge tube that had been cut off ~5 cm from the bottom. The ejaculated sperm was deposited in the bottom of this cup.

After being allowed to equilibrate in various solutions, oocytes were washed in two 35 mm dishes containing IVF medium and then placed into 100 μl droplets of sperm suspension under warmed mineral oil (4008-5; Sage Biopharma, Pasadena, CA) in an incubator at 38°C in 5% CO_2 in air for ~12 hours. The IVF medium consisted of a Tyrode's salt solution (T-2397) supplemented with 0.3% BSA, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)lactic acid, 100 IU/ml of penicillin G (P-3032) and 100 mg/ml of streptomycin sulfate (S-9137) (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm).

After being fertilized, presumptive zygotes were washed with in vitro culture-1 (IVC-1) medium. IVC-1 medium consisted of Tyrode's salt solution supplemented with 0.3% BSA, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)lactic acid, 1% MEM nonessential amino acids (M-7145), 50 μg/ml of gentamicin sulfate and 1 μl/ml of amphotericin B (A-2942) (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm). These zygotes were then transferred to a fresh well containing 800 μl of IVC-1 medium in a 4-well culture dish. These dishes were placed into a humidified, gassed (5% O_2, 5% CO_2 and 90% N_2) sealed bag in an incubator (38°C) for 2 days.

On day 2 post-insemination, fertilization rates were determined by evaluating for cleavage. Embryos not dividing were removed from culture and cleaved embryos were incubated in IVC-1 + EAA (IVC-1 supplemented with 1% MEM essential amino acids; M-5550) for an additional 2 days. On day 5, embryos were washed twice with in vitro culuture-2 (IVC-2) medium. IVC-2 medium consisted of Tyrode's salt solution supplemented with 10% FBS, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)lactic acid, 1% MEM nonessential amino acids, 1% MEM essential amino acids, 50 µg/ml of gentamicin sulfate and 1 µl/ml of amphotericin B (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm). Embryos were then transferred into 800 µl of IVC-2 and incubated 38ºC for an additional 2 days. On days 7 and 8 post-insemination, blastocyst development was assessed. Blastocyst hatching and expansion were recorded. Embryo development was further monitored for an additional 2 days.

4.3.6 Statistical Analysis

In Experiment 1, a linear regression was calculated on the average relative volumes of oocytes exposed to each saccharide solution prepared in mM199, as a function of the average osmotic pressure of the solution. In Experiment 2, cleavage and blastocyst rates of Control IVP and saccharide-treated (Sucrose and Trehalose) oocytes were compared using a Chi-square analysis ($P \leq 0.05$ statistically different) (Instat Graphpad, Version 3.0, San Diego, CA).

4.4 Results

4.4.1 Volumetric Response to Dehydration

In the first experiment, the effects of dehydration were examined using two saccharides, sucrose and trehalose, prepared in mM199. Oocytes exposed to sucrose and trehalose solutions behaved osmotically when transferred from an isotonic solution into increasingly concentrated solutions of saccharides. A total 17 oocytes were exposed to sucrose solutions (0.15, 0.30, 0.50, 0.65 and 0.99 M) and 18 oocytes were exposed to trehalose solutions (0.13, 0.25, 0.35, 0.55 and 0.65 M). Each of these oocytes was first suspended in isotonic solution (mM199 alone) and its image was recorded. The effect of increasingly concentrated saccharide solutions is shown in Figures 4.2 and 4.3.

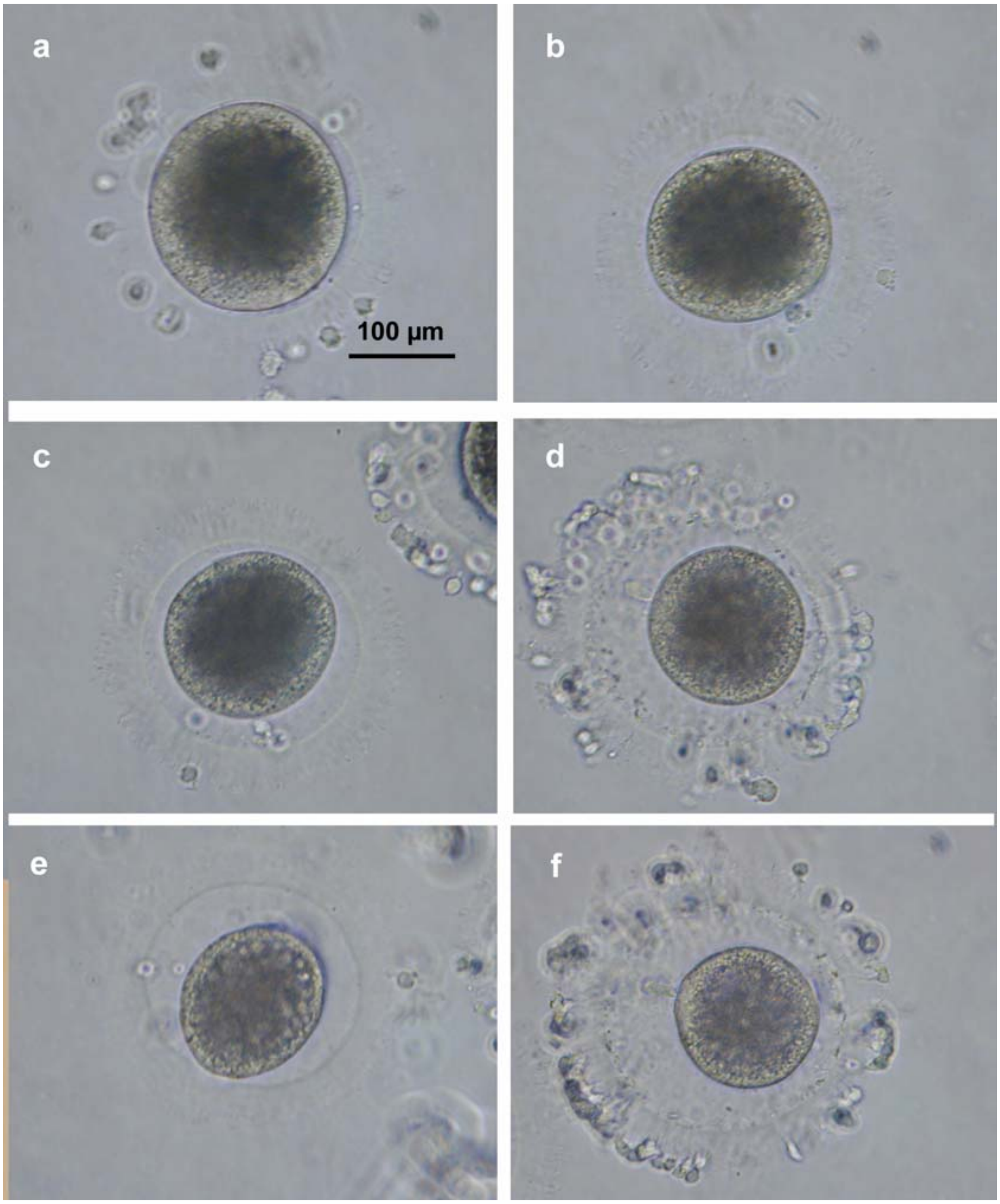

Figure 4.2 Photomicrographs of feline oocytes at various concentrations of trehalose prepared in mM199 medium. (a) Oocyte exposed to mM199 medium alone for 10 minutes at room temperature in air, (b) 0.13 M trehalose, (c) 0.25 M, (d) 0.35 M, (e) 0.55 M and (f) 0.65 M. Note the increasing perivitelline space between the oocyte and the zona pellucida. Images were obtained using a digital camera attached to an inverted Nikon microscope with 20X objective. Images were adjusted for brightness and contrast using Adobe Photoshop Version 6.0. (Scale bar = 100 µm, set for all images).

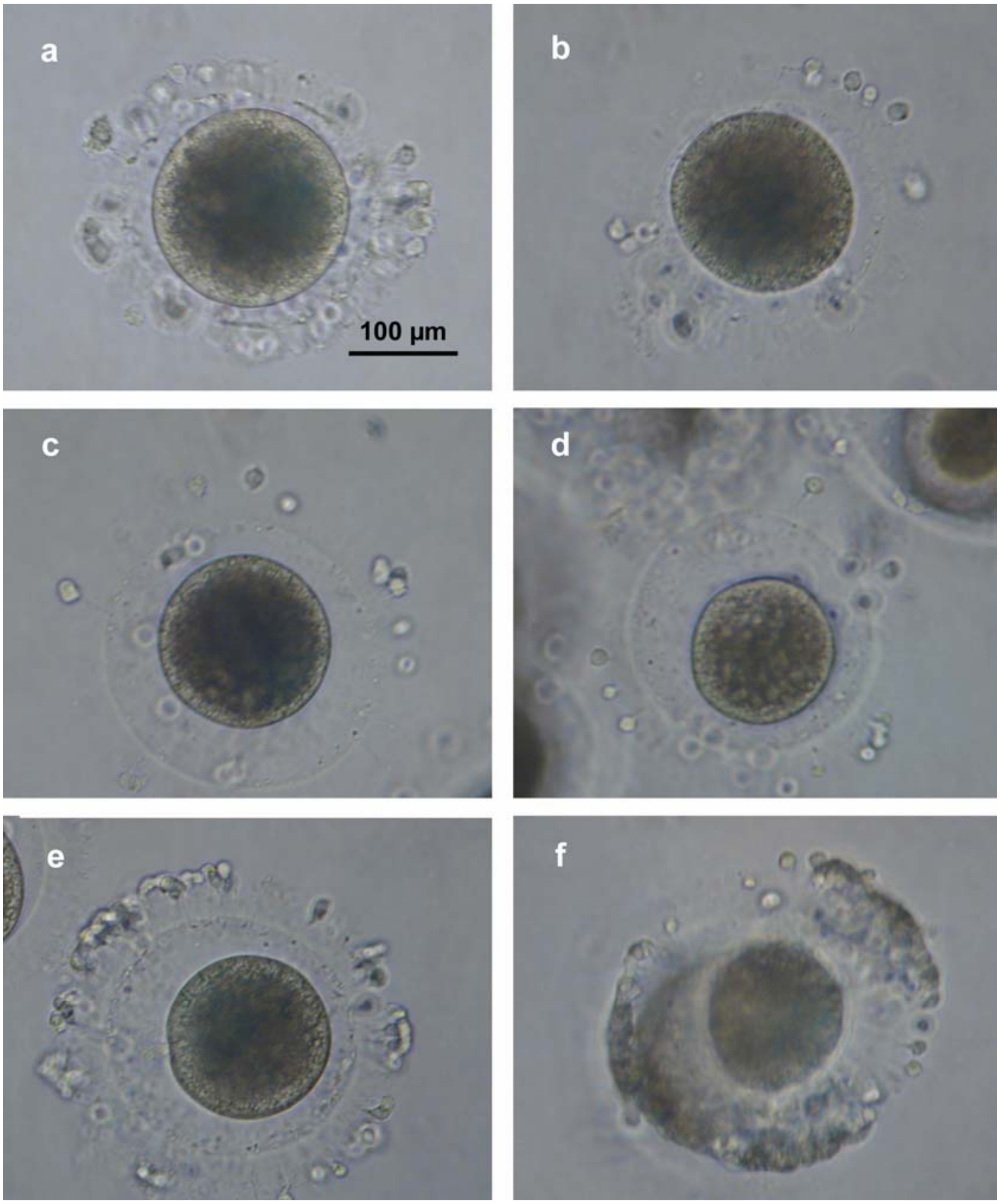

Figure 4.3 Photomicrographs of feline oocytes at various concentrations of sucrose prepared in mM199 medium. (a) Oocyte exposed to mM199 medium alone for 10 minutes at room temperature in air, (b) 0.15 M sucrose, (c) 0.30 M, (d) 0.55 M, (e) 0.65 M and (f) 0.99 M. Note the increasing perivitelline space between the oocyte and the zona pellucida. Images were obtained using a digital camera attached to an inverted Nikon microscope with 20X objective. Images were adjusted for brightness and contrast using Adobe Photoshop Version 6.0. (Scale bar = 100 µm, set for all images).

The results of the dehydration are presented in Figure 4.4, a Boyle van't Hoff plot for feline oocytes exhibiting a linear decrease in volume as a function of the reciprocal of osmolality. The osmolality of the mM199 was 297 mOsm. Extrapolation of the values to the x-intercept determines the nonosmotic volume for the oocytes in each saccharide solution. For trehalose this value was 32% and it was 22% for sucrose prepared in M199 medium. Both sucrose and trehalose in solution resulted in a reasonably good fit to the linear regression, with high R^2 values of 0.94 for each. There was some lysis in both groups of dehydrated oocytes (sucrose = 2 oocytes; trehalose = 4 oocytes). Only intact oocytes were placed into culture.

4.4.2 In Vitro Fertilization of Dehydrated Oocytes

In the second experiment, feline oocytes equilibrated with either sucrose or trehalose were placed into in vitro insemination and then cultured to determine the effect of the saccharide on subsequent embryonic development. Of the 14 trehalose-treated oocytes, 29% cleaved compared with 20% of those treated in sucrose (n = 15). Of the 28 control oocytes remaining in mM199 for the duration of the experiment, 25% cleaved and 25% developed into blastocysts. The cleavage rates of the dehydrated oocytes were not significantly different from that of control oocytes. No blastocysts resulted from oocytes that had been exposed to saccharides.

4.5 Discussion

Oocytes and embryos of all animal species studied to date are known to behave osmotically when exposed to increasing concentrations of hypertonic solutions, such as

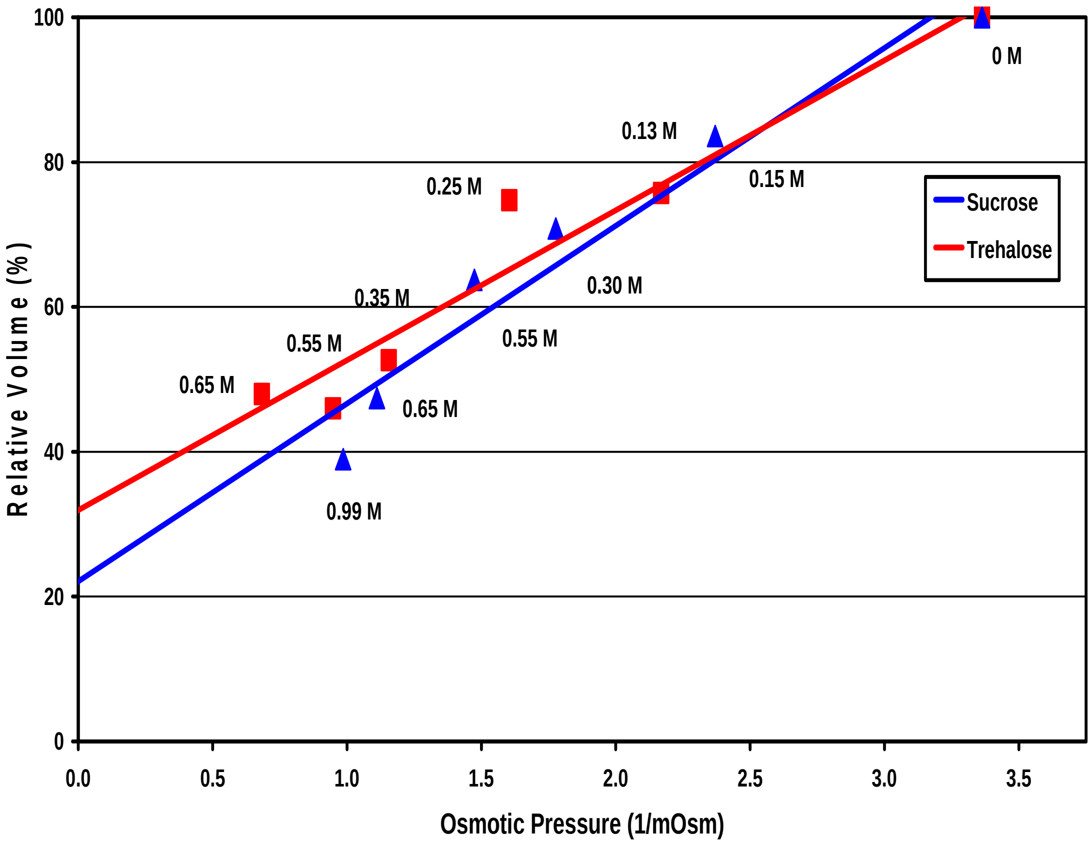

Figure 4.4 Boyle van't Hoff plots of feline oocytes exposed to various solutions of trehalose or sucrose prepared in mM199 medium. The points represent the mean relative volumes of oocytes. The correlation coefficients (R^2) for trehalose and sucrose prepared in mM199 medium were both 0.94.

the mouse (Leibo, 1980; Oda et al., 1992), cow (Mazur and Schneider, 1986; Ruffing et al., 1993; Agca et al., 1998), goat (Le Gal et al., 1994), nonhuman primates (Songsasen et al., 2002b) and fish (Valdez et al., 2005). When the osmotic response of a cell occurs the relative volume of a cell decreases in relation to the reciprocal of the osmolality of the solution to which it is exposed (Ruffing et al., 1993). Oocytes are very sensitive to volume excursions, which occur during cryopreservation as the cell is exposed to CPAs, freezing, warming and removal of CPAs (Oda et al., 1992). If the cell cannot withstand these volume changes, the cellular membranes often rupture and cell lysis will occur. Therefore, careful consideration should be made in preparing protocols for oocyte preservation so that these permutations are minimized.

In the first experiment of this study, volumetric measurements of feline oocytes were made during their dehydration when exposed to two saccharides prepared in mM199. Oocytes undergo dehydration by a loss of water in an attempt to restore the osmotic pressure between intracellular and extracellular compartments. The efflux of water from the cell reaches a maximum when the cell is dehydrated to 13% to 23% of their isotonic volume, as reported in mouse and human oocytes (McWilliams et al., 1995). This maximal level of dehydration refers to the nonosmotic volume of an oocyte that is made up of cellular components and solutes.

In the cat oocyte, a nonosmotic volume of 22% to 32% was determined using two different saccharides. This is similar to the nonosmotic volumes of bovine oocytes (24% to 32%) and Rhesus monkey oocytes (23%) and only slightly higher than that reported for mouse and goat oocytes (18% and 20%, respectively). These values are calculated by extrapolating the cell volume exposed to an infinitely concentrated solution.

In the second experiment, dehydrated oocytes were subjected to in vitro fertilization and cultured to assess their development. This experiment was done to determine the effects of the vitrification and warming solutions on the ability of oocytes to be fertilized and subsequently to develop in vitro. No significant difference was detected in cleavage rates among all treatment groups (control oocytes, sucrose -treated oocytes and trehalose-treated oocytes). Based on cleavage rates, there is no direct evidence of an effect of either of the cryoprotectants (sucrose or trehalose) on the in vitro fertilization process. However, blastocyst development was severely impaired when oocytes were exposed to these cryoprotectants compared with that of the control oocytes. Although the low rates of cleavage were similar for all treatments, events occurring during the fertilization process can not be ruled out as a potential cause of the subsequent embryo development in vitro. Furthermore, one can not overlook that some oocytes may have become parthenogenetically activated in this study.

Studies designed to determine the sensitivity of feline oocytes to CPA exposure have been limited. Comizzoli et al. (2004) examined the resumption of meiosis, fertilization and embryonic development of immature feline oocytes. It was observed that high concentrations of PrOH and EG decreased the ability of feline oocytes to mature in vitro. Of those oocytes that did mature (47% to 70%, respectively), they contained disrupted meiotic spindles. Cleavage, however, was not affected, similar to the results obtained in the current experiment. In the cow, oocytes exposed to CPAs alone demonstrated chromosome dispersal and clumping, microtubule depolymerization and discontinuities in the actin cytoskeleton (Saunders and Parks, 1999). This is in contrast to mouse oocytes that exhibit little or no spindle disruption after exposure to either PrOH or

DMSO (van der Elst et al., 1992). Unfortunately, due to the limited availability of feline oocytes and the Hurricane Katrina, this experiment could not be repeated.

4.6 Conclusions

Feline oocytes behave osmotically when exposed to increasingly concentrated solutions. There was no difference in the effect on feline oocytes of being exposed to sucrose or trehalose at the concentrations evaluated in this study. Embryonic development of these dehydrated oocytes was also assessed and it was found that cleavage rates were not affected by exposure of feline MII-stage oocytes to cryoprotectants, although blastocyst development was adversely affected and when compared with control oocytes. To date, very few studies have examined the effects of various permeating and nonpermeating cryoprotectants on feline oocytes. Further study is needed to determine the source of the detrimental effects noted on blastocyst development.

CHAPTER 5
EMBRYO DEVELOPMENT FOLLOWING IVF OF VITRIFIED FELINE OOCYTES WITH TWO DIFFERENT DILUENTS

5.1 Introduction

Assisted reproductive techniques (ART), such as artificial insemination, in vitro fertilization, embryo transfer, gamete preservation, intracytoplasmic sperm injection and nuclear transfer have been used in both domestic and nondomestic species in an effort to increase genetically valuable animals. Although often used routinely in various commercial settings for domestic species, the use of ART for endangered species has been less effective. Genome resource banking is a valuable resource for endangered species but necessitates the ability to preserve sperm, oocytes and/or embryos. This resource can then be used to ensure representation of each individual in the genetic pool, thus preventing inbreeding due to small, fragmented animal populations.

Currently, all species of felines, with the exception of the domestic cat, are considered either threatened or endangered within some part of their range. To preserve their genetics, sperm and oocyte cryopreservation techniques need further examination. Although a number of studies of feline sperm cryopreservation have been reported, very few studies of feline oocyte cryopreservation have been published to date. Therefore, the objective of this experiment was to determine the ability of vitrified domestic cat oocytes to undergo fertilization and embryonic development after warming.

5.2 Literature Review

5.2.1 Current Status of Feline Oocyte Preservation

The first report of live offspring produced using ART in domestic felids used cryopreserved sperm in conjunction with artificial insemination (AI) (Platz et al., 1978).

Since then, a few of the nondomestic species such as, the ocelot (Swanson et al, 1996a), jaguar (Swanson et al, 1996b), cheetah (Swanson et al, 1996b, Howard et al, 1997), tiger (Donoghue et al., 1996) and the Clouded leopard (Howard et al, 1997) have produced offspring after being inseminated surgically with cryopreserved sperm (for a review see Howard, 1999). However, oocyte cryopreservation has been much less successful. Only two studies to date have reported in vitro development from either cryopreserved or vitrified-warmed domestic cat oocytes (Luvoni and Pellizzari, 2000; Murakami et al., 2004). There have been no reports found on oocytes of endangered felids being successfully cryopreserved and offspring produced.

The relatively low success of felid oocyte preservation methods may be due to the high lipid content and the decreased permeability of the oocytes plasma membrane to various cryoprotectants (Luvoni, 2000). In contrast, cryopreservation of cleavage-stage embryos has had greater success in the domestic cat and to some extent in one nondomestic species (tiger) (Crichton et al., 2003; Gómez et al., 2003). In an attempt to increase the low success in feline oocyte preservation, Comizzoli et al. (2004) evaluated the effect of cryoprotectant additives (CPAs) on feline oocytes. Exposure to both ethylene glycol (EG) and propanediol (PrOH) was found to cause a high incidence of abnormal spindle morphologies and decreased blastocyst development, with the exception of 1.5 M PrOH at 25ºC. Luvoni et al. (1997) and Luvoni (2000) also reported detrimental effects of both CPAs and the cryopreservation process on feline oocytes.

5.2.2 Use of Cryoprotectants and Diluents

Various components that make up the vitrification solution for oocytes includes the base medium that is composed of a buffered-saline solution, usually TCM-199 or phosphate-buffered saline (PBS). These solutions contain sodium, which is detrimental to embryonic development in mouse oocytes during cryopreservation due to the increased cation load during the dehydration and cooling process (Stachecki et al., 1998b). To reduce this detrimental effect, Stachecki et al. (1998a) have developed a choline-based medium (CJ2) where the sodium was replaced by choline. A 40% increase in blastocyst development was found with choline compared with the sodium-based medium. This choline-based medium has also been successfully used to preserve human oocytes (Quintans et al., 2002).

Besides the base medium, vitrification solutions often contain CPAs, such as EG, PrOH, dimethylsulfoxide (DMSO) and propylene glycol (PG). In addition to the permeating CPAs, nonpermeating CPAs such as sucrose, glucose or trehalose have been used in the cryopreservation/vitrification medium. In the cat, 40% EG and 0.3 M sucrose were used to vitrify oocytes but only a 4% blastocyst rate was obtained by Murakami et al. (2004). These researchers used a two-step dilution beginning with only 20% EG followed by the vitrification solution containing 40% EG and 0.3 M sucrose. Two-step dilutions allow the oocyte to dehydrate slowly avoiding osmotic shock due to the high concentrations of CPAs used. It should be noted that Luvoni et al. (1997) found no significant difference in the resumption of meiosis of feline oocytes that were cryopreserved in either DMSO or EG solution.

5.2.3 Instruments Used for Vitrification

Various instruments have been used for oocyte cryopreservation. For slow-cooling, standard 0.25 ml straws are used and loaded into a controlled rate freezer. Different instruments have been used for vitrification, such as open pulled straws (OPS)

Vajta et al., 1998), electron microscope grids (Martino et al., 1996), cryoloops (Lane et al., 1999a) and CryoTops (Kuwayama et al., 2005).

Cryoloops and the CryoTops markedly improved the success of embryonic development post-vitrification by decreasing the minimum volume required and subsequently increasing the cooling rates in mouse and human oocytes and embryos (Lane et al., 199a,b; Kuwayama et al., 2005). For example, the OPS method makes use of a 1.5 µl volume whereas, the CryoTop method uses a <0.1 µl volume during the proceedure. This volume difference is due to the instrument surface area, the vitrification solution and type of cells used in the process. Pulled straws have a larger surface area to fill whereas, the slender tip of the CryoTop allows a much smaller volume to be used during the process. Consequently, the cooling and warming rates increase from ~16,000ºC/minute to 23,000ºC/minute for cooling and 14,000ºC/minute to 42,000ºC/minute for warming, respectively (Kuwayama et al., 2005). Only 0.25 ml French plastic straws have been reported to date for oocyte vitrification in the domestic cat (Murakami et al., 2004).

5.3 Materials and Methods

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise stated.

5.3.1 Experimental Design

In the first experiment (Experiment 1), oocytes were graded and then randomly divided into one of two treatments groups: Control metaphase-II (MII) stage oocytes (n = 102) that were subjected to standard IVP (Control IVP) and MII-stage oocytes (n = 214) that were vitrified using a choline-based medium CJ2 (Vitrified MII). Control IVP and

Vitrified MII treatments were classified into two oocyte quality treatment groups (Grade A & B and Grade C). After vitrification all oocytes were fertilized and cultured to assess their embryonic development. There were five replicates used in this experiment.

In the second experiment (Experiment 2), oocytes were again graded and then randomly divided into one of three treatments groups: Control MII-stage oocytes (n = 204) that were subjected to standard IVP (Control IVP), MII-stage oocytes (n = 548) that were vitrified using a standard oocyte maturation medium, M199 Earle's salts solution (Vitrified MII) and MII-stage oocytes (n = 86) subjected to the vitrification solution alone and then fertilized and cultured (CPA Control). Control IVP and Vitrified MII treatments were classified into two oocyte quality treatment groups (Grade A & B and Grade C). As in Experiment 1, all oocytes were fertilized in vitro and cultured to assess their embryonic development. There were eight replicates of this experiment.

5.3.2 Oocyte Collection and Maturation

Ovaries were collected at local veterinary clinics during regular ovary/ hysterectomies of queens at various stages of their reproductive cycle. The ovaries were placed in a plastic 100 ml sample vial (9853Q47; Thomas Scientific, Swedesboro, NJ) containing 50 ml of TL HEPES (04-616F; Cambrex Bio Science, Walkersville, MD) supplemented with 50 μg/ml of gentamicin (G-1397) and transported at room temperature to the laboratory at the Audubon Center for Research of Endangered Species in New Orleans, Louisiana. Fresh ovaries were processed within 2 to 8 hours post-collection or were stored at 4°C overnight (refrigerated ovaries) and processed the next morning. Oocytes collected from refrigerated ovaries, matured in vitro, have been reported to produce blastocysts and live young in the domestic cat (Wolfe and Wildt, 1996; Pope et al., 2003). Both fresh and refrigerated ovaries were processed as described by (Gómez et al., 2000), with only minor modifications.

Ovaries were separated from their connective tissue and tract remnants using fine scissors, washed through two 60 mm petri dish (351029; Falcon - Becton Dickinson, Franklin Lakes, NJ,) containing 2 to 3 ml of oocyte holding medium (He199) and then held in the same medium until processed. He199 consisted of Medium 199 (M-3769) supplemented with 71 mM sodium bicarbonate (S-5761), HEPES buffer (H-6147), 0.4% BSA (A-8412) and 1 mM L-glutamine (G-8540), 0.36 mM pyruvic acid (P-4562), 2.22 mM L(+)lactic acid (L-4388) and 50 µg/ml of gentamicin sulfate (G-1264) (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm). A few ovaries at a time were transferred to a 60 mm petri dish containing 2 to 3 ml of He199, where they were measured and the number of visible follicles assessed and recovered. Each ovary was held with a pair of serrated, curved forceps in a 35 mm petri dish (351008; Falcon - Becton Dickinson) containing He199 and bisected along its longitudinal axis using a (# 10) scalpel blade. Each half was then scrapped with the blade to puncture all follicles. This caused cumulus-oocyte-complexes (COCs) to be released into the medium. COCs were collected and transferred to a new 35 mm dish containing He199. The ovaries were minced a second time and COCs again collected.

After the COCs had been collected, they were washed in another 35 mm dish containing He199 and then washed through two successive dishes containing in vitro maturation (IVM) medium. The IVM medium consisted of M199 Earle's salts solution (9102; Irvine Scientific, Santa Ana, CA) supplemented with 100 IU/ml of human chorionic gonadotropin (hCG) (CG-10), 200 IU/ml of equine chorionic gonadotropin (eCG) (G-4877), 100 IU/ml of Epidermal Growth Factor (EGF) (E-9644), 0.3% BSA Fraction V (81-068-2, Serological Proton, Kankakee, IL), 2 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM lactic acid, 1.12 mM L-cysteine (C-6852) and 50 µg/ml of gentamicin (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm). All solutions were filtered through a 0.2 µm pore acrodisk.

Oocytes were graded based on the number of cumulus cell layers. Oocytes with two or more layers of cumulus cells and an even dark cytoplasm were assigned a quality score of Grade of A & B. Oocytes with less than one layer of cumulus cells and/or an uneven or light distribution of cytoplasm were assigned a score of Grade of C.

Groups of 20 to 30 oocytes were transferred to 800 µl of IVM medium in a 4-well dish for culture (176740; Nunclon - VWR International, West Chester, PA). These dishes were placed into a humidified, gassed (5% O_2, 5% CO_2 and 90% N_2) in a sealed plastic bag (0181216; Kapak sealpak pouches - Fisher Scientific, Pittsburg, PA) in an incubator at 38°C for 22 to 24 hours.

5.3.3 Oocyte Vitrification

MII-stage oocytes were processed for vitrification by one of two protocols. In the first experiment, the vitrification protocol used was modified from a protocol that used a choline-substitute medium (CJ2) for bovine oocytes. In the second experiment, this protocol was further modified by using M199 Earle's salts solution to prepare the vitrification and warming solutions. All solutions and steps of oocyte vitrification and warming were conducted at 28 ºC to 30ºC. For the first experiment, oocytes (n = 214) were removed from the maturation medium and placed into mCJ2 medium (CJ2 prepared as described in Stachecki et al., 1998a) then supplemented with 10% FBS (osmolality = 290; pH = 7.4).

The vitrification solutions were prepared by adding increasing concentrations of EG (E-9129) and DMSO (D-4540) to the prepared mM199 and mCJ2. Vitrification was done using a three-step dilution. The first vitrification solution consisted of 5% EG + 5% DMSO, the second contained 10% EG + 10% DMSO and the final solution contained 20% EG + 20% DMSO plus 0.65 M trehalose D(+)trehalose (T-5251). In the second experiment, the vitrification solutions were prepared as described in Experiment 1 except the diluent used was mM199 (Medium 199 Earle's salts solution supplemented with 10% FBS) instead of mCJ2.

In the first experiment, 7 to 10 oocytes were transferred into a 35 mm dish containing the first vitrification solution and allowed to equilibrate for 75 seconds. The oocytes were then sequentially transferred through the second and final vitrification solutions and equilibrated for 75 seconds each time. Once equilibrated in the final vitrification solution, the oocytes were picked up in minimal medium (<0.1 μl) using a pulled glass micropipette (i.d. 200 μm) and transferred onto a film created on the cryoloop (HR4-963; Hampton Research, Aliso Vieho, CA) (Figure 5.1).

The cryoloop was immediately plunged into a LN_2 bath and placed into a plastic holder submerged in LN_2 until all cryoloops were loaded. Another group of 7 to 10 oocytes was then transferred to the first vitrification solution and moved through the next two solutions and then loaded onto cryoloops. Once all oocytes had been plunged, the cryoloops were placed into cryovials (HR4-911; Hampton Research). The cryovials containing the cryoloops were then placed onto canes and then transferred into a LN_2 storage tank.

In the second experiment, oocytes (n = 548) were vitrified as described for Experiment 1 with the following differences. The medium used was mM199 for all solution preparations and after equilibration in the final vitrification solution, the oocytes were picked up in minimal medium (<0.1 µl) and transferred onto the tip of a CryoTop (provided by Dr. Masashige Kuwayama, Kato Ladies Clinic, Tokyo, Japan). The CryoTop was then immediately plunged into LN_2. The rest of the oocytes were processed in a similar manner until all of the oocytes had been vitrified. To protect the oocytes from damage during storage, protective sheaths were affixed onto CryoTops using large forceps being careful to keep the entire CryoTop submerged in LN_2 (Figure 5.1). The CryoTops with sheaths were placed onto canes and transferred into a LN_2 storage tank until needed.

5.3.4 Controls for the Effect of the CPAs

To determine the effect of the vitrification and warming solutions alone on embryonic development, feline MII-stage oocytes (n = 86) were processed for vitrification. These oocytes were subjected to the vitrification solutions (but not vitrified) and then subjected to IVF and cultured in vitro (CPA Control). Briefly, oocytes were sequentially moved through the vitrification and warming solutions. After being exposed to the final warming solution of the vitrification proceedure, the oocytes were placed into mM199 and then inseminated (IVF) and cultured in vitro as described for the Control IVP group.

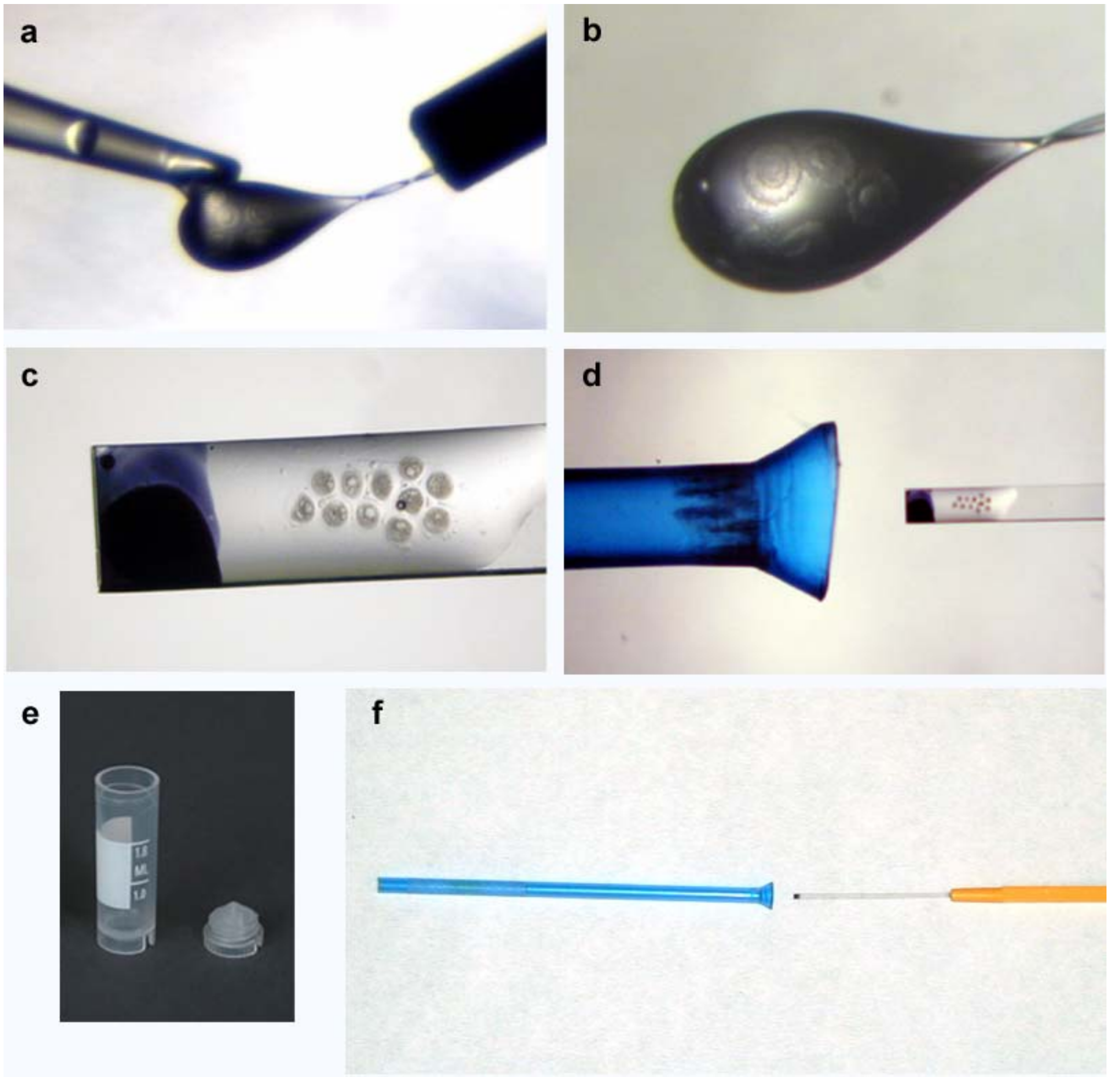

Figure 5.1 The cryoloop and CryoTop instruments used for feline oocyte vitrification. (a) Feline oocytes loaded onto a film created on a nylon loop attached to a metal holder, (b) Close-up of oocytes loaded onto the cryoloop, (c) Oocytes loaded onto the tip of a CryoTop in minimal volume, (d) CryoTop with oocytes being placed into its protective sheath, (e) Cryovial and cap in which the cryoloop is placed before storage in liquid nitrogen and (f) Lower magnification of CryoTop showing holder (yellow) and protective sheath with plug (blue).

5.3.5 Oocyte Warming

The same diluent used for oocyte vitrification media preparation was used for the warming solution (mCJ2 or mM199). The three warming solutions were prepared by the addition of trehalose to the modified diluent previously prepared. The first warming solution contained 0.25 M trehalose, the second warming solution contained 0.19 M trehalose and the final warming solution contained 0.13 M trehalose. Each of these solutions was filtered through a 0.2 μm pore acrodisk. In the first experiment, the cryovial caps were unscrewed one at a time and the cryoloop placed directly into a 35 mm dish containing ~2 ml of the first warming solution (0.25 M trehalose). In the second experiment, the plastic sheaths were removed and one at a time, the CryoTops containing oocytes were placed directly into a 35 mm dish containing ~2 ml of the first warming solution (0.25 M trehalose). The rest of the protocol was the same for both Experiments 1 and 2. The oocytes were equilibrated for 1 minute in the first warming solution.

The recovered oocytes were transferred to the second solution (0.19 M trehalose) and allowed to equilibrate for 1 minute. The oocytes were then transferred to the final warming solution (0.13 M trehalose) and equilibrated for 1 minute. The oocytes were transferred to mM199, where they remained until all the oocytes were recovered. The second cryoloop or CryoTop was then warmed as described above and repeated until all oocytes were recovered and placed into mM199. The number of oocytes lysed was counted and the intact oocytes were incubated for ~1 hour before in vitro fertilization (IVF).

5.3.6 Collection and Processing of Sperm

Ejaculated sperm was collected from toms (n = 3) housed at the Audubon Center for Research of Endangered Species using a teaser female and artificial vagina (AV). The animal handeling procedures used in this study were pre-approved by the Audubon's Institutional Animal Care and Use Committee. The AV consisted of a small glass bottle filled with 40 ml of warm water (42°C to 45°C), with a balloon with the tip cut out and a small plastic cup was inserted. The balloon was placed into the bottle and the top was stretched over the neck of bottle. A small amount of K-Y lubricant was applied to the balloon edge to ease penetration. A teaser female in natural estrus was used to collect the sperm sample. Briefly, the tom was allowed to mount her and his penis was guided into the AV while a technician held the bottle at a 45°angle. Once the tom had ejaculated, the AV was gently removed and the sperm sample was transferred into a 1.5 ml microcentrifuge tube and held in a 500 ml water bottle at ~30°C until processed.

The motility and sperm concentration of each ejaculate were assessed by use of a hemocytometer. The volume of each ejaculate ranged from 22 µl to 130 µl. The sperm were diluted to the desired concentration of ~ 50×10^4 sperm/ml with sperm washing medium (HeTy) consisting of Tyrode's salt solution (T-2397) containing 1.5% M199 HEPES, 0.4% BSA, 1 mM L-glutamine (G-8540), 0.36 mM pyruvic acid (P-4562), 2.22 mM L(+)-lactic acid, 50 µg/ml of gentamicin sulfate (G-1264) and 1.12 mM L-cysteine (C-6852) (pH = 7.3 - 7.4; osmolality = 285 - 295 mOsm).

Fresh or refrigerated ejaculates were used for IVF. The sample to be refrigerated was placed into a 500 ml bottle filled with water at ~30°C and then placed into a refrigerator (4°C) where it was held for use for up to 1 week. The desired volume of the sample to be refrigerated was transferred to a 1.5 ml microcentrifuge tube and an equal volume (1:1) of standard refrigeration medium (90129, Irvine Scientific) was added drop-wise with gentle agitation.

5.3.7 In Vitro Fertilization and Embryo Culture

For Control IVP oocytes, (n = 102 for Experiment 1; n = 204 for Experiment 2) COCs were removed from maturation at 22 hours evaluated and assigned an oocyte quality score of Grade A & B or Grade C. They were then assigned by grades to the IVF procedure. The oocytes were then washed twice in 35 mm dishes containing IVF medium and then placed into 100 µl droplets of sperm suspension under warmed mineral oil (4008-5; Sage Biopharma, Pasadena, CA) in an incubator at 38°C in 5% CO_2 in air for 12 hours. IVF medium consisted of Tyrode's salt solution supplemented with 0.3% BSA, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)-lactic acid, 100 IU/ml of penicillin G (P-3032) and 100 mg/ml of streptomycin sulfate (S-9137) (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm).

Vitrified-warmed oocytes in Experiments 1 and 2 were transferred from mM199 into a 35 mm dish containing IVF medium and then were placed into 100 µl droplets of sperm suspension for ~18 hours. After fertilization, presumptive zygotes were washed once in IVC-1 medium and any remaining cumulus cells were removed manually using a 200 µm glass micropipette and then transferred into a fresh well containing 800 µl of IVC-1 medium in a 4-well dish for culture. IVC-1 medium consisted of Tyrode's salt solution supplemented with 0.3% BSA, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)-lactic acid, 1% MEM nonessential amino acids (M-7145), 50 µg/ml of gentamicin sulfate and 1 µl/ml of amphotericin B (A-2942) (pH =

7.7 - 7.9; osmolality = 285 - 295 mOsm). These dishes were placed into a humidified, gassed (5% O_2, 5% CO_2 and 90% N_2) in a sealed bag in an incubator (38°C) for 2 days.

On day 2 post-insemination, fertilization was determined by cleavage rates. Nondividing embryos were removed and dividing embryos were incubated in IVC-1 + EAA (IVC-1 supplemented with 1% MEM essential amino acids; M-5550) for an additional 2 days. On day 5, embryos were washed twice in IVC-2 and then transferred into a well containing 800 µl of IVC-2 and incubated again for 2 days. IVC-2 medium consisted of a Tyrode's salt solution supplemented with 10% FBS, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)-lactic acid, 1% MEM nonessential amino acids, 1% MEM essential amino acids, 50 µg/ml of gentamicin sulfate and 1 µl/ml of amphotericin B (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm). On days 7 and 8 post-insemination, blastocyst development was evaluated. The number of embryos that had developed into blastocysts and the number that had hatched were recorded and the embryos were cultured in vitro for an additional 2 days.

5.3.8 Statistical Analysis

Cleavage and blastocyst rates were analyzed for statistical significance across treatment groups using Chi-square analysis and $P \leq 0.05$ was considered statistically different in this study (Instat Graphpad Version 3.0, San Diego, CA).

5.4 Results

5.4.1 Vitrification of Oocytes in CJ2 as Diluent

In the first experiment, the choline-based medium CJ2 used for bovine oocytes was used to prepare the vitrification and warming solutions for feline oocyte vitrification. Embryonic development was compared with oocytes subjected to a standard IVF and in vitro cultured (IVP controls) (Table 5.1). Cleavage rates of Control IVP oocytes was 27% (Grades A & B; n = 75) and 41% (Grade C; n = 27). There was no cleavage or embryonic development in any of the vitrified-warmed oocytes (Grades A & B; n = 144 and Grade C; n = 70) using CJ2. Although five replicates of this experiment were performed, no signs of embryonic development were observed in the vitrified-warmed treatment group.

5.4.2 Vitrification of Oocytes with M199 Medium as the Diluent

In the second experiment, M199 Earle's salts medium was used to prepare the vitrification and warming solutions for feline oocyte vitrification. Since this medium is used to prepare the IVM medium for cats it was selected for oocyte manipulations. The results of Experiment 2 are listed in Table 5.2. In this experiment, the cleavage rate of Control IVP oocytes (22%) was not significantly different from vitrified MII-stage oocytes (24%) for the higher quality oocytes Grades A & B. Although these rates were not significantly different, blastocysts were only produced in the Control IVP groups (4% and 3%) and none in the Vitrified MII groups. Vitrified oocytes cleaved after insemination (24% and 11%), however, no blastocysts were produced up to 8 days of culture. Cleavage and blastocyst development of oocytes exposed to the vitrification and warming solutions alone (CPA Control) were not significantly different from IVP controls. This suggests that the M199 medium had no detrimental effect on embryonic development.

5.5 Discussion

The preservation of genetic material in genome resource banks and frozen zoos is a valuable tool guarding against extinction of endangered species (Karow and Critser,

Table 5.1 Summary of embryonic development of control and vitrified feline oocytes using mCJ2

			Oocytes/embryos developing (%)	
Treatment	Oocyte grade	No. of oocytes	Cleavage (d 2)	Blastocysts (d 8)
Control IVP	A & B	75	27	0
Control IVP	C	27	41	0
Vitrified MII	A & B	144	0	0
Vitrified MII	C	70	0	0

Values were not significantly different at P≤0.05.

Table 5.2 Summary of embryonic development of control and vitrified feline oocytes using mM199

			Oocytes/embryos developing (%)	
Treatment	Oocyte grade	No. of oocytes	Cleavage (d 2)	Blastocysts (d 8)
Control IVP	A & B	74	22	4
Control IVP	C	130	28	3
Vitrified MII	A & B	216	24	0
Vitrified MII	C	332	11	0
CPA Control	A & B	19	37	5
CPA Control	C	67	19	3

Values were not significantly different at P≤0.05.

1997; Watson and Holt, 2001; Holt et al., 2003). This material can be used to re-introduce a nonrepresented individual to a population, help avoid inbreeding by introducing new genetics into a fragmented population and to re-establish a small population after a disease outbreak or environmental disaster. In the future, the use of ART procedures may be useful in re-establishing these populations.

Sperm cryopreservation has been relatively successful in many domestic and nondomestic species, but oocyte cryopreservation has been less successful. In any genome bank, both the male and female of a species must be represented and therefore embryo cryopreservation has been used as an alternative to oocyte preservation in many species. To date, oocyte vitrification has been achieved in at least 10 species but most with relatively low efficiency. Therefore, the objective of this experiment was to attempt to derive an efficient method of feline oocyte vitrification that could be used to preserve oocytes of endangered felines.

In the first experiment, no embryonic development was observed using the CJ2 medium as the diluent for the vitrification and warming solutions. This was unexpected because CJ2 medium has been successful in the vitrification of mice (Stachecki et al., 1998a), human (Quintans et al., 2002) and bovine oocytes. One possible cause is the increased cytoplasmic lipid content of the cat oocyte compared with the other species, such as the pig and cow (Fujihira et al., 2004). There was a significant increase in blastocyst development with CJ2 medium found in the mouse and human (Quintans et al., 2002; Stachecki et al., 2002) whereas, there was no increase in blastocyst development noted in the cow. This may also be due to its higher oocyte lipid content (Kim et al., 2001). Oocyte quality may also be one of the reasons for the poor results observed in the cat because the several replicates were done using different sets of ovaries obtained from cats of various ages and at various stages of their reproductive cycle. Due to the lack of success in embryo development of the CJ2 medium in the cat, no further replications were done.

In the second experiment, M199 Earle's salts solution was used instead of CJ2 as the diluent for the vitrification and warming solutions. Compared with the results obtained using CJ2 medium in the first experiment, the use of M199 for feline oocyte vitrification was substantially improved. With high quality oocytes (Grade A & B), 24% of these cleaved after vitrification using M199 medium and 11% of the lower quality oocytes cleaved (Grade C) compared with 0% (all Grades combined) using CJ2. Also, in Experiment 2, the CryoTop was used for vitrification instead of the cryoloop, which reduced the amount of vitrification solution required and subsequently, increased the cooling rate of the proceedure. This may also be a factor in the increased oocyte survival post-warming. However, no blastocysts were produced from the oocytes vitrified using M199 compared with ~ 4% blastocysts in the Control IVP group. Luvoni et al. (1997) observed no resumption in meiosis after vitrification of immature cat oocytes using DMSO or EG as the CPA, but did report some resumption of meiosis if oocytes were cryopreserved using standard slow-cooling proceedure.

The cleavage rate from vitrified feline oocytes in Experiment 2 was higher (24%) than the 1% cleavage rate reported by Murakami et al. (2004). No blastocysts were obtained in their experiment until these researchers subjected vitrified oocytes to 0.5 M sucrose post-liquefaction. In the later case, Murakami et al (2004) reported 19% cleavage, which is comparable with the 24% in the present study. However, these researchers also obtained a 2% blastocyst rate per 200 oocytes while we did not obtain any blastocyst development from the vitrified oocytes. It is possible that with further research, our proceedure may also produce blastocysts.

To attempt to address this lack of blastocyst production, a group of oocytes was vitrified using EG only (following the protocol described in Experiment 2), by increasing its concentration to that of EG and DMSO together. In this trial, one blastocyst was produced from 93 cat oocytes (~1%) vitrified and warmed compared with 4% blastocyst for IVP controls. Further experiments are required to determine if this technique could significantly improve the rates of embryonic development in the vitrified oocytes.

To further investigate the effect of the medium on the oocytes without vitrification, control oocytes were exposed to the vitrification and warming solutions alone. No significant differences in the cleavage and blastocyst rates between these CPA Control and IVP Control oocytes suggest that M199 and the CPAs used did not have any detrimental effect on the ability of the oocytes to be fertilized and develop. The permeating CPAs used in this experiment were EG and DMSO. Both of these have been used in various concentrations for cat oocyte vitrification previously (Luvoni et al., 2000; Murakami et al., 2004). Also, a short period of exposure to the CPAs, especially to the high concentration of a saccharide, has been shown to be less detrimental to subsequent embryonic development (Murakami et al., 2004). These investigators found that a 1-minute exposure of cat oocytes to 0.5 M sucrose was less toxic than 5 minutes of exposure. For this reason, the time of equilibration in each vitrification solution was set at 75 seconds and the time for equilibration in the warming solution was 1 minute each. Also, we used trehalose instead of sucrose in both experiments. Trehalose is a nonreducing saccharide that is thought to confer protective properties to the cellular membranes by stabilizing the membrane bound proteins (Crowe et al., 2001). Also, because it is a dissacharide and not a monosaccharide, it is less likely to leak through the cell membranes, which would increase the likelihood of cell lysis or toxicity.

5.6 Conclusions

In the present study, M199 was a better medium than CJ2 medium for the preparation of oocyte vitrification medium. It appears that CJ2 is not an acceptable alternative. Also, the CryoTop is a useful instrument for feline oocyte vitrification. The method used for bovine oocyte vitrification was successful in the cat, as assessed by their ability to cleave post-vitrification. However, further study is needed to determine an optimal protocol that would allow blastocyst development of vitrified-warmed feline oocytes before this methodology can be transferred to nondomestic feline species.

CHAPTER 6
BLASTOCYST DEVELOPMENT FROM FELINE OOCYTES INJECTED WITH DEHYDRATED FELINE SPERMATOZOA

6.1 Introduction

In an effort to maintain the genetic diversity of endangered species, genome resource banks containing sperm, ova and embryos have been established in recent years (Karow and Critser, 1997; Watson and Holt, 2001; Holt et al., 2003). Maintaining a large group of samples requires space to house numerous liquid nitrogen (LN_2) tanks Furthermore, these containers must be monitored for leaks and filled regularly to make up for LN_2 evaporation loss. Recently, sperm dehydration has been proposed as an alternative to sperm cryopreservation as a more costeffective means of preserving the many transgenic strains of mice produced for research (Kusakabe et al., 2001).

Desiccation would allow sperm to be stored at room temperature or in a refrigerator, rather than at ultra-low temperatures. Also, dehydration does not require the use of cryoprotectants and, therefore, avoids any toxic effects due to these additives commonly used in cryopreservation protocols. In addition, because the technique of dessication does not require controlled rate freezers, this may be a more practical technique for field work, where access to such instruments is not usually possible.

Since the first report of live young produced using freeze-dried sperm in mice using intracytoplasmic sperm injection (ICSI) (Wakayama and Yanagimachi, 1998), dehydration of sperm in rats (Hirabayashi et al., 2005), rabbits (Liu et al., 2004), pigs (Kwon et al., 2004) and cattle (Keskintepe et al., 2002) has been examined. Therefore, the objective of the present study was to determine whether the dehydration methodology might be applied to feline sperm. The approach was to determine the capability of dehydrated domestic cat sperm to fertilize oocytes after ICSI.

6.2 Literature Review

6.2.1 History of Sperm Dehydration

Natural dehydration of organisms, by removal of water, was first described by Antoine van Leeuwenhoek in 1702 when he observed that adding water could rehydrate tiny organisms that he called “animalcules” (McGinnis et al., 2005). The technique of dehydration of sperm was first attempted by Polge et al. (1949) using fowl sperm. They exposed sperm to a 20% or 30% glycerol solution and cooled the samples to -79°C, warmed them to -25°C, attached the sample to a freeze-dryer, rehydrated and warmed the sample to 40°C before they assessed motility. A maximum of 50% motility was reported although the fertility of the sperm was not assessed at that time.

There have been a few reports of offspring in rabbits (Yushchenko, 1957 as stated in Wakayama and Yanagimachi, 1998) and pregnancies in cattle (Meryman and Kafig, 1963; Larson and Graham, 1976) after AI using freeze-dried sperm, but these studies have not been repeatable by others. The earlier reports of successful freeze-drying of sperm have been considered suspect because the freeze-drying process normally renders the sperm immotile (Wakayama and Yanagimachi, 1998).

It was not until the advent of intracytoplasmic sperm injection (ICSI) that viability of immotile, dried sperm was re-examined. Uehera and Yanagimachi (1976) reported the first embryonic development in hamsters by directly injecting a single fresh epididymal sperm into a mature oocyte. After more than two decades later, the first widely accepted report of live young produced using freeze-dried sperm was reported using ICSI in mice (Wakayama and Yanagimachi, 1998). Since this report, there have been only a few studies involving the use of freeze-dried or air-dried sperm. Most of the studies to date have been done in the mouse (e.g., Kusakabe et al., 2001; Pangestu et al., 2002; Bhowmick et al., 2003; Kaneko et al., 2003a,b; Ward et al., 2003). Embryonic development has also been achieved in the cow (Keskintepe et al., 2002) and pig (Kwon et al., 2004) and live young have been produced after fertilization with freeze-dried sperm in the rabbit (Liu et al., 2004) and the rat (Hirabayashi et al., 2005).

6.2.2 Types of Sperm Dehydration

There two main categories of sperm dehydration are convective or evaporative drying and freeze-drying. Convective drying is a simple procedure of drying a liquid sample at room temperature. In convective or evaporative drying, the sample is placed onto a surface across where an inert gas (e.g., nitrogen) is passed (forced convection) or the sample is left out in the open (passive convection or air-drying) (Bhowmick et al., 2003). In contrast, freeze-drying involves a three-step process including freezing, sublimation and diffusion/desorption (for a review see Acker et al, 2004). In both cases, as long as the vapor pressure of the surrounding environment is less than that at the surface of the sample, water evaporates, drying the sample. Although natural convection is a simple technique, it has a much slower drying rate compared with other drying methods, because it is dependent upon the relative humidity level of the sample environment.

The major disadvantage of this type of drying is that the final moisture level is unknown and there is always humidity left in both open and closed environments.

Forced convection can provide more rapid drying rates than the former by blowing dry air or a gas over the sample. The rate of drying depends on the temperature, velocity of gas flow and the shape and size of the container holding the sample (Bhowmick et al., 2003). Because of the continuous gas flow over the sample, a greater level of dehydration occurs compared with natural convection, because the moisture released from the sample is immediately removed from the environment.

Freeze-drying involves the freezing of the sample to separate the unbound water from the cells (~80% of water in the original solution) in the form of ice (Meyers, 2006). The removal of frozen water from the system by sublimation is called the primary drying phase. Usually the container in which the sample is placed is kept below 0ºC and at a high vacuum (<100 mbar pressure). This drying occurs until the sample undergoes the glass phase transition at which point diffusion or desoption will occur (Crowe et al., 1990). The temperature of the container is then raised above the glass transition point to allow removal of the bound water (20% water of hydration) during the last phase of drying. This phase allows the remaining water to be removed by desorption (for a review see Acker et al., 2004). This last step is very sensitive to permutations, since the temperature must be raised in a controlled manner and not exceed the critical temperature at which the sample looses its ability to rehydrate properly. The resulting product is a lyophilized sample that can be stored by refrigeration or at room temperature.

6.2.3 Advantages and Dissadvantages of Sperm Dehydration

There are advantages but also disadvantages of preserving sperm by various types of dehydration. Evaporative or convective drying has the advantage of being simple and requires less expensive instruments than does freeze-drying, since all steps can be performed at room temperature. The main disadvantage of this method of dehydration is unequal drying rates of the sample within the container. To minimize this effect, the flow rate can be increased and the container size decreased. With the freeze-drying process, the removal of the water of hydration by desorption enables a much higher level of dehydration compared with evaporative or convective drying. The rate of drying is dependant on the sample temperature and the vacuum pressure produced by the freeze-drying apparatus. The major problem with this type of drying it that it is very time consuming, since drying may take from several hours to a few days to complete. Although this technique does require the use of LN_2 for the freezing process, it is still more efficient than standard cryopreservation because the dried samples can be stored at ~20ºC or at 4ºC.

6.2.4 Previous Studies of Sperm Dehydration

Since the first offspring were produced from dehydrated sperm (Wakayama and Yanagimachi, 1998), various methods of sperm drying have been attempted. Although simple methods of sperm desiccation, such as convective drying (Bhowmick et al., 2003) and evaporative drying (Pangestu et al., 2000) have been reported, the most common method of sperm dehydration is the freeze-drying approach (e.g., Kusakabe et al., 2001; Keskintepe et al., 2002; Bhowmick et al., 2003; Kaneko et al., 2003a,b; Ward et al., 2003; Kusakabe and Kamiguchi, 2004; Kwon et al., 2004; Liu et al., 2004; Hirabayashi et al., 2005). Briefly, a 100 µl volume of sperm suspension is transferred into a glass ampule (or microcentrifuge tube) that is immediately plunged into LN_2 (20 to 30 seconds up to 10 minutes). The ampule is then connected to a freeze-dryer unit for 4 hours (Kusakabe et al., 2001) to 18 hours (Wakayama and Yanagimachi, 1998; Keskintepe et al., 2002) and then the ampule sealed. The inlet pressure of the drying unit reported varies for each study (e.g., 32 - 40 x 10^{-3} mbar; 1 to 30 mbar; 190 x 10^{3} mbar), which likely depends on the manufacturer's specifications of the freeze-drying apparatus used. In one case, a much higher value (190 x 10^{-3} mbar) is reported than all the other studies (Keskintepe et al., 2002).

To date, only two species, the rabbit (Liu et al., 2004) and the rat (Hirabayashi et al., 2005), have successfully produced live young by fertilizing oocytes with dehydrated sperm. In several other species, various levels of embryonic development have been observed, such as blastocyst development of 30% in the cow (Keskintepe et al., 2002) and 10% blastocysts development in the pig (Kwon et al., 2004). There has been no report of feline sperm dehydration and embryo development to date.

6.2.5 Fertilization with Dehydrated Sperm

In all species examined to date, the dehydration of sperm causes the loss of motility, not allowing in vitro fertilization to occur. Thus, it was not until ICSI was used that embryo production from dehydrated sperm was successful in mice (Wakayama and Yanagimachi, 1998). In this study and others, the sperm heads of mice are separated from the tails to facilitate the microinjections. In species other than laboratory rodents (mouse and rat), the microtubule organizing centers needed for pronuclear formation are provided by the mid-piece of the sperm (Schatten et al., 1991, Palmero et al., 1997). In the mouse, microtubule organizing centers are provided by the oocyte and therefore, only the sperm head is necessary for fertilization.

Briefly, ICSI is performed by selecting a single sperm and injecting it directly into the cytoplasm of a mature oocyte. The injection bypasses the early processes of fertilization, including the acrosome reaction and membrane fusion. These reactions are usually necessary to initiate pathways responsible for oocyte activation. Activation is needed for the oocyte to resume meiosis and allow extrusion of the second polar body. In some species, such as the mouse (Kimura and Yanagimachi, 1995), hamster (Uehara and Yanagimachi, 1976), rabbit (Keefer, 1989), pig (Kolbe and Holtz, 1999), cat (Pope et al., 1998) and the human (Palmero and Joris, 1992), the mechanical damage to the oocyte is sufficient to induce activation. Other species (e.g., sheep and cattle), not properly activated by the mechanical injury of the injection, require chemical or electrical activation protocols (Gómez et al., 1998; Ock et al., 2003).

6.2.6 Types of Oocyte Activation

In all mammals studied to date, proper oocyte activation is critical for fertilization to occur. Oocyte activation is the process by which a metabolically quiescent oocyte is converted into a metabolically active embryo, which can undergo DNA synthesis, RNA translation and cell division (for a review see Ozil and Huneau, 2001). Activation can be attained naturally, as in normal fertilization (in vivo or in vitro) or due to oocyte ageing. Alternatively, oocytes can be artificially activated by subjecting them to mechanical, chemical or electrical stimulation. In artificially activated oocytes, the normal sperm-oocyte interactions, including sperm-zona binding, the acrosome reaction, plasma membrane binding, are bypassed. In this case, oocyte activation needs to be initiated by another factor(s). The resulting cascade of oocyte events mimics those that occur in natural fertilization (for a review see Williams, 2002).

Although much has been learned in the past decade about oocyte activation, the complete signaling pathway is not yet known. Natural activation begins with a trigger when the spermatozoon binds to an oolema. This initiates a cascade of events involving the release of intracellular calcium stores and an increase in pH, which leads to the exocytosis of cortical granules from within the ooplasm and the resumption of meiosis (for a review see Williams, 2002). A few candidates that may act in the triggering process are a tyrosine kinase receptor on the sperm membrane or a cytosolic sperm factor within the sperm (Swann and Parrington, 1999). During normal fertilization, a spermatozoon binds to the zona pellucida of an oocyte, undergoes an acrosome reaction and then binds to the oolema. The contents of the sperm enter the ooplasm and activation occurs (Williams, 2002).

Oocytes that have remained unfertilized for a prolonged period of time are referred to as ‘aged oocytes’. These oocytes can undergo spontaneous activation, or if fertilized, they will become activated and cleave. However, the resulting embryos exhibit fragmentation, apoptosis and poor embryonic development (Fissore et al., 2002).

Activation protocols are used in conjunction with reproductive techniques including the use of ICSI, nuclear transfer and parthenogenesis. All three types of activation, mechanical, chemical and electrical are used in conjunction with ICSI. The most common type of mechanical activation is the damage occurs during ICSI. It should be noted that improper handling of the oocytes can also induce activation. Chemical activation is achieved with a variety of chemicals that promote the release of internal calcium stores, such as calcium ionophores, strontium chloride, phorbol esthers, ethanol and ionomycin followed by 6-dimethylaminopurine (DMAP) (Alberio et al., 2001). Also, an electrical DC pulse although not used much today, has also been reported to induce oocyte activation (Ozil, 1990).

6.3 Materials and Methods

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise stated.

6.3.1 Experimental Design

In this experiment, matured feline oocytes were randomly assigned to one of five treatments as follows: Control oocytes (n = 36) subjected standard in vitro fertilization (IVF) and cultured in vitro (Control IVP), oocytes (n = 74) injected with fresh or refrigerated sperm and cultured in vitro (Control ICSI), oocytes (n = 57) injected with air-dried sperm and cultured in vitro (Air-Dried Sperm ICSI) and oocytes (n = 45) injected with freeze-dried sperm and cultured in vitro (Freeze-Dried Sperm ICSI). Six replicates of this experiment were performed. All inseminated or injected oocytes were then placed into culture and embryonic development was assessed.

6.3.2 Collection and Processing of Sperm

Ejaculated sperm were collected from toms housed at the Audubon Center for Research of Endangered Species by the use of a teaser female and artificial vagina (AV) as prior approved by the Audubon's Institutional Animal Care and Use Committee. The AV consisted of a small glass bottle filled with 40 ml of warm water (42°C to 45°C), and then a balloon with the tip cut out and a small plastic cup was inserted. A small amount of K-Y lubricant was applied to the balloon edge to ease penetration. The tom was allowed to mount a teaser female in estrus and his penis was guided by a technician into the AV, holding the bottle at a 45°angle. Once the tom had ejaculated, the AV was removed and the sperm sample was transferred to a 1.5 ml microcentrifuge tube and held in a 500 ml water bottle at ~30°C until processed. Sperm motility and concentration were assessed using a hemocytometer. Samples with little or no sperm or motility below 40% were not used in this study.

6.3.3 Sperm Dehydration Procedure

Ejaculated sperm samples were collected and processed individually twice a week. A 30 µl volume of sample was gently pipetted into the bottom of a 1.5 ml microcentrifuge tube containing 1 ml of HeTy + EGTA. This medium was prepared from a sperm washing medium (HeTy) consisting of Tyrode's salt solution (T-2397) and containing 1.5% M199 HEPES, 0.4% BSA, 1 mM L-glutamine (G-8540), 0.36 mM pyruvic acid (P-4562), 2.22 mM L(+)-lactic acid, 50 µg/ml of gentamicin sulfate (G-1264) and 1.12 mM L-cysteine (C-6852) (pH = 7.3 - 7.4; osmolality = 285 - 295 mOsm). To this HeTY medium, 50 mM EGTA (ethylene glycol-bis [β-animoethyl ether]-N,N,N',N'-tetraacetic acid) (03778; Fluka) to was added and the pH was adjusted to between 8.2 to 8.4 (HeTY + EGTA; osmolality = ~400 mOsm). The solution was then sterile filtered using a 0.2 µm pore acrodisk syringe filter.

The sperm sample in the microcentrifuge tube was allowed to swim-up for 12 minutes at 38°C, then the top 600 µl of sperm suspension was collected and transferred to a new 1.5 ml microcentrifuge tube. The sperm motility and concentration were assessed. Each sample was then divided into four 100 µl aliquots for freeze-drying and 4 to 12 aliquots (10 µl each) for air-drying. For freeze-drying, 100 µl aliquots of the collected sample were transferred into 2 ml glass ampules (12-009-36; Wheaton - Fisher Scientific, Pittsburg, PA). Ampules were fixed to canes and plunged into LN_2 for transportation to the Department of Biological Sciences in Baton Rouge, Louisiana. Once the freeze-dryer

(Labconco, Kansas City, MO) was set, one ampule at a time was quickly removed from the LN_2 and placed into a rack submerged in a LN_2 bath until all ampules were in the rack. The rack was then placed inside the machine and the vacuum was re-initiated. The temperature of the chamber was between -43ºC to -45ºC with an internal pressure between 44 and 76 x 10^{-3} mBar. Samples were dried for 4 hours.

The ampules were then filled with an inert gas (N_2) to avoid continued drying and then quickly sealed using silicone corks and then flame sealed to avoid rehydration and transported cool back to the Audubon Center in New Orleans where they were stored at 4°C (Figure 6.1).

For air-dried samples, 10 µl of sperm suspension was placed on a glass microscope slide (12-544-15; Superfrost - Fisher Scientific) and allowed to dry at room temperature in the dark for 30 minutes. The slides were then transferred to a desiccator and stored at room temperature and in the dark until use (Figure 6.2). The desiccator was sealed by applying a vacuum for 1 minute.

6.3.4 Oocyte Collection and Maturation

Ovaries were collected from ovary/hysterectomies at veterinary clinics located in the Baton Rouge and New Orleans areas. The ovaries were obtained from queens of various ages and at various stages of their reproductive cycle. The ovaries were transported in a cooler at room temperature in a 100 ml plastic sample vial (9853Q47; Thomas Scientific, Swedesboro, NJ) containing 50 ml of TL HEPES (04-616F, Cambrex Bio Science, Walkersville, MD) supplemented with 50 µg/ml of gentamicin (G-1397) to the laboratory at the Audubon Center for Research on Endangered Species in New Orleans, Louisiana.

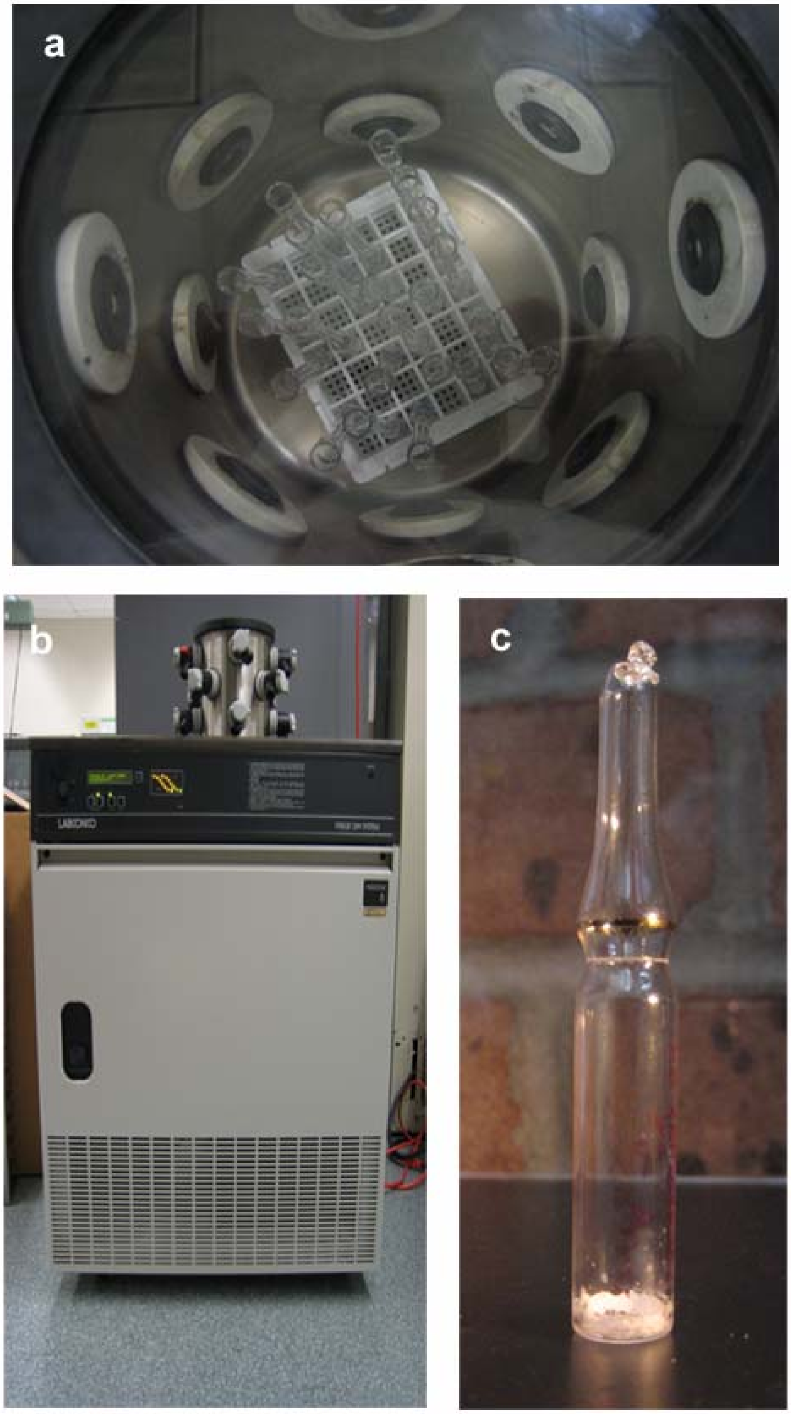

Figure 6.1 Feline sperm being processed for freeze-drying. (a) glass ampules inside the manifold of a freeze-dryer held in a plastic centrifuge tube rack, (b) front view of the freeze-dryer while running and (c) heat sealed glass ampule containing lyophilized feline spermatozoa.

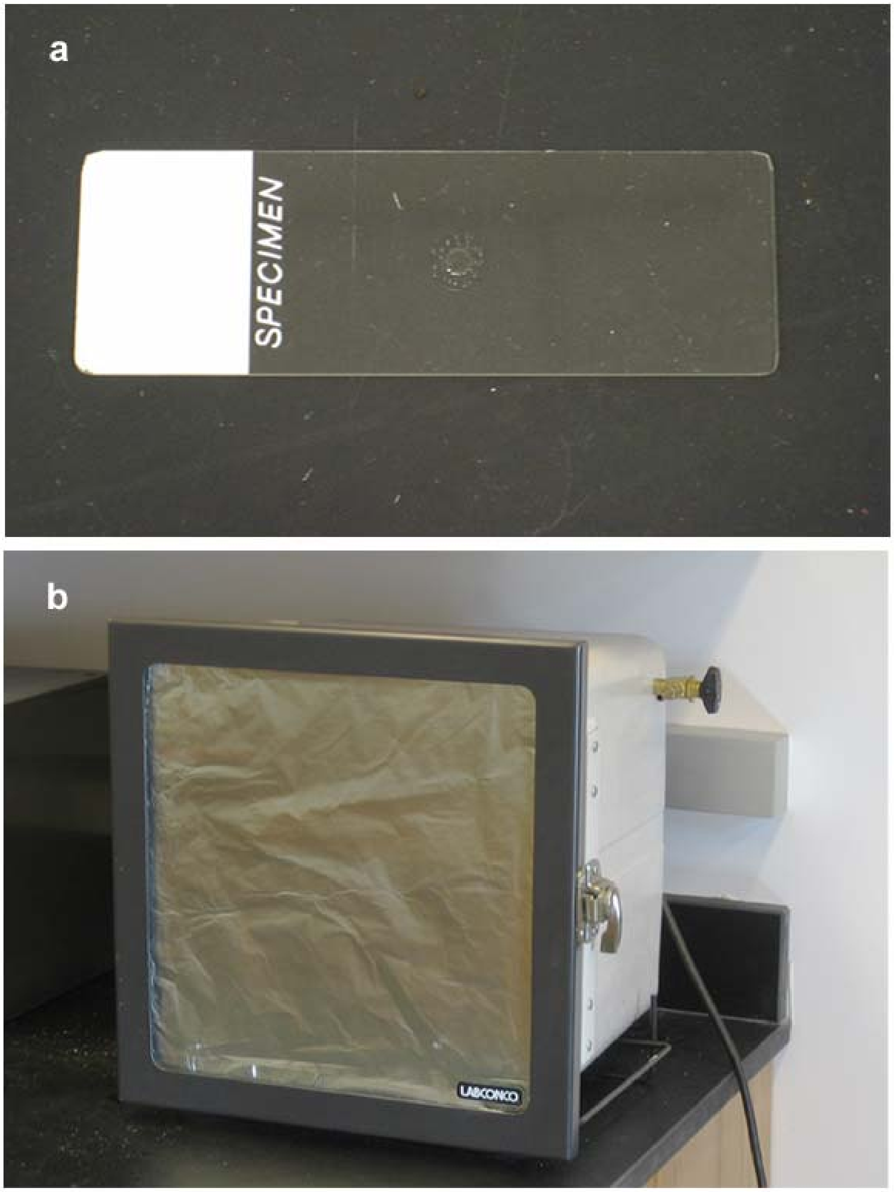
a
SPECIMEN
b
LABCONCO

Figure 6.2 (a) A glass microscope slide with feline air-dried sperm and (b) the dessicator for storing the air-dried samples.

Both fresh and refrigerated ovaries were used in this study. Fresh ovaries were processed within 6 hours post-collection while ovaries for cool storage were placed in a refrigerator once at the laboratory and processed the next morning for in vitro production (IVP) as described, with only minor modifications, by Gómez et al. (2000).

Ovaries were separated from their connective tissue using a fine pair of scissors and then washed in two 60 mm petri dish (351029; Falcon - Becton Dickinson, Franklin Lakes, NJ) containing 2 to 3 ml of oocyte holding medium (He199) and then held in the same medium until processed. The holding medium consisted of Medium 199 (M-3769) supplemented with 71 mM sodium bicarbonate (S-5761), HEPES buffer (H-6147), 0.4% BSA (A-8412) and 1 mM L-glutamine (G-8540), 0.36 mM pyruvic acid (P-4562), 2.22 mM L(+)lactic acid (L-4388) and 50 μg/ml of gentamicin sulfate (G-1264) (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm).

The ovaries were transferred to a 60 mm petri dish containing 2 to 3 ml of He199 where they were measured and the number of visible follicles was recorded. Then each ovary was held with a pair of serrated, curved forceps in a 35 mm petri dish (351008; Falcon - Becton Dickinson) containing He199 and then bisected along its longitudinal axis using a scalpel blade (# 10). Each half was then scraped with the blade to puncture all follicles. This caused the cumulus-oocyte-complexes (COCs) to be released into the medium.

COCs were collected and transferred to a new 35 mm dish containing He199. The ovaries were minced a second time and COCs again collected. After the COCs had been collected, they were washed in another 35 mm dish containing He199 and then washed in two successive dishes containing in vitro maturation (IVM) medium. The

IVM medium consisted of M199 Earle's salts solution (9102; Irvine Scientific, Santa Ana, CA) supplemented with 100 IU/ml of human chorionic gonadotropin (hCG) (CG-10), 200 IU/ml of equine chorionic gonadotropin (eCG) (G-4877), 100 IU/ml of Epidermal Growth Factor (EGF) (E-9644), 0.3% BSA Fraction V (81-068-2; Serological Proton, Kankakee, IL), 2 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM lactic acid, 1.12 mM L-cysteine (C-6852) and 50 µg/ml of gentamicin (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm).

Oocytes were graded based on the number of cumulus cell layers. Oocytes with two or more layers of cumulus cells and an even dark cytoplasm were given a quality score of Grade A & B. Oocytes with less than one layer of cumulus cells and/or a light or uneven distribution of cytoplasm were given a quality score of Grade C.

Groups of 20 to 30 oocytes were transferred to 800 µl of IVM medium in a 4-well culture dish (176740; Nunclon - VWR International, West Chester, PA). These dishes were placed into a humidified, gassed with 5% O_2, 5% CO_2 and 90% N_2 and placed in a humidified sealed bag (0181216; Kapak sealpak pouches - Fisher Scientific) in an incubator at 38°C for 24 hours. All media were filtered through a 0.2 µm pore acrodisk.

6.3.5 Re-Suspension of Dried Sperm

Just prior to use, all dried sperm samples were rehydrated by adding an equal volume of water to the dehydrated samples (10 µl/slide or 100 µl/ampule). The sample was mixed and then transferred to a 1.5 ml microcentrifuge tube and kept at room temperature until needed.

6.3.6 Oocyte Fertilization by Intracytoplasmic Sperm Injection (ICSI)

After 24 hours of maturation, feline COCs were ramdomly assigned to one of three treatment groups as follows: Control ICSI (n = 74), Freeze-Dried Sperm ICSI (n = 45) and Air-Dried Sperm ICSI (n = 57). All COCs to be injected were stripped of their cumulus cells by transferring them into a 1.5 ml microcentrifuge tube containing 0.05% hyaluronidase (H-4272) prepared in IVM medium. The tube was vortexed for 75 seconds and then the contents were transferred to a 35 mm petri dish to recover the denuded oocytes in a heated room at 28ºC to 30ºC. Manually stripping of coronal cells was done using a 200 μm micropipette, if needed. Oocytes were washed through two 35 mm dishes containing ~3 ml of He199.

Oocytes were then transferred into a 35 mm petri dish containing ~3 ml of He199 and incubated at 38°C (5% CO_2 in air) for at least 30 minutes before performing ICSI. The injection preparation in shown in Figure 6.3. Holding pipettes were hand made from borosilicate glass (B100-50-10; Sutter Instruments Co., Novato, CA). Pipettes were pulled and cut (i.d. 180 μm) at a 20ºangle was made using a microforge (Model # MI-9; Narishige, Tokyo, Japan). The injection pipettes were obtained commercially (4-6 μm with a spike and a 20ºangle, # IC-SP-20; Conception Technologies, San Diego, CA).

First, a single spermatozoon was immobilized in the sperm suspension droplet and then it was aspirated tail-first into the tip of the microinjection pipette. The injection pipette was then moved to the oocyte droplet where the oocyte maturity was assessed. Only metaphase-II (MII) stage oocytes were used for injection. The oocyte was held by the holding pipette with the polar body in the 12 o’clock or the 6 o’clock position by

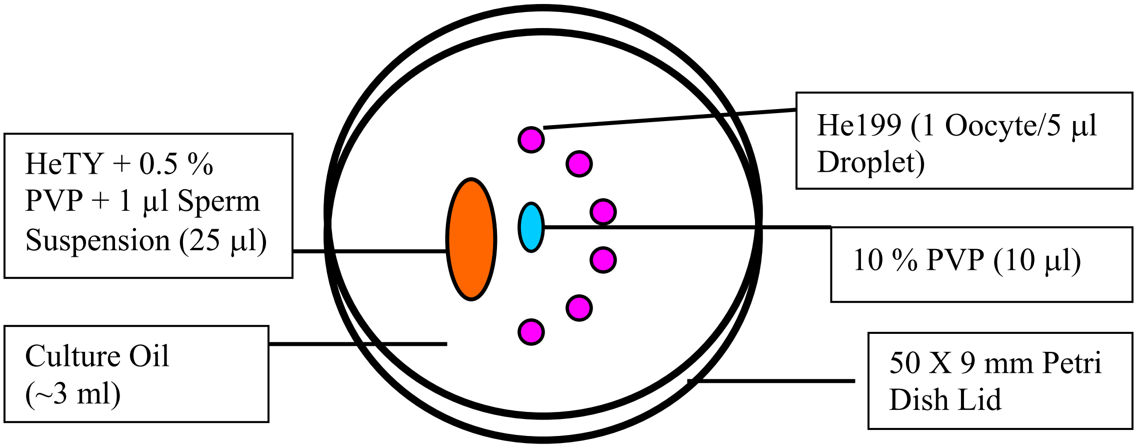
HeTY + 0.5 % PVP + 1 µl Sperm Suspension (25 µl)
Culture Oil (~3 ml)
He199 (1 Oocyte/5 µl Droplet)
10 % PVP (10 µl)
50 X 9 mm Petri Dish Lid

Figure 6.3 Plate preparation for the injection of feline oocytes (ICSI). Sperm were immobilized in the HeTY + PVP + Sperm suspension (orange) droplet. The injections took place in the He199 droplets containing a single oocyte each. The center mineral oil droplet was used to clean the pipette between injections and load the injection pipette before starting to give better control. A new dish was prepared for each group of 6 oocytes to be injected.

applying suction. The injection pipette was then inserted into the oocyte and the suction was increased to aspirate the ooplasm until the membrane ruptured. The ooplasm and the sperm were then slowly injected back into the oocyte with minimal volume. The injection pipette was gently withdrawn and the suction on the holding pipette released. The injection pipette was returned to the sperm suspension droplet and another sperm was selected. The injections were repeated in groups of 6 oocytes.

Once all of the injections were completed, the presumptive zygotes were washed in two 35 mm dishes containing ~3 ml IVC-1 and then transferred into 800 µl of IVC-1 in a 4-well dish. The dish was then placed into a plastic bag filled with a gas mixture of 5% CO_2, 5% O_2 and 90% N_2 and incubated at 38°C as described for standard feline IVP (day 0).

Sham injections were also preformed in a preliminary study to assess the level of parthenogenetic development associated with the injection alone. These injections were done as previously described by injecting only a small amount of medium and no sperm into the oocyte.

6.3.7 In Vitro Fertilization and Culture

For standard IVP control (Control IVP), COCs (n = 36) were processed by the procedure described by Gómez et al. (2004) with only minor modifications. COCs were washed in two 35 mm dishes containing in vitro fertilization (IVF) medium and then placed into 100 µl insemination droplets under warmed mineral oil (4008-5, Sage Biopharma, Pasadena, CA) in an incubator at 38°C in 5% CO_2 in air for 12 hours. To prepare insemination microdrops, 2 µl of the refrigerated pellet were resuspended in 400 µl of pre-warmed HEPES-Tyrode’s solution (HeTY) medium.

Sperm progressive motility and concentration were determined using a hemocytometer to determine the amount of sperm to be added to the IVF droplets (50 x 10^4 sperm/ml). The IVF medium consisted of Tyrode's salt solution (T-2397) supplemented with 0.3% BSA, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)lactic acid, 100 IU/ml of penicillin G (P-3032) and 100 mg/ml of streptomycin sulfate (S-9137) (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm).

After fertilization, presumptive zygotes were washed once in IVC-1 medium and then transferred to a fresh well containing 800 µl of in vitro culture-1 (IVC-1) medium in a 4-well culture dish. IVC-1 medium consisted of Tyrode's salt solution supplemented with 0.3% BSA, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)lactic acid, 1% MEM nonessential amino acids (M-7145), 50 µg/ml of gentamicin sulfate and 1 µl/ml of amphotericin B (A-2942) (pH = 7.7 - 7.9; osmolality = 285 - 295 mOsm). These dishes were placed into a humidified, gassed (5% O_2, 5% CO_2 and 90% N_2) sealed bag in an incubator (38°C) for 2 days. On day 2 post-insemination, fertilization rates were determined based on cleavage rates. Nondividing ova were removed and dividing embryos were incubated in IVC-1 + EAA (IVC-1 supplemented with 1% MEM essential amino acids; M-5550) for another 2 days (day 5 of culture).

On day 5, embryos were washed twice with in vitro culture-2 (IVC-2) medium and then transferred to a well containing 800 µl of IVC-2 and incubated for an additional 2 days. IVC-2 medium consisted of Tyrode's salt solution supplemented with 10% FBS, 0.1% sodium bicarbonate, 1 mM L-glutamine, 0.36 mM pyruvic acid, 2.22 mM L(+)lactic acid, 1% MEM nonessential amino acids, 1% MEM essential amino acids, 50 µg/ml of gentamicin sulfate and 1 µl/ml of amphotericin B (pH = 7.7-7.9; osmolality =

285-295 mOsm). On days 7 and 8 post-insemination, blastocyst development was assessed and blastocyst hatching and expansion were noted. Further development was monitored for an additional 2 days.

6.3.8 Calcium Ionophore Activation

One replicate of activation was performed on injected oocytes (n = 18) 2.5 hours post-injection using freeze-dried sperm. Briefly, oocytes were processed as described for ICSI and placed into IVC-1 for 2.5 hours. The injected oocytes were then transferred into a 4-well culture dish containing 400 µl of 10 µM calcium ionophore (C-7522) prepared in He199 without BSA and placed in an incubator for 5 minutes. The oocytes were removed from the activation medium and washed twice in IVC-1 before being placed back into in vitro culture. Embryonic development was assessed as previously described.

6.3.9 Statistical Analysis

Cleavage and blastocyst rates of control and injected feline oocytes across treatment groups were analyzed for statistical significance using Chi-square analysis ($P \leq 0.05$ statistically different) (Instat Graphpad Version 3.0, San Diego, CA).

6.4 Results

In a preliminary study, sham injections were completed on feline oocytes (n = 45) with fresh cat sperm and subsequent embryonic development was noted during in vitro culture. In this trial, 40% of the injected oocytes cleaved in culture, however, none of these embryos produced blastocysts. All of the sham-produced early stage embryos exhibited fragmentation and had unequal blastomere size after the first two divisions. The sham injections were conducted to assess the oocyte activation protocol for feline oocytes prior to the main experiment.

In the main experiment, embryonic development of feline oocytes injected with sperm was assessed in six replications. Cleavage rates on day 2 of in vitro culture were 56% for the Control IVP, 59% for the Control ICSI groups, 35% for the Air-Dried Sperm ICSI and 60% for the Freeze-Dried Sperm ICSI groups, respectively (Table 6.1). The embryo cleavage rates for the Control IVP, Control ICSI and the ICSI with freeze-dried sperm (Freeze-Dried Sperm ICSI) groups were significantly higher ($P \leq 0.05$) than the cleavage rate obtained after ICSI with air-dried sperm (Air-Dried Sperm ICSI).

Blastocyst development on day 8 of in vitro culture in all the oocyte injected groups (Control ICSI, Air-dried ICSI and Freeze-dried ICSI) was lower (9%, 2% and 0%) ($P \leq 0.05$) than that obtained in the Control IVP group at 25%. Only one blastocyst was produced using air-dried sperm and no blastocysts resulted in the freeze-dried injected oocytes (Figure 6.4).

In an attempt to increase the potential for blastocyst development using freeze-dried sperm, an oocyte activation protocol using calcium ionophore was evaluated in a subsequent experiment. In this study, feline MII-stage oocytes ($n = 18$) were injected with freeze-dried sperm or these oocytes ($n = 42$) were subjected to the standard IVP protocol. The cleavage rate of freeze-dried sperm was higher (61%) than that for the control IVP group (24%) (data not shown). Also, one hatching blastocyst (6%) was

Table 6.1 Embryonic development resulting from feline oocytes subjected to standard IVP and oocytes injected with fresh, freeze-dried or air-dried spermatozoa

		Oocytes/embryos developing (%)	
Treatment	No. of oocytes	Cleavage (d 2)	Blastocysts (d 8)
Control IVP	36	20 (56)	9 (25)
Control ICSI	74	44 (59)	7 (9)*
Freeze-Dried Sperm ICSI	45	27 (60)	0 (0)*
Air-Dried Sperm ICSI	57	20 (35)*	1 (2)*

*Values with an astericks within columns are statistically different from the remaining values (P≤0.05; Chi-square analysis).

produced when oocytes obtained from one pair of ovaries were exposed to calcium ionophore and injected with freeze-dried sperm (Figure 6.4). No blastocysts were obtained from the IVP control oocytes.

6.5 Discussion

In this study, we found that air-dried and freeze-dried feline sperm can fertilize in vitro matured oocytes using ICSI and could result in embryonic development to the blastocyst stage. Previous reports have demonstrated that, although the sperm resulting from dehydration are immotile or ‘dead’ in conventional terms and that they retain their ability to undergo decondensation, pronuclear formation and syngamy when injected into mature oocytes (Wakayama and Yanagimachi, 1998, Kwon et al., 2004, Liu et al., 2004). This has been attributed to a sperm-specific factor located within the sperm that is maintained during the drying process (Ward et al., 2003).

This sperm-specific factor was initially reported in mammals by Stice and Robl (1990), when they injected mouse oocytes with rabbit sperm. This factor is known to be a protein and to induce calcium oscillations in mammalian oocytes (releases Ca^{2+} from intracellular stores in the oocyte) across species (Wu et al., 1997). Numerous candidates for this sperm-specific factor have been identified including: oscillin (Parrington et al., 1996), a truncated c-kit receptor (tr-kit) (Sette et al., 1997), Src-like tyrosine kinase (Parrington et al., 2002) and sperm-specific phospholipase C (PLCζ) (Saunders et al., 2002). The latter protein is currently thought to be the most likely candidate because it most closely imitates the normal sequence of events that lead to syngamy during

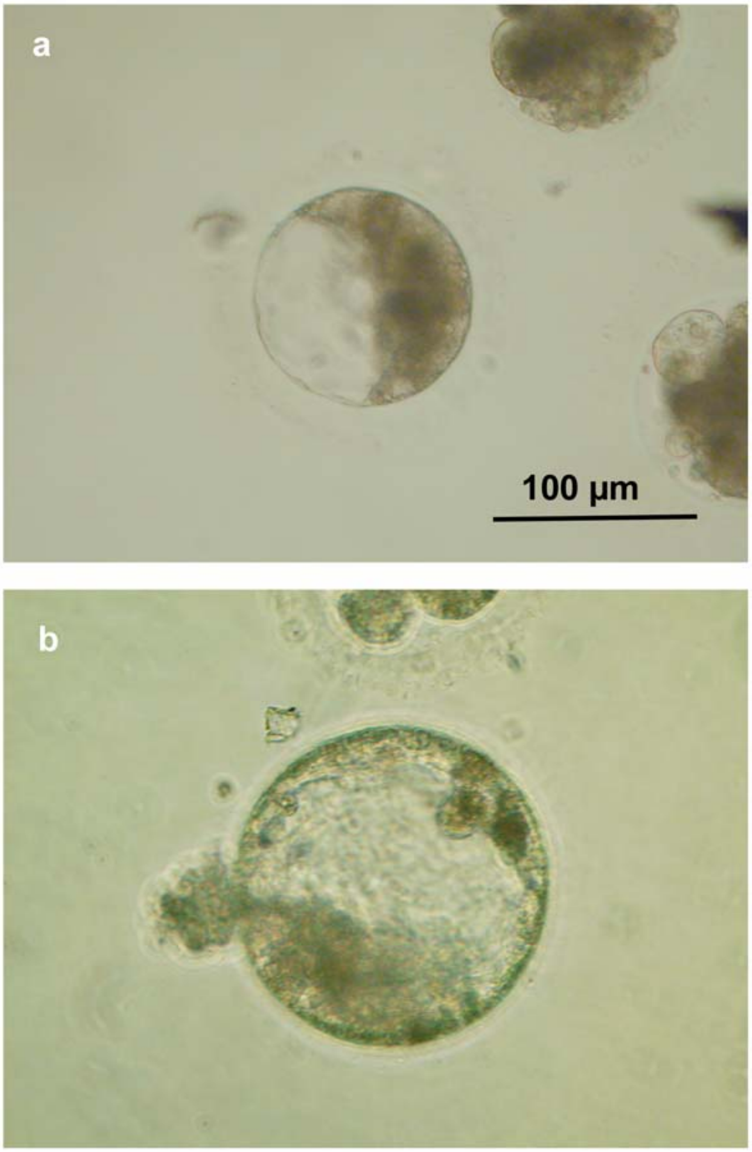

Figure 6.4 Blastocysts (day 9) produced by injecting (a) air-dried sperm or (b) freeze-dried sperm into matured feline oocytes. (a) Air-dried sperm. Note the thin trophectoderm overlying the blastocoel. (b) Freeze-dried sperm. Note the mass of cells hatching from the zona pellucida and thick trophectoderm. Photographs were taken at the same magnification using an inverted Nikon microscope with a 20X objective.

fertilization. This sperm factor has been found in all species examined thus far, including, fish, lower vertebrates and mammals (for a review see Swann et al., 2004).

Freeze-dried sperm has been used to produce live offspring in mice, rabbits and rats when combined with the ICSI procedure (Wakayama and Yanagimachi, 1998, Liu et al., 2004, Hirabayashi et al., 2005). In this study, feline freeze-dried and air-dried sperm injected into in vitro mature feline oocytes cleaved at rates of 60% and 35%, respectively. These cleavage rates are less than those obtained when freeze-dried sperm was injected into mouse oocytes (80% to 99%) (Kaneko et al., 2003b) and rabbit oocytes (70%) (Liu et al., 2004). However, the results of this study were comparable to the cleavage rates obtained in the cattle at 44% to 63% (Keskintepe et al., 2002) and in the pig at 50% to 52% (Kwon et al., 2004) when injected with freeze-dried sperm. In the rat, Hirabayashi et al. (2005) reported only an 8% cleavage rate, yet they obtained live young after embryo transfer. Therfore, although the cleavage rate was low, these developing embryos were still competent.

Blastocyst rates of oocytes injected with dried feline sperm (2%) were low in our study compared with 10% to 69% in mice (Kaneko et al., 2003b), 11% to 30% in cattle (Keskintepe et al., 2002), 24% in rabbits (Liu et al., 2004) and 11% in the pig (Kwon et al., 2004). In the present study, however, no blastocysts were derived from feline oocytes injected with freeze-dried sperm without chemical activation. One possible reason for this is that the oocytes may not have been properly activated. This is not uncommon as the oocytes of those species must be artificially activated in conjunction with ICSI to induce the resumption of meiosis (Ock et al., 2003). With freeze-dried rabbit and pig sperm, oocyte activation can be accomplished by exposing injected oocytes to 10 µM calcium ionophore or 10 μM ionomycin with and without DMAP (Kwon et al., 2004; Liu et al., 2004).

In the present study, the effect of activation was examined using the same concentration of calcium ionophore reported in the rabbit and pig studies using freeze-dried sperm. No marked increase in cleavage rates resulted, however, the first feline blastocyst was obtained only after oocyte activation with calcium ionophore was used. This finding is similar to that for the pig, where no blastocyst development was observed until an oocyte activation protocol was established (Kwon et al., 2004). However, the ultimate test of sperm function is its ability to fertilize an oocyte and the resulting transferred embryo that produces a viable offspring. To date, no transfers of feline embryos produced using freeze-dried or air-dried sperm have been attempted.

The ability to compare and contrast the results of this experiment to those of others has been limitated due to variables involved during the sperm drying process. These variables include the sperm type, the medium used for dehydration, the type of dehydration used, the time of drying, storage and temperature of all of these processes. Two types of sperm have been used for freeze-drying studies, ejaculated and epididymal. The level of maturation on the sperm head membranes likely affects their stability during the dehydration process. The types of dehydration used are freeze-drying and air-drying. Freeze-drying has been the only type that has successful resulted in embryonic development and live young (Wakayama and Yanagimachi, 1998, Liu et al., 2004, Hirabayashi et al., 2005). In the present study, the only embryo to completely hatch was produced from freeze-dried sperm. The freeze-drying process results in a lower level of humidity remaining in the dried sample compared with the air-drying approach.

Inconsistencies in reporting the vacuum pressure exerted on the samples and differences in time allowed to dry have lead to ambiguities relating to the best approach to sperm dehydration. Most reports indicate that dehydrated sperm have ~5% humidity and that levels below 5% can result in chromosomal damage or embryonic abberations (Jeyendran et al., 1981; Bhowmich et al., 2003). However, this humidity issue has not yet been fully evaluated. Finally, storage time has varied from a few hours to a year but further confusion matters is the various storage temperature and their affect on genetic integrity.

6.6 Conclusions

This experiment shows that feline sperm (both air-dried and freeze-dried) can maintain their ability to fertilize in vitro matured feline oocytes that can undergo early stage embryonic development in vitro. To date, this is the first report of successful preservation of domestic cat sperm using freeze-dried and air-dried sperm dehydration. However, at this stage the efficiency of this technique is low. Molecular-based analyses are needed in the future studies to eliminate the possibility that the resulting embryos from this process were parthenogenetic. Further study is recommended to optimize this methodology before it may be used as a tool for the preservation of sperm from domestics and endangered cats.

CHAPTER 7
ASSESSMENT OF DNA INTEGRITY OF DEHYDRATED FELINE SPERMATOZOA USING THE COMET ASSAY

7.1 Introduction

Recently, Wakayama and Yanagimachi (1998) discovered that mouse spermatozoa can be dehydrated, stored at temperatures above 0ºC and produce live offspring (n = 14) when injected into mouse oocytes. Since this first offspring produced with freeze-dried intracytoplasmic sperm injection (ICSI) in the mouse, two other species, the rabbit (Liu et al., 2004) and the rat (Hirabayashi et al., 2005), have also been used to produce live young with freeze-dried sperm in conjuction with ICSI.

The freeze-drying technique is more cost efficient than standard cryopreservation that requires storage at ultra-low temperatures. In addition, standard cryopreservation may induce sperm DNA damage (Lindford and Meyers, 2002). It is also possible that severe dehydration (e.g., freeze-drying) may also cause damage to the sperm nuclear material. Therefore, the objective of this study was to determine the effect of two types of dehydration (freeze-drying and air-drying) on the DNA integrity of feline spermatozoa.

7.2 Literature Review

7.2.1 Applications of Sperm Dehydration

Freeze-drying (lyophilization) is commonly used in the food and drug industries. It has also been used to preserve bacteria and other microrganisms, but only recently has this technique been applied to mammalian cells and tissues. Because one first must determine optimal freezing protocols for freeze-drying, tissue preservation using this approach has not been very successful to date. However, erythrocytes and human fibroblast cells have been successfully freeze-dried and rehydrated (Goodrich et al., 1992; Puhlev et al., 2001). With the success of Wakayama and Yanagimachi (1998), using freeze-dried mouse sperm, this technique has been proposed as a cost-effective means of preserving the numerous transgenic strains of mice produced for biomedical research (Kusakabe et al., 2001).

This technique may provide an effective method to preserve sperm of inbred mouse strains that, with conventional freezing, exhibit poor post-thaw recovery. Desiccation might allow for sperm to be stored at room temperature instead of at ultra-low temperatures as in the case for cryopreserved sperm samples. Dessication would result in a drastic decrease in the cost of maintaining large storage facilities of liquid nitrogen (LN_2) tanks. Furthermore, sperm dehydration might also be more practical for field work, where access to cooling units and LN_2 is limited or unavailable. Transport of dried genetic material is also far less expensive and would require less special handling, allowing for easier movement of specimens between different locations.

7.2.2 Media Used for Dehydration

Various types of media have been used to preserve the cells during dehydration. In the original publication by Wakayama and Yanagimachi (1998), they used two types of media; CZB medium without EDTA and DMEM supplemented with 10% fetal bovine serum (FBS). For bovine sperm, Keskintepe et al. (2002) also used DMEM supplemented with 10% FBS but added glutamine, sodium pyruvate, nonessential amino acids and nucleosides. The most common medium used to protect spermatozoa during desiccation was originally published for mouse sperm by Kusakabe et al. (2001). It consists of a 10 mM Tris-HCl buffer containing 50 mM EGTA (ethylene glycol-bis [β-animoethyl ether]-N,N,N',N'-tetraacetic acid) in 50 mM NaCl with a high pH (8.0 - 8.4). In that study, the authors compared the effects of CZB medium with the Tris-HCl buffer and found that the latter resulted in a higher percentage of karyotypically normal embryos.

High concentrations of a calcium-chelating agent, such as EGTA, are used routinely to help maintain chromosome integrity in DNA preparations from eukaryotic cells. This Tris-HCl solution has subsequently been used in various studies for sperm dehydration from a variety of different species (Bhowmick et al., 2003; Kaneko et al., 2003a,b; Ward et al., 2003; Liu et al., 2004). Other solutions used for sperm dehydration include distilled water (Hoshi et al., 1994). Chemical agents used to cause sperm desiccation are 100% ethanol, dithiothreitol (DTT), methanol, acetone or a chloroform-methanol (2:1) mixture (Katayose et al., 1992). The rehydration procedures of sperm samples used in all dehydration studies to date has been very similar. The samples are rehydrated by simply adding an equal volume of ultra-pure water to the volume of the sample of dried sperm (Wakayama and Yanagimachi, 1998). It has been reported that an increase in the time of injection after rehydration is correlated to a decrease in activation and fertilization in mice (Wakayama and Yanagimachi, 1998).

7.2.3 Storage of Dried Sperm Samples

The length of storage and the temperature at which the dried sperm samples are stored vary. For freeze-dried rabbit sperm, the longest period of storage reported has been for more than 2 years at ambient temperature (Liu et al., 2004). Storage time (up to 1 year) was not shown to have a deleterious effect on the genetic integrity of freeze-dried samples of mouse sperm (Ward et al., 2003). Various temperatures used for storage of dehydrated sperm have been at room temperature (22°C to 25°C), in the refrigerator (4°C), in a -80°C freezer or in LN_2 (-196°C).

By applying the theory of accelerated degradation kinetics on freeze-dried sperm, it has been estimated that samples stored above -80°C for >10 years would result in a 0% blastocyst rate in mice (Kawase et al., 2005). However, in the short term (<1 year), there were no significant differences if freeze-dried sperm were stored at 4°C or -80°C (Kawase et al., 2005). Until sperm samples have been stored for many years at various temperatures, the real effects on genetic integrity will not be known.

7.2.4 Assessment of DNA Integrity

In addition to studies on embryonic development, investigators have examined the effect of sperm dehydration on DNA integrity and/or cell ploidy. It is well known that freeze-drying causes severe damage to the acrosome and plasma membranes. The deleterious effect of drying on spermatozoa has been examined by use of chromosomal spreads of zygotes (Kaneko et al., 2003a,b; Ward et al., 2003; Kaneko and Nakagata, 2005) and most recently, using the comet assay (Kawase et al., 2005).

Briefly, chromosome spreads are made on zygotes because oocyte chromosomes seldom show chromosomal aberrations at the metaphase-II (MII) stage, and any abnormal chromosomes are considered to be of paternal origin (Kaneko et al., 2003b). In the mouse, when freeze-dried sperm samples were stored at 4ºC for up to 5 months the amount of abnormal sperm was not significantly different from that of control sperm (56% to 76%) (Kaneko et al., 2003b; Kaneko and Nakagata, 2005). However, Ward et al., (2003) found a similar percentage of chromosomal abnormalities in freeze-dried mouse sperm but it was significantly lower than the abnormalities noted in the control samples. The number of chromosomal abnormalities found in mouse embryos produced by using freeze-dried sperm was found not to be significantly different than that of fresh sperm used with ICSI (Kusakabe et al., 2001).

The comet assay, is a single-cell gel electrophoresis procedure that is used to examine the comet 'tails' produced by the sperm heads in the presence of unbound or fragmented DNA (for a review see Collins, 2004). It was developed by Ostling and Johanson in 1984 to assess DNA damage incurred in somatic cells after irradiation (Fairbairn et al., 1995). Since then, many reports have used the comet assay to assess damage in a variety of cell and tissue types (for a review see Fairbairn et al., 1995).

This technique requires that the chromosomes first be decondensed after the cells are embedded in an agarose matrix and then the cells are lysed to allow for unbound DNA to migrate away from the sperm head when exposed to a weak electrical current (for a review see Olive, 2002). The presence of comet tails has been observed in samples of freeze-dried sperm stored at 4ºC for several months but not found when samples were fresh or freeze-dried and stored at -80ºC (Kawase et al., 2005).

Most recently, the comet assay has been used in human fertility clinics as a predictor of male infertility (Morris et al., 2002). The biggest problem associated with this comet assay results is the inability to directly compare results from different laboratories due to the numerous image analysis programs and varied protocols. DNA integrity is an important but sometimes forgotten a factor that needs to be examined to ultimately determine the success of any desiccation protocol.

7.3 Materials and Methods

All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise stated.

7.3.1 Experimental Design

In the first experiment (Experiment 1), evaporation rates of a medium (HeTy + EGTA) used for dehydration and of feline sperm suspensions prepared in the same medium (HeTY + EGTA + Sperm) prepared in this solution were examined. Three samples of HeTY + EGTA (a, b, c) were evaluated as well as two samples of feline sperm suspensions, HeTY + EGTA + Sperm (a, b).

In the second experiment (Experiment 2), four treatments for feline sperm were as follows: Fresh sperm (n = 9), sperm exposed to HeTy + EGTA only (n = 9), sperm exposed to HeTy + EGTA and then vitrified (n = 4) (LN_2 only), air-dried (n = 4) and freeze-dried sperm (n = 4). In this experiment, membrane integrity, or viability, of these sperm samples was assessed.

In the third experiment (Experiment 3), sperm DNA damage was assessed using the comet assay. The five treatments were: Fresh ejaculated sperm (n = 8) (Fresh Sperm), sperm exposed to HeTy + EGTA and then vitrified (n = 10) (LN_2 only), sperm processed for air-drying and then rehydrated (n = 9) (Air-Dried), ejaculated and epididymal sperm processed for freeze-drying and then rehydrated (n = 16 and n = 12) (Freeze-Dried EJ and Freeze-Dried EP).

7.3.2 Sperm Collection and Processing for Drying

Both epididymal and ejaculated feline sperm were compared in Experiment 3. Only ejaculated feline sperm was evaluated in Experiments 1 and 2. Epididymal sperm were collected from testes obtained from local veterinary clinics and transported in a cooler in a plastic 100 ml sample vial (9853Q47; Thomas Scientific, Swedesboro, NJ) containing 50 ml of TL HEPES (04-616F; Cambrex Bio Science, Walkerville, MD) supplemented with 50 µg/ml of gentamicin (G-1397) and transported at room temperature to the laboratory at the Audubon Center for Research of Endangered Species, New Orleans, Louisiana. Testes were kept at room temperature and processed within 2 to 4 hours after being surgically removed. The testes were recovered from the sample vials and rinsed twice in Dulbecco's phosphate-buffered saline (PBS) (D-8662) in a 35 mm petri dish. One testis at a time was transferred to a new 35 mm dish containing ~3 ml of sperm washing medium (HeTy) consisting of a Tyrode's salt solution (T-2397) and containing 1.5% M199 HEPES, 0.4% BSA, 1 mM L-glutamine (G-8540), 0.36 mM pyruvic acid (P-4562), 2.22 mM L(+)lactic acid, 50 µg/ml of gentamicin sulfate (G-1264) and 1.12 mM L-cysteine (C-6852) (pH = 7.3 - 7.4; osmolality = 285 - 295 mOsm).

The epididymis was carefully dissected free from the testis and placed into a new 35 mm dish containing HeTy plus EGTA. The dehydration medium (HeTy + EGTA) was prepared by adding 50 mM EGTA (03778; Fluka) to HeTy medium and adjusting the pH between 8.2 to 8.4 (osmolality = ~400 mOsm). The solution was filter-sterilized through a 0.2 µm pore acrodisk syringe filter. A longitudinal opening was cut into the epididymis with a scalpel blade (# 10) and then it was placed on a slide warmer for 10 minutes, allowing the sperm to swim into the medium. The sperm suspension was collected and then transferred to a 1.5 ml microcentrifuge tube. Motility and sperm concentration were assessed and then the sample was processed for drying.

Ejaculated sperm were collected from healthy toms of known fertility housed at the Audubon Center in New Orleans. Samples were collected by use of a teaser female and an artificial vagina (AV) twice a week. Ejaculates volumes ranged from 23 µl to 130 µl. All procedures were pre-approved by the Audubon's Institutional Animal Care and Use Committee. The AV consisted of a small glass bottle filled with 40 ml of warm water (42°C to 45°C). In this bottle, a balloon with the tip cut out was inserted and a small plastic cup was placed at the tip to hold the sample. The tom was allowed to mount the teaser female and his penis was guided into the AV by the investigator, holding the bottle at a 45°angle. Once the sample was produced, the AV was removed and the sperm sample was transferred to a 1.5 ml microcentrifuge tube and held in a 500 ml water bottle at ~30°C until processed.

Both ejaculated and epididymal sperm were processed for dehydration as follows. For ejaculated sperm, a volume of ~30 µl of each individual ejaculate was gently pipetted into the bottom of a 1.5 ml microcentrifuge tube containing 1 ml of HeTy + EGTA. The sample was allowed to swim-up for 12 minutes at 38°C and then the top 600 µl of sperm suspension were collected and transferred to a new 1.5 ml microcentrifuge tube. For epididymals sperm, 800 µl of the sperm suspension was collected after the swim-out and then transferred to a 1.5 ml microcentrifuge tube. The sperm progressive motility and concentration were then assessed. The sample from each tom was then divided with four 100 µl aliquots for freeze-drying and four to twelve 10 µl aliquots for air-drying.

For freeze-drying, 100 µl aliquots of the collected sample were transferred into a 2 ml glass ampules (12-009-36; Wheaton - Fisher Scientific, Pittsburg, PA) with a sterile, glass Pasteur pipette. Ampules were fixed to canes and plunged into LN_2 for transportation to the Department of Biological Sciences in Baton Rouge, Louisiana. One ampule at a time was quickly removed from the LN_2 and placed into a rack submerged in a LN_2 bath until all ampules were in the rack. The rack was then placed inside the freeze-dryer (Labconco, Kansas City, MO) and the vacuum was initiated. The temperature of the chamber was between -43ºC and -45ºC with an internal pressure between 44 and 76 x 10^{-3} mBar. Samples were then dried for 4 hours. The ampules were filled with an inert gas (N_2) to avoid rehydration and then quickly sealed with silicone corks. The ampules were then flame-sealed to prevent the samples from being rehydrated and transported back to the Audubon Center in New Orleans where the dried samples were stored at 4°C until use.

For air-dried samples, 10 µl of sperm suspension for each male was placed on a glass microscope slide (12-544-15; Superfrost - Fisher Scientific) and allowed to dry at room temperature in the dark for 30 minutes. The slides were then transferred to a desiccator and stored at room temperature and in the dark until use. The desiccator was sealed by applying a vacuum for 1 minute.

Just prior to use, freeze-dried and air-dried sperm samples were rehydrated by adding an equal volume of ultra-pure water to the dehydrated samples (10 µl/slide or 100 µl/ampule, respectively). The rehydrated sample was then transferred to a 1.5 ml microcentrifuge tube and kept at room temperature until needed.

7.3.3 Measurement of Evaporation Rates

Evaporation rates of various media, as well as sperm suspensions, were examined to determine the appropriate drying time required for air-dried sperm samples. Briefly, a 10 µl sample was placed on a glass microscope slide and the total was weighed (time =

0). The slide was left on the balance (± 0.0001) and the weight was recorded every 10 minutes until two successive identical weights were recorded (dried sample). This was done three times for each sample analyzed.

7.3.4 Viability Assessment of Sperm

In Experiment 2, viability of feline spermatozoa was assessed at various stages of the drying process to determine the stage at which the membranes were being disrupted. A commercially available kit (541-465-8300; Live/Dead Sperm Viability Kit - Molecular Probes, Eugene, OR) containing propidium iodide (PI) and Syber 14 (SYB14) was used to make this determination. Briefly, a 1:50 dilution of SYB14 in HeTy was prepared. Then, 10 µl of the sperm sample were diluted with 5 µl of the SYB14 + HeTY (1:50) and placed in a 38°C water bath for 5 minutes. After this, 5 µl of PI were added to the sample, mixed and the sample was placed onto a clean glass microscope slide. The slides were examined immediately with a long pass filter block (B-2A; Ex 450-490 DM 505 BA 520) attached to a Nikon fluorescence microscope. For each sample, 200 sperm cells were counted in randomly selected microscopic fields, being careful to avoid clumps of sperm cells. Spermatozoa that fluoresce red were designated as 'dead' while those that fluoresce green were designated as 'live'.

7.3.5 DNA Analysis Using the Comet Assay

In Experiment 3, the protocol used for the comet assay was slightly modified from that of Kawase et al. (2005). Dried sperm samples were individually rehydrated as previously described (100 µl of ultra-pure water for freeze-dried and 10 µl for air-dried samples; no water was added to samples that had only been frozen). Sperm samples that were processed for freeze-drying that were not placed in the drying machine were designated as frozen only. They remained frozen in liquid nitrogen until use as controls (LN_2 Only) for the medium and the freezing process alone.

Samples were centrifuged and the sperm were re-suspended in 100 µl of chilled phosphate-buffered saline (PBS without Ca^{2+} and Mg^{2+}) (D-8537). Pellets of sperm were re-suspended in either 800 µl of PBS (Fresh Sperm) or in 100 µl of PBS (Freeze-Dried, Air-Dried and LN_2 Only). Then, the sperm suspension was combined with the Comet LMAgarose (LMA) (4250-050-02; Trevigen Inc., Gaithersburg, MD) at a ratio of 1:10 (v/v). A 25 µl of sample was evenly spread onto a Trevegen HT Comet slide (4252-200-01; Trevigen Inc.). Each sample, including a control sample was placed in duplicate on each comet slide to be analyzed.

The slides were refrigerated for 15 minutes and then immersed in a large jar containing chilled lysis solution (4250-050-01; Trevigen, Inc.) at 4°C for 1 hour. To the 299 ml of lysis solution, 32.5 ml of 20 mM DTT (15508-013; Invitrogen, Carlsbad, CA) were added and incubated at 4°C for 30 minutes and then 32.5 ml of 8 mM lithium diiodosalicylate (D-3635) was added to the previous solution and further incubated at room temperature for 90 minutes. The slides were then transferred to an alkaline solution (pH>13) for 1 hour at room temperature in the dark. The alkaline solution consisted of 1.5 ml of 200 mM EDTA, pH 10 (4250-050-04; Trevigen, Inc.) and 0.03 M NaOH (S–8045) prepared in water. The slides were rinsed twice in 1X TBE buffer (15581-044; Invitrogen) and then subjected to electrophoresis in 1X TBE buffer at 25 v for 15 minutes (1v/cm). Then, the slides were fixed in 70% ethanol for 8 minutes and air-dried at room temperature for ~30 minutes.

Staining was done by adding 25 μl of a 1:1000 dilution of SYBR Green I nucleic acid gel stain diluted in TE Buffer (10 mM Tris (pH 7.5), 1 mM EDTA) solution and 5 μl of antifade solution (S-2828; Component B - Molecular Probes) on each slide. Slides were examined with an inverted Nikon microscope and images were captured using video cassette and a computer.

7.3.6 Assessment of Comet Lengths

Comet tail lengths were measured by first video taping a micrometer scale with the same objective used to visualize the sperm cells. Then, this scale was recorded on a piece of tape by playing the video of the micrometer on a 28-inch television set. Each experiment was analyzed using this scale in micrometers. At least 50 sperm were counted per treatment per male and each treatment was recorded twice. Both comet tail length and width (halos) were recorded for each sperm. Comets near the edges of the wells of the slide and in clumps were not analyzed.

7.3.7 Statistical Analysis

The mean tail length of fresh, frozen only, freeze-dried and air-dried sperm samples was recorded and t-tests were used to compare comet tail lengths. In this study, $P \leq 0.05$ was considered as statistically different (Instat Graphpad Version 3.0, San Diego, CA).

7.4 Results

7.4.1 Evaporation Time of Sperm Solutions

In the first experiment, feline sperm samples were weighed to assess the time required for them to reach a constant weight (evaporation rates) at room temperature. All samples in each treatment group had completely dried to a constant weight within 40 minutes (Figure 7.1). The effects of two sperm mixtures in HeTY were also assessed and the maximum amount of time required for drying was 40 minutes for each of these samples. From these data it was determined that the desired time for air-drying should be ≥30 minutes.

7.4.2 Viability of Sperm During and After Drying

To determine the stage at which the feline ejaculated sperm membranes were disrupted, membrane integrity tests were performed in the second experiment. Three replicates were performed on each individual male sperm sample. There was little membrane disruption (decreased membrane integrity) noted when sperm were exposed to the HeTY + EGTA solution alone (n = 9 individual male samples). After 120 minutes of exposure at 30ºC, all samples examined retained membrane integrity of 57% to 68%. However, there was a rapid increase of membrane-disrupted sperm with increased drying times. With air-dried sperm samples (n = 6), after only 5 minutes of drying, the sperm membrane integrity decreased from 95% intact sperm to less than 13%. At 15 minutes of drying in air, there was 0% membrane intact cells in all sperm samples.

Post-freezing, cryopreserved sperm (n = 4) had only 0.1% with intact membranes after storage in LN_2 for 10 to 14 days as assessed by membrane integrity tests. In addition, an examination of membrane integrity was made of both freeze-dried sperm (n = 4 samples) and air-dried sperm (n = 4 samples). All samples had 0% intact membranes. See Figure 7.2 for an example of intact and nonintact sperm samples.

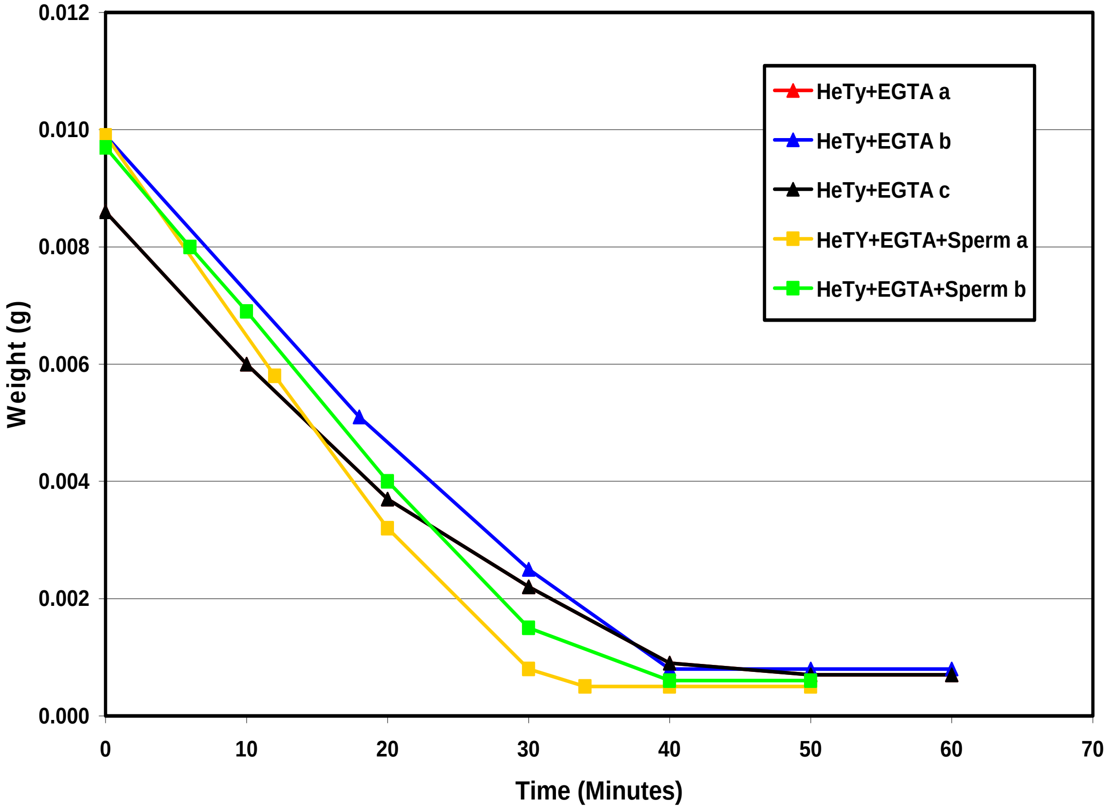

Figure 7.1 Evaporation rates of the dehydration medium alone (HeTY + EGTA) and sperm suspensions prepared in HeTY + EGTA. Each point on the graph represents a single weight measurement of medium or feline sperm suspension in medium. Three different slides with HeTy + EGTA were measured per sample (a, b and c). Two slides with sperm suspensions in HeTy + EGTA were also measured per sample (a and b). The black and red points are identical and therefore overlap.

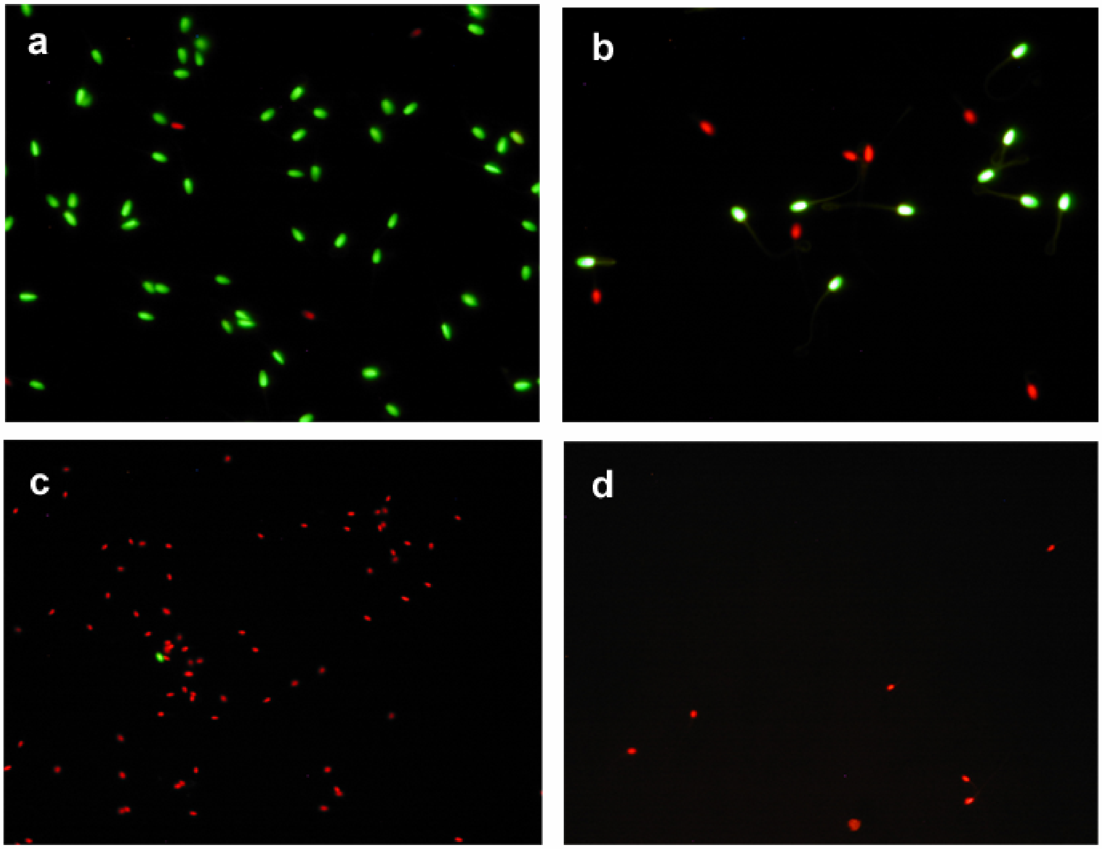

Figure 7.2 Photographs of feline sperm membrane integrity of fresh and freeze-dried samples. (a) Fresh sperm with many viable sperm (green = intact) and only a few dead sperm (red = disrupted). (b) Sperm assessed after 120 minute of exposure to HeTY + EGTA medium. (c) Mainly disrupted sperm heads after sperm was processed for drying and vitrified-warmed (frozen only). (d) Freeze-dried sperm after rehydration demonstrating all disrupted sperm membranes. Figures (a) and (b) were taken with a 40X objective and figures (c) and (d) were taken with a 20X objective for a larger field of view.

7.4.3 Comet Analysis of Dried Sperm

In the third experiment, comet tail lengths were calculated for: fresh sperm, frozen only sperm (vitrified but not dehydrated), freeze-dried (epididymal and ejaculated assessed separately) and air-dried cat sperm treatment groups. At least eight replicates of the comet assay were completed. For fresh sperm samples (total of 8 samples collected from 3 toms), the mean (± SE) comet tail length was 20 ± 4.9 µm (minimum = 10 µm and maximun = 61 µm). Frozen only sperm samples (n = 10) had an average tail length of 35 ± 11.2 µm (minimum = 10 µm and maximun = 93 µm) (Figure 7.3). After being freeze-dried, ejaculated sperm samples (n = 16) had a mean comet length of 110 ± 8.0 µm (minimum = 33 µm and maximun = 167 µm) and epididymal sperm samples (n = 12) had a mean length of 134 ± 9.5 µm (minimum = 40 µm and maximum = 181 µm). Finally, air-dried sperm samples (n = 9) had no verifiable comet tails in any of the samples examined. All measurments were ~10 µm (size of the feline sperm head). Representative images of comet tails are shown in Figure 7.4. There was no significant difference in comet tail lengths of fresh and frozen only sperm samples, although both had significantly shorter tail lengths than both the freeze-dried ejaculated and epididymal sperm.

7.5 Discussion

In this study, dehydration of feline sperm resulted in complete membrane disruption and DNA damage. This finding appears to challenge the earliest reports of the successful use of artificial insemination of rabbits (Yushchenko, 1957 as cited by Wakayama and Yanagimachi, 1998) and cattle (Larson and Graham, 1976) with immotile freeze-dried sperm. Without the use of ICSI, these sperm should not have been able to

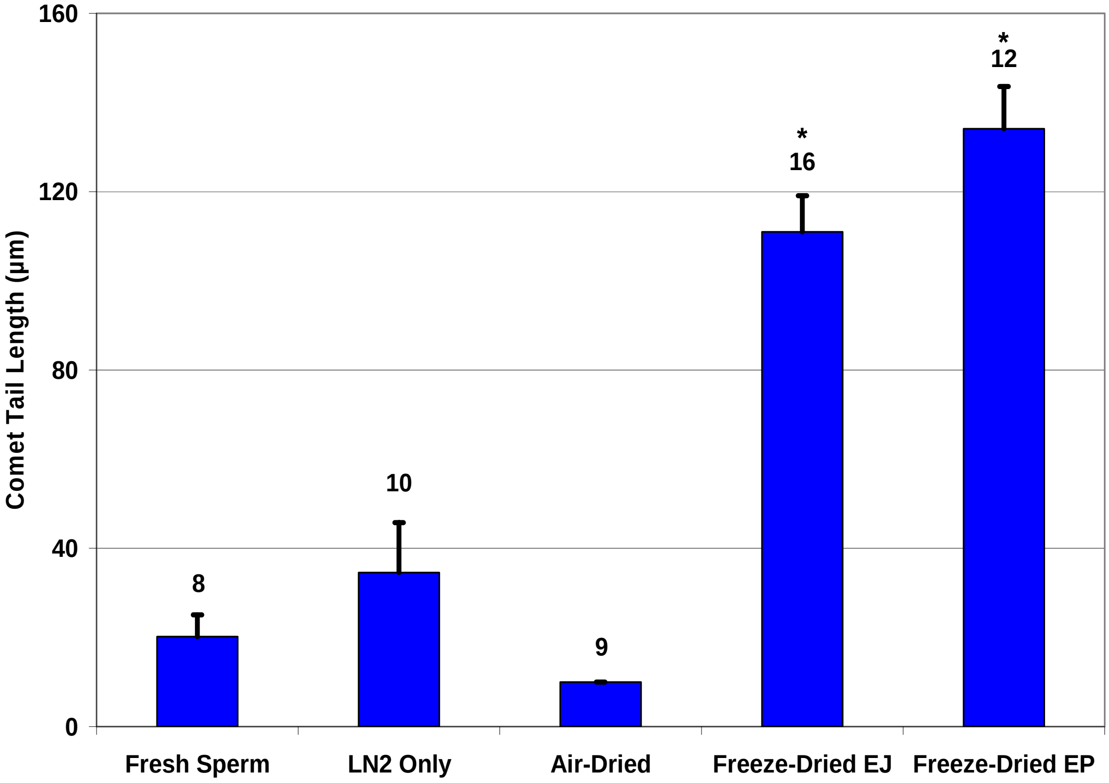

Figure 7.3 Mean (± SE) comet tail lengths of fresh, frozen and dried feline sperm. The numbers above the bars represent the number of sperm samples analyzed for each treatment. LN2 Only = sperm processed for freeze-drying that were vitrified but not attached to the dehydration machine (frozen alone). EJ = Ejaculated; EP = Epididymal. Asterices indicate statistical significance between fresh sperm and all other treatments using t-tests, P≤0.05.

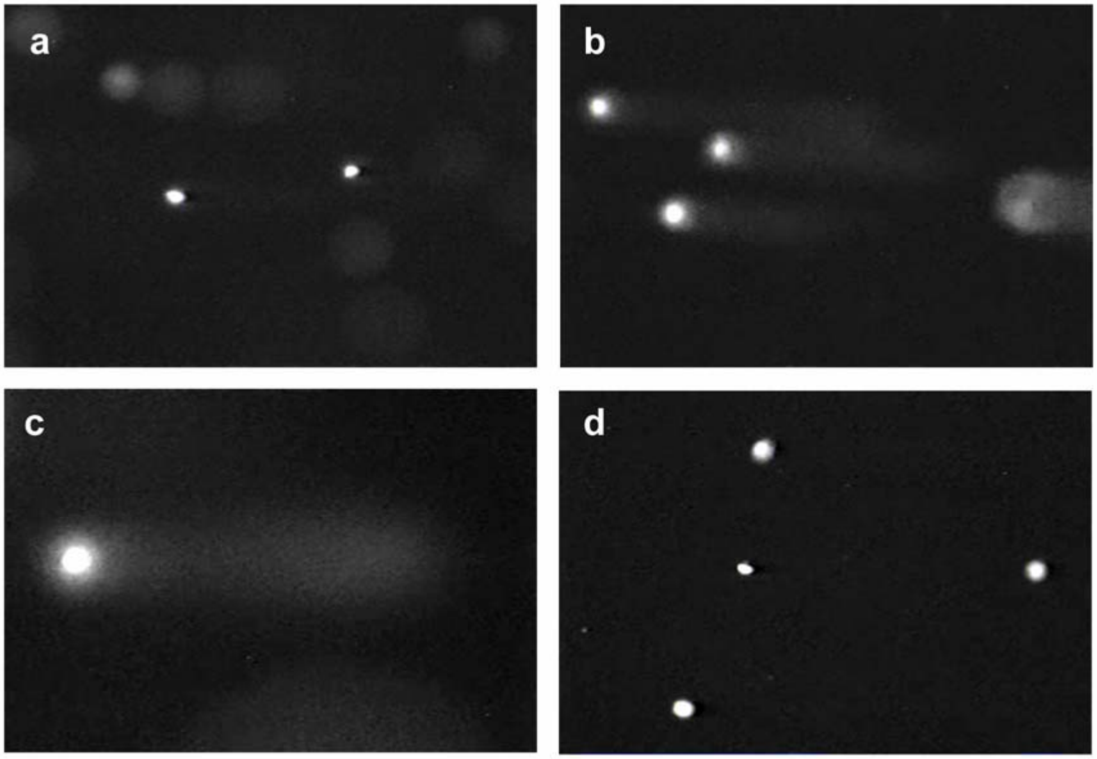

Figure 7.4 Digital images of comet tails of various types of fresh,freeze-dried and air-dried feline sperm. (a) Fresh sperm with no tails present. (b) Frozen only sperm with no or little tails present. (c) Freeze-dried ejaculated sperm demonstrating a very long comet tail trailing away from the sperm head region. (d) Air-dried sperm that did not decondense and therefore, exhibited no comet tails.

fertilize oocytes in vivo. The first report to be widely accepted of the successful production of mice offspring resulting from freeze-dried sperm was that of Wakayama and Yanagimachi (1998).

The ability to preserve sperm without cryoprotection and to store samples at higher temperatures than conventional techniques out-weighs the problem associated with the need for a technically demanding procedure, such as ICSI for fertilization. There are many strains of research mice where the sperm cannot currently be preserved using standard cryopreservation methodologies. This is also the case for numerous exotic and endangered species. With this simple sperm dehydration technique, there is potential for these endangered species to be successfully preserved for future generations. However, there remains a need to test the feasibility of preserving sperm by dehydration and examine the potential damage to the DNA incurred during storage.

In the present study, it was also determined that the minimum drying time for air-dried feline sperm samples to be 30 minutes or greater. This information was then used in planning subsequent experiments. The optimum drying time for freeze-dried sperm samples was previously reported to be 4 hours (Kwon et al., 2004). Furthermore, the medium selected for sperm dehydration was modified from that described in previous reports where there was a supplementation of standard sperm handling medium with 50 mM EGTA and the pH was adjusted to 8.2 to 8.4. This high pH was reported in mice after examining the genetic integrity (DNA fragmentation) of the sperm used in ICSI with mouse oocytes (Kaneko et al., 2003b). Sample size and rehydration conditions in the present study were also similar to those previously used in sperm dehydration studies (e.g., Kusakabe et al., 2001; Keskintepe et al., 2002; Bhowmick et al., 2003; Kaneko et al., 2003a,b; Ward et al., 2003; Kusakabe and Kamiguchi, 2004; Kwon et al., 2004; Liu et al., 2004; Hirabayashi et al., 2005).

Comet analyses on sperm are commonly used in human fertility clinics in an attempt to try to diagnose the cause of infertility in men. In freeze-dried sperm studies, only one report has used the comet assay to evaluate the effects of dehydration on mouse sperm. In that study, increased tail length was used as an indicator of sperm DNA damage. Thus, the longer the comet tail length, the more damage has occurred.

Other studies in humans have used sophisticated software to determine the tail length, tail moment (tail length X % tail DNA) and percent tail DNA (total DNA that migrates away for the nucleus into the comet tail). Unfortunately, this requires expensive computer analysis systems and results in little more information. In one study, it is suggested that simply using an eyepiece micrometer to measure the tail lengths was sufficient to make an assessment (Duty et al., 2002). In our study, there was significant differences found in comet tail lengths. Although percent tail DNA could be calculated using the halo sizes of sperm heads, the sperm halo sizes were so uniform in our treatments that this parameter was not included in this data set.

Fresh and frozen only feline sperm samples had only a few or no comet tails, therefore, these sperm were considered to have suffered little or no DNA damage. However, freeze-dried feline sperm (epididymal and ejaculated) had significantly longer comet tails lengths than that of the fresh sperm controls. These findings indicate that there were a number of DNA fragments or single stranded DNA present in the freeze-dried sperm. This level of sperm head DNA damage was 10 fold higher than their fresh sperm counterparts. The lack of comet tails in the air-dried sperm samples was thought to be due to a lack of sperm head decondensation. Without decondensation of the tightly wound DNA in the sperm head, the fragmented chromosomes do not migrate away from the sperm head under the in vivo fertilization process. Further study is needed to determine the true level of DNA damage in these freeze-dried and air-dried samples. The sample stored for the longest time (13 weeks) at 4ºC was freeze-dried ejaculated sperm. This sample did not show an increase in DNA damage compared with freeze-dried sperm stored for only 2 weeks. Therefore, it appears from this study that time of dry storage up to 13 weeks did not have an obvious effect on chromosomal damage in freeze-dried feline spermatozoa.

The major component in all cells is water. Cell membranes rely on the presence of water to maintain their structure and function therefore in most cases, dehydration leads to cell damage or even death (Puhlev et al., 2001). Yet, many plants and invertebrate animals can tolerate severe desiccation under natural conditions. The ability to dehydrate mammalian cells (especially sperm) would increase the efficiency of storing biological samples. The impact of dehydration on the food and drug industries, which rely on the ability to safely store a multitude of compounds, alone makes this prodeedure economically desirable.

Recent improvements of the sperm medium to protect the genetic integrity of the sample and the ability to produce live offspring makes both freeze-drying and air-drying potentially efficient methods of sperm preservation. However, the question relating to the gradual degradation of the sperm maintained for extended periods has yet to be answered. One major factor involved in the loss of genetic stability is the temperature of storage, which increases the possibility of free radicals in the sperm DNA (Puhlev et al., 2001). Before freeze-drying and air-drying procedures can be used for routine sperm preservation, further improvements must be made in methodologies.

7.6 Conclusions

It was concluded that the freeze-drying and air-drying procedures can be done with cat sperm and that the comet assay can be used to assess cat sperm DNA damage. Also, freeze-drying and air-drying of feline sperm resulted in a marked increase in the level of sperm head DNA damage. However, it appears as if dried feline sperm retain their ability to fertilize oocytes and these will undergo embryonic development. This leads to the question of how much DNA damage can sperm endure and still maintain their physiological function. Unfortunately, there is no simple way to evaluate this question. Many replications of injecting dried sperm into oocytes followed by embryo transfer will be the only true test of the ability of these ‘damaged’ sperm to produce live offspring.

CHAPTER 8
SUMMARY AND CONCLUSIONS

8.1 Why Preserve Gametes and Embryos?

In recent years, people have become more aware of their effects on their environment. Factors that have lead to the listing of hundreds of species on the endangered species list include increased human population worldwide, habitat destruction for timber, agriculture and mining industries. Increased environmental pollution and over hunting have also contributed to the loss of species (Sunquist and Sunquist, 2002). Because in situ conservation is very difficult and sometimes impossible, the ex situ conservation approach has become a primary focus area for animal conservation researchers. One of the conservation strategies is the establishment of a genome resource bank for both plants and animals and initially these resource banks were created to store sperm, oocytes and embryos of mammalian species, with the initial focus to preserve endangered or threatened species (Karow and Critser, 1997; Watson and Holt, 2001; Holt et al., 2003). This genome bank would be a safeguard against disease, disaster or other factors that may destroy an animal population. Maintaining valuable genomes would help provide protection against genetic drift and loss of genetics.

Cryobiology of living cells teeters on a fine line between cell injury and cell survival. Two main causes of cryoinjury incurred by cells result from intracellular ice formation and osmotic shock or solute toxicity (Mazur et al., 1972). The former can be avoided by inducing cell dehydration either by extracellular ice formation or by using nonpermeating compounds, such as saccharides. The latter form of cryoinjury is not as easy to define or even to avoid. However, many cell types, including mammalian gametes of a host of species, have now been successfully cryopreserved using standardized protocols.

The two main types of cryopreservation are equilibrium and nonequilibrium freezing procedures (Mazur, 1990). Slow cooling (equilibrium freezing) was used in the first successful cryopreservation of male gametes and remains the most common method used today. It has also been used to preserve embryos and to a lesser extent, oocytes of various species. Nonequilibrium freezing requires that the cells be dehydrated before cooling occurs whereas, equilibrium cooling causes dehydration to occur during the cooling process. This nonequilibrium approach has been successful for sperm preservation but not as effective for embryo cryopreservation. In general, most cryopreservation protocols require the use of a cryoprotective agent and that the cell undergo dehydration.

An alternative to standard cryopreservation is vitrification, which uses high concentrations of cryoprotectant agents and ultra-rapid cooling rates (Rall and Fahy, 1985). Vitrification also requires cell dehydration but avoids the damaging ice formation that occurs in standard cryopreservation techniques. Furthermore, recent studies on sperm vitrification have demonstrated the ability to successfully preserve spermatozoa without the need for potentially harmful cryoprotective agents (Nawroth et al., 2002; for a review see Isachenko, 2003).

In the present study, the ability to preserve both sperm and oocytes using alternative methods to standard cryopreservation was examined. Both bovine and feline oocytes were preserved using vitrification (an ultra-rapid cooling process) to avoid the formation of intracellular ice. This is done by using high concentrations of cryoprotectant additives (CPAs) such as dimethylsulfoxide (DMSO), ethylene glycol (EG) and saccharides, as well as, using very small volumes of CPAs to achieve very rapid cooling and warming rates (Vajta and Kuwayama, 2006). Together these factors allow the vitrification solution containing the oocytes to transform from the liquid state to a solid ‘glassy’ state.

Sperm preservation in this study was achieved by dehydration either by air-drying or by freeze-drying. Freeze-drying has successfully produced live young of three species: mouse (Wakayama and Yanagimachi, 1998), rabbit (Liu et al., 2004) and rat (Hirabayashi et al., 2005) and embryonic development to the blastocyst stage in cattle (Keskintepe et al., 2002) and pigs (Kwon et al., 2004). Freeze-dried sperm samples may be stored at room temperature and do not need to be stored in large containers of liquid nitrogen. However, when rehydrated, sperm are immotile and then ICSI can be used to fertilize oocytes. Both sperm drying methods developed in our studies will likely have advantages over standard cryopreservation procedures in the future but need to be evaluated before they can be used to preserve gametes and embryos of endangered species.

8.2 Volumetric Responses of Oocytes to Various Saccharide Solutions

In Chapters 2 and 4, volumetric measurements in response to increasing concentrations of solutes were made using bovine and feline oocytes. Oocytes from both species behaved osmotically although the feline oocytes ($R^2 = 0.94$) did not fit the linear regression as tightly as did the bovine oocytes ($R^2 = 0.98$ to 0.99). These results were considered good fits to the regression lines. Lysis was observed in feline mature oocytes at the highest concentration of saccharides examined, while no lysis was noted in the bovine matured oocytes. This was likely due to the higher cytoplasmic lipid content of the feline oocyte or increased sensitivity of feline oocytes to osmotic permutations.

Furthermore, the minimal volumes of feline oocytes from 32% to 22% calculated by extrapolation of the linear regression to an infinitely concentrated solution was very similar to that found in bovine oocytes from 32% to 18%. This is not surprising as the nonosmotic volume of most cell types has been determined to be within this range. It was concluded that bovine oocytes could serve as a prediction model for the effect of dehydration on feline oocytes.

8.3 Vitrification of Bovine and Feline Oocytes

In Chapters 3 and 5, vitrification of oocytes and embryos was examined using a standard oocyte and embryo handling medium and a choline-substituted medium (CJ2). Both bovine and feline oocytes were vitrified-warmed, fertilized and cultured to assess subsequent embryonic development. The cleavage rate following in vitro fertilization was significantly higher for the bovine oocytes (54%) when compared with the feline oocytes (22%). The bovine blastocyst development rate was 4% compared with 0% for the feline oocytes. This difference between bovine and feline oocytes may be due to differences in chilling sensitivity of the oocytes. This can also be due to the differences in oocyte lipid content between the two species. Another factor that may be involved in the low survival of feline oocytes post-vitrification was that the feline oocytes used were obtained from both young and old queens. The age of the oocyte donor will have an effect on the quality of oocytes available whereas, the commercial source of bovine oocytes used does allow for more selection to be placed on the oocytes prior to treatment.

What was somewhat surprising was the difference in embryo development success rates using CJ2 for vitrification. There was no significant difference between bovine oocytes vitrified using the standard handling medium (TCM-199) or CJ2 whereas, there was no survival of feline oocytes resulted following vitrification. Although choline-based media have successfully been used for oocytes of mice, humans and now cattle, this type of medium apparently has a different effect on feline oocytes. What causes this difference is not yet understood. In our studies, bovine oocytes were relatively effective as models for feline oocyte vitrification, with the noted exception of the use of CJ2 medium for feline oocyte vitrification.

8.4 Sperm Dehydration and Analyses

In Chapters 6 and 7, feline sperm were dehydrated, their DNA integrity assessed and they were used to fertilize feline oocytes and embryonic development was evaluated in vitro. In these experiments, freeze-dried and air-dried feline sperm injected into mature feline oocytes produced embryos with cleavage rates of 60% and 35%, respectively. These cleavage rates were comparable to those reported in both cattle at 44% to 63% (Keskintepe et al., 2002) and pigs at 50% to 52% (Kwon et al., 2004). However, the results from this present experiment were generally lower than the cleavage rates using freeze-dried sperm in mice (80% to 99%) (Kaneko et al., 2003b) and in rabbits (70%) (Liu et al., 2004).

Blastocyst rates obtained for freeze-dried feline sperm (2%) were lower than those found using bull sperm (11% to 30%) and markedly lower than in the rat, mouse, and rabbit evaluated to date. It should be noted that there are morphological similarities of both feline and bovine sperm, such as their paddle-shaped heads and similar overall surface area.

Another factor that may be similar in the two species is the need for activation after injection of dried sperm. Although the cat oocyte is normally activated by the injection process alone, it may be beneficial to in vitro activate all injected oocytes. It is important that the sperm activating factor needs be maintained in the sperm during drying. It should be noted, however, that there was embryonic development to the blastocyst stage using dried feline sperm in our studies and this occurred without any in vitro chemical activation. Therefore, we can conclude that the sperm activating factor was preserved in the air-dried feline sperm and that it was reduced during the freeze-drying process. This may explain why the feline oocytes had acceptable cleavage rates but the embryos became arrested soon after reaching the 8- to 16-cell stage. Furthermore, the time of genomic activation of embryos has not been determined in the domestic cat.

Although promising results were obtained in our studies using activation with freeze-dried feline sperm, further study is necessary to determine the efficiency of this technique. Assessment of DNA integrity of freeze-dried and air-dried feline sperm indicates that the medium and vitrification process used in this study did not have obvious deleterious effects on the sperm, however, the freeze-drying procedure apparently created DNA strand breaks.

It should be noted that no difference in cleavage rates has been found between both fresh and cryopreserved testicular sperm in humans (Thompson-Cree et al., 2003), however, there was a significant decrease in pregnancy rates that was attributed to the higher level of DNA damage found in the cryopreserved sperm. These results have a similar pattern to what was noted in this study, where cleavage rates were not affected by sperm dehydration, however, blastocyst development was impaired. This likely indicates that although there was no effect of the increased level of DNA damage evident in early embryonic development, problems associated with such damage likely occurred at a later stage of embryonic development. Any detrimental effect of this DNA damage can not be fully assessed, since freeze-dried and air-dried feline sperm were able to produce embryos that developed beyond the 8- to 16-cell stage following sperm injection. In addition, this study demonstrates that the comet assay can be used as a tool to assess DNA damage in felid sperm.

8.5 Conclusions

In conclusion, bovine oocytes could be used as a model for studying the cryopreservation of feline oocytes. Since bovine oocytes can be purchased commercially, they are more available to perform various experiments than could not be completed with the limited availability of feline oocytes. Oocyte dehydration demonstrated that both bovine and feline oocytes behaved osmotically in solutions. It should also be noted that the effects of the CPAs on the meiotic spindle and embryonic development after in vitro fertilization were only slightly disrupted. Vitrified bovine and feline oocytes fertilized in vitro, cleaved and developed to the blastocyst stage. Bovine oocytes and embryos were successfully vitrified with relatively adequate efficiency that could be used in a commercial environment. However, the low efficiency of this technique in felids, will make oocyte vitrification remain a research technique, with emphasis on its use in endangered species.

It is proposed that freeze-drying and air-drying feline sperm could be an alternative method of sperm preservation in the future. Blastocyst development rates with both freeze-dried and air-dried sperm indicate that further optimization is needed. Factors such as poor quality and aged oocytes, length of time sperm is rehydrated before injection, the medium used and storage temperature all can affect the optimization of the sperm drying procedure. This study is the first report of blastocyst development in felids using freeze-dried and air-dried sperm. It places the cat on the limited list of animal species to date to have sperm successfully dried, injected into mature oocytes and resulting embryos undergo development in vitro.

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VITA

Allison Erna Fex was born on January 16, 1974, in the little northern Ontario town of Lively, Canada. She attended both primary and secondary school in French and then headed off to Ottawa, Ontario, Canada, for her first year of University in September, 1993. At the University of Ottawa, she studied biology in French for 1 year after which she transferred to Laurentian University in Sudbury in the same department to be closer to family and friends.

After 2 years at Laurentian, she then changed her early thoughts on becoming a veterinarian and transferred to the University of Guelph, Guelph, Ontario, Canada, to study in the Zoology Department. Studying zoology allowed her to focus on becoming a zoologist and then eventually working in a zoological park or a national park. It was here she completed her first research project studying the feeding behavior of hatchling snapping turtles. She then graduated with her Bachelor of Science with honors in April, 1998, and, at that time, she decided to pursue a career in reproductive biology. Dr. Keith Betteridge at the University of Guelph was approached with the proposal of studying the reproductive biology of sugar gliders (a small marsupial). He kindly introduced her to Dr. Ann Hahnel and the idea of studying germ cells in mice and goats at the University of Guelph. This led to a fantastic 3 years of studying the transplantation of both mouse and exogenous caprine germ cells into mouse testes, research that culminated in her obtaining a Master of Science Degree in biomedical sciences in November, 2001. After finishing her degree, she worked in an infertility clinic in Toronto, Ontario, Canada, for a year while she decided what path to take in further graduate studies. During that time, she married her high school sweetheart of 11 years, Reynald Jean-Marc Moisan in Lively, Ontario, Canada, on September 30th, 2000.

In the spring of 2001, she visited many of the centers for research on endangered species to decide where she wanted to go to pursue her doctoral degree. After visiting the Audubon Center for Research of Endangered Species in New Orleans and the Embryo Biotechnology Center at Louisiana State University in Baton Rouge, she decided that this was the appropriate place for her to continue her graduate studies. She began her training in August, 2001 at Louisiana State University at the LSU Embryo Biotechnology Laboratory in Baton Rouge and after 1 year, then moved to New Orleans to finish her dissertation research. During this interval she studied the reproduction of domestic cats and evaluated the ability to preserve oocytes and sperm using novel methodologies. Unfortunately, at the end of her degree, her house was destroyed by Hurricane Katrina and she was forced to finish writing her dissertation at home in Toronto, Canada.