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Cryopreservation of bovine and caprine oocytes by vitrification

Sabrina Marie Luster

Louisiana State University and Agricultural and Mechanical College, luster7777@yahoo.com

CRYOPRESERVATION OF BOVINE AND CAPRINE
OOCYTES BY VITRIFICATION

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF NOMENCLATURE

BSA	Bovine Serum Albumin
FBS	Fetal Bovine Serum
CPA	Cryoprotective Additive
HES	hydroxyethyl starch
PVP	polyvinylpyrrolidone
PEG	polyethylene glycol
EG	Ethylene glycol
LN_2	Liquid Nitrogen
IVF	IVF
SCNT	Somatic Cell Nuclear Transfer
Me_2SO	Dimethylsulfoxide
THP	thermal hysteresis proteins
OPS	Open Pulled Straw
IVM	In Vitro Maturation
IVC	In Vitro Culture
OPU	ovum pick-up
TCM-199	Tissue Culture Media-199
FITC	fluorescein isothiocyanate

ABSTRACT

Cryopreservation of animal oocytes will permit germplasm of valuable or unique females to be preserved for extended times. The objective of this research was to derive a procedure to cryopreserve bovine oocytes by vitrification to be used as recipients for somatic cell nuclear transfer (SCNT). Caprine oocytes vitrified by the same procedure were assayed by cytological examination of microtubules.

In the first two of three experiments, bovine oocytes matured in vitro were vitrified in a mixture of ethylene glycol (EG), dimethylsulfoxide (Me_2SO) and trehalose, and then subjected to in vitro fertilization (IVF) or SCNT. For vitrification, oocytes were first exposed to increasing concentrations of EG + Me_2SO, placed into the vitrification solution composed of 2.8 M Me_2SO + 3.6 M EG + 0.65 M trehalose for 20 sec, immediately loaded onto 20-μm cryoloops, and finally plunged directly into liquid nitrogen (LN_2). Vitrified oocytes were warmed by direct immersion of cryoloops into 0.25 M trehalose prepared in TCM-199 medium at 37°C, rinsed briefly, and then assayed. In Experiment I, of 327 bovine oocytes subjected to IVF after being vitrified, 267 cleaved and 32 (9.8%) formed blastocysts, compared to 32.1% blastocysts for control oocytes. In Experiment II, of 266 bovine oocytes enucleated after vitrification and subjected to SCNT, 248 formed couplets, 152 of which cleaved and 31 (12.5%) developed into blastocysts, compared to 33.0% blastocysts for controls. During the course of Experiment II, 20 of 31 blastocysts derived by SCNT of somatic cells from a Brahman cow into vitrified oocytes were transferred into recipients, resulting in three pregnancies and the birth of one Braham calf that has survived to adulthood. In Experiment III, cytological analysis of caprine oocytes vitrified by the same procedure used for bovine oocytes demonstrated that their

microtubules were normal, suggesting that this same procedure can also be used for the former species.

The results demonstrate that bovine oocytes can be successfully vitrified and warmed, yielding normal embryos after fertilization or SCNT. Additional research is needed to verify that caprine oocytes vitrified by this method can also develop into kids.

CHAPTER I.

REVIEW OF LITERATURE

INTRODUCTION

Cryobiology is the study of the effects of low temperatures on living organisms. People long believed that very low temperatures would only exert negative effects on cells and tissues. They could not possibly imagine the advancements in cryobiology to be achieved and possibilities of the future in this area. In the future, it may be possible to cryopreserve human cells, whole human organs, such as kidneys, hearts and livers for subsequent transplantation, preserve corneas and other delicate tissues with minimal damage long enough to allow them to be shared all over the world and protect fragile and rare plants from extinction through ice-free preservation.

The history of cryobiology dates back to the late 1600s. Henry Power froze a jar of vinegar eels in salt water and after thawing, he found that they were still as active as they were prior to freezing. Power was the first to theorize that cold did not have so-called "killing properties" that are possessed by heat (Sittig, 1963). Robert Boyle wrote a monograph entitled "New Experiments and Observations Touching Cold" in 1683 in which he described the effects of freezing on living animals (Parkes, 1960). Another pioneer in cryobiology was Lazzaro Spallanzani who conducted extensive studies on tissues of several species and their reaction to low temperatures in the late 1700s (Sittig, 1963). In 1940, Father B.J. Luyet and P. M. Gehenio published a book entitled "Life and Death at Low Temperatures" in which they outlined the basic components for the study of cryobiology (Luyet and Gehenio, 1940).

In the late 1940s, Christopher Polge and his colleagues at the University of Cambridge accidentally discovered the protective capabilities of glycerol when they used bottles of chemicals that had been inadvertently mislabeled. This accidental discovery enabled them to successfully cryopreserve spermatozoa of chickens and cattle (Polge et al., 1949). Discovery of the ability of glycerol to protect cells against freezing damage led to the derivation of the science of low temperature biology. In 1951, the first calf produced by artificial insemination with frozen-thawed bovine spermatozoa was born (Stewart, 1951). In the 1960s, Peter Mazur conducted extensive experiments to model the responses of microorganisms when subjected to low temperatures and freezing. These early studies resulted in the development of the discipline that is now known as cryobiology (Mazur, 1963, 2004).

Successful cryopreservation of mammalian cells is dependent on several variables. These variables include the type of cell itself, the solution in which the cell is suspended and whether or not the solution contains a cryoprotective additive (CPA), the rate at which the cell is cooled to low subzero temperatures, the minimum subzero temperature to which the cell is cooled, the rate at which the cell is warmed, and the conditions under which the cryoprotectant is removed from the cell. Depending on the suspending solution, different types of cells exhibit different optimum cooling rates that may vary from a low rate of ~0.2ºC/min to a high of 1,000ºC/min. Under certain conditions, cells may even survive after being cooled at rates >100,000ºC/min. For a given type of cell, identification of an appropriate CPA, an optimum cooling rate and a corresponding optimum warming rate are keys to successful cryopreservation. This thesis describes experiments of this sort for bovine and caprine oocytes to be used for somatic cell nuclear transfer (SCNT).

In the field of reproductive physiology, extensive research has been done on the cryopreservation of gametes and embryos. The earliest studies involved the cryopreservation of spermatozoa. Examples of such large molecular weight compounds used as supplements include polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES), Ficoll, and polyethylene glycol (PEG). Although these compounds usually will not protect cells against freezing damage when used alone in solution, they will often enhance the protective effect of other low molecular weight CPAs. For example, Leibo and Oda (1993) found that survival of mouse embryos after freezing could be improved by using a mixture of PVP and ethylene glycol (EG) (See reviews by Watson, 1990, 1995; Holt, 2000a,b; Leibo and Bradley, 1999). Successful storage of spermatozoa made the technique of artificial insemination in numerous species extremely practical. Today, it is common for most cattle producers to own liquid nitrogen (LN$_2$) tanks full of cryopreserved semen straws of different sires from all over the country and the world. Advancements in cryobiology have promoted the increase in genetic diversity among herds.

Whittingham et al. (1972) were the first to report the successful cryopreservation of embryos of any animal species, including the births of mice from frozen-thawed embryos. They froze almost 1,000 mouse embryos in LN$_2$ at -196°C and transferred hundreds of these cryopreserved embryos into recipient foster mothers, producing hundreds of normal full-term fetuses and dozens of live mouse pups. After this first demonstration, embryo cryopreservation was applied to other species and it developed into a standard procedure for cattle producers in the 1980s. A human pregnancy was first reported by Trounson and Mohr (1983), although it spontaneously aborted. The first births in humans from frozen embryos were described by Zeilmaker et al. (1984). This allowed embryo cryopreservation to become commonplace in the

human field. Over the years, various protocols have been established for the cryopreservation of embryos and oocytes (see review by Rall, 1992).

Although the cryopreservation of cleavage-stage embryos is now a standard procedure, the mammalian oocyte has proven to be much more difficult to cryopreserve successfully. Early attempts with mouse oocytes used the conventional cryopreservation protocols for embryos, but these resulted in only 6 to 14% of these oocytes developing into fetuses or offspring after in vitro fertilization (IVF) (Parkening et al., 1976; Whittingham, 1977; Glenister et al., 1987; Schroeder et al., 1990). Cattle oocytes cryopreserved by slow-cooling exhibited low fertilization rates after IVF and fewer than 13% developed to 2-cell embryos (Schellander et al., 1988; Lim et al., 1991). Nevertheless, offspring have been produced from oocytes that have been successfully cryopreserved in mice (Parkening et al., 1976; Whittingham, 1977), rabbits (Al-Hasani et al., 1989), cattle (Fuku et al., 1992; Otoi et al., 1992) and humans (Chen, 1988).

In recent years, cryopreservation of mammalian oocytes has become much more successful for a number of reasons. However, these latest accomplishments are most likely due to viewing the differences between oocytes and embryos, rather than their similarities. Oocytes are difficult to cryopreserve because of their large size, low surface area to volume ratio, high water content and low hydraulic conductivity (Leibo, 1980). This has led to increased investigation of the vitrification of oocytes, as an alternative to cryopreservation by slow-cooling methods.

When fully optimized, cryopreservation of oocytes will have multiple applications. In livestock, producers could preserve valuable genetic blood lines from females and could market oocytes rather than embryos whose sire has already been chosen. Endangered species could also

4

benefit from oocyte cryopreservation by conserving the female genetic material of these vulnerable animals (Watson and Holt, 2001). Currently, there is insufficient information regarding the cryobiology of all endangered species. Cryopreservation of the oocytes of these rare species would allow one to preserve oocytes until the assisted reproductive technologies become available for use. Furthermore, oocyte cryopreservation could aid in the production and longevity of transgenic animal lines. Transgenic mice tend to have poor reproductive capabilities and production of these lines is a lengthy and costly process (Cecim et al., 1995). Oocytes from laboratory animals, such as those from valuable transgenic strains of mice, could also be preserved through oocyte cryopreservation.

In humans, the long-term storage of oocytes rather than embryos would help reduce legal, ethical, and moral issues associated with storing embryos (Gosden and Nagano, 2002; Grundy et al., 2001). Storage of oocytes would also allow cancer patients without partners to preserve their genetics for later use (Fabbri et al., 2001; Porcu et al., 2004)

The overall objective of this study was to develop an effective procedure to cryopreserve bovine and caprine oocytes for later use in SCNT. Specific objectives of the three parts of this study were (1) to vitrify bovine oocytes and determine their developmental capacity after IVF, (2) to cryopreserve bovine oocytes and determine their capacity to serve as cytoplasts for SCNT and (3) to evaluate caprine oocytes after vitrification and warming for microtubule damage.

BASICS OF COOLING AND CRYOPRESERVATION

Principal Variables

Cryopreservation involves five critical steps: (1) exposure of cells or tissues to cryoprotectants, (2) cooling specimens to temperatures below 0°C, (3) storage at the "glass"

transition temperature of water below -130°C, (4) warming and thawing (5) and finally, dilution and removal of cryoprotectants prior to incubation (Luyet and Rapatz, 1970; Mazur, 1988, 2004; Leibo, 1986, 2004b; Lebio and Songsasen, 2002). There are innumerable protocols that have been used to cryopreserve cells and tissues of many types. A comprehensive textbook has been published recently in which the fundamental and applied aspects of cryobiology have been described in detail (Fuller et al., 2004). Variations in the type of cryoprotectant, the concentrations of these solutions and also the interactive rates of cooling and warming can be adjusted. A universal protocol is impractical for all types of cells due to differences among species and types of material.

The causes for cellular damage and death from cryopreservation are not completely understood. During the cryopreservation process, cells experience several changes in their milieu: water is removed from the solution in the form of ice; consequently solutes become more concentrated and can precipitate; the cell responds osmotically by losing water. These processes can also be caused by changes in temperature, except for the precipitation of solutes. Researchers have debated whether changes in temperature, several solution effects or both are the cause of cellular damage and death during cryopreservation (Mazur, 1970; Karow and Critser, 1997; Fuller et al., 2004).

<u>Cooling Rates</u>

Cooling rate is one of the principal determinants of cell survival during cryopreservation. Cooling too slowly may kill cells by exposing them to concentrated solutions, whereas cooling them too quickly can cause cell death by ice crystal formation. Mazur (Mazur, 1965; 1970; Mazur et al., 1972; Leibo and Mazur, 1971) proposed that cell survival in respect to cooling rate

6

can be plotted as a bell-shaped curve. Essentially, cell survival is low at low cooling rates, increases to a maximum at an optimal cooling rate, and finally declines at high cooling rates. Each type of cell has its own optimal cooling rate. Cryoprotectant permeability also changes with changes in temperature (Mazur, 1977).

When cells in suspension are cooled to subzero temperatures, ice crystals first form in the extracellular solution and the cell cytoplasm supercools. As the cell cytoplasm is cooled to lower temperatures (below -10°C or -15°C), ice crystals may form abruptly in the cytoplasm itself, a phenomenon referred to as intracellular nucleation. This is often, but not inevitably lethal to the cells. If cells that have frozen intracellularly are warmed very rapidly, the cells may be "rescued" from this damage (Mazur, 1970). In contrast, when cells are cryopreserved by vitrification, they are cooled in such high concentrations of CPA solution and at such high cooling rates that intracellular ice crystals do not form.

Warming Rates

The warming rate is also very important for successful cryopreservation of mammalian cells. The optimum warming rate for a given type of cell is highly dependent on the optimum cooling rate that preceded it. Early investigators assumed that rapid warming of mammalian cells after cryopreservation was always better because cells had shorter times to recrystalize and were exposed for less time to CPAs. However, the first investigations of mouse embryo freezing by Whittingham et al. (1972) proved that there are exceptions to this rule. Their study showed that embryos cryopreserved by slow-cooling had greater post-thaw survival when they were warmed slowly. In fact, they reported that embryo survival was dependent on a slow warming

rate. They concluded that poor survival using faster warming rates is most likely due to osmotic effects.

The most common method for warming of oocytes after they have been vitrified is a rapid and direct method. Usually oocytes are placed into warming solutions at 20° to 37°C. After warming, oocytes must rehydrate and CPAs used for vitrification must be removed. This is also done quickly but the debate on whether step-wise dilution is necessary still remains.

Cryoprotectants

When mammalian cells suspended in dilute saline solutions are cooled and frozen, they are inevitably damaged and destroyed. As mentioned above, however, it was accidentally discovered in 1949 that fowl spermatozoa would survive freezing when suspended in a solution of 10% glycerol plus albumin (Polge et al., 1949). Later, calves were born from artificial insemination with frozen-thawed sperm (Stewart, 1951; Polge and Rowson, 1952). These were the first explicit demonstrations that mammalian cells can be successfully frozen with the addition of a supplement to the culture medium.

Since those first discoveries, it has been found that many low molecular weight compounds protect cells against freezing damage. These compounds, now referred to as CPAs, are all miscible with water in all proportions. These compounds are also non-toxic to cells and have low molecular weights that allow them to permeate the cell membrane. Cryoprotective additives are added to solutions used to freeze cells to lower the freezing point. Table 1 lists some of the common injuries observed after cryopreservation which may also lead to cell death (Shaw et al., 2000a). Cryoprotective additives also make the cell membrane more elastic by affecting lipids within the cell membrane.

8

Table 1. Factors Associated with Cooling and Cryopreservation that Contribute to Cellular Injury and Death in Biological Systems

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

Adapted from Shaw et al. (2000a)

Many compounds act as CPAs to protect cells against freezing damage. Examples of CPAs are methanol, ethylene glycol (EG), dimethylsulfoxide (Me_2SO), propylene glycol (1-2, propanediol) and glycerol. Leibo et al. (1970) reported that <2% of mouse marrow cells survive when cooled at rates varying from 0.3° to 600°C/min in a salt solution. However, they found that when glycerol was added to the solution, more than 70% of the cells survived cryopreservation. Glycerol was the only compound used as a CPA until the protective effects of Me_2SO were discovered by Lovelock and Bishop in 1959 (Mazur, 1970). Since then, many other compounds have been reported to act as CPAs.

When cells are first exposed to multimolar solutions of CPAs, the cells contract by water loss. As the CPA permeates the cells, water re-enters and the cell returns to its initial isotonic volume. Cryoprotective additives have different rates of diffusion into the cell. For example, the rate of diffusion for propylene glycol into oocytes is relatively fast (5-7 min) compared to the rate of diffusion for Me_2SO (20-30 min) or glycerol (>60 min) (Jackowski et al., 1980; Renard and Babinet, 1984).

Supplements to CPA Solutions

Supplements to CPA solutions are compounds of various sizes that by themselves do not protect the cell from freezing damage. There are several classes of compounds that have been used as supplements to CPA solutions, including saccharides, other large molecular weight compounds and proteins.

Various saccharides have been used as supplements. These include mono-, di- and trisaccharides that have molecular weights ranging from ~180 to 540. Examples of monosaccharides include fructose, glucose, and galactose. Examples of disaccharides include

sucrose, trehalose and lactose, and an example of a trisaccharide is raffinose. Trehalose has been reported to be very effective as a CPA supplement for vitrification of oocytes by several groups (Dinnyes et al., 2000; Lj et al., 2002; Begin et al., 2003). Arav et al. (1993) showed that bovine oocytes exposed to trehalose had a higher rate of survival than oocytes exposed to sucrose and they also reported a very high rate of fertilization after exposure to 0.25M trehalose (70%).

There are other large molecular weight compounds that are often added to CPA solutions as supplements. Several reports have shown that PVP is not successful for cryopreserving embryos and that the compound is highly toxic to embryos (Wilmut and Rowson, 1973; Fahy et al., 1984). Polyethylene glycol has also been used to supplement CPA solutions (Rall and Fahy, 1985).

Large proteins can also be used as supplements to CPA solutions. Examples include bovine serum albumin (BSA) or fetal bovine serum (FBS). Another less common type of CPA supplement is thermal hysteresis proteins (THPs). Arctic fish live in icy polar waters that drop to temperatures of -1.8°C. DeVries and Wohlschlag (1969) isolated glycoproteins from the serum of these fish, and found that the glycoproteins changed the freezing point without changing the melting point, and named these glycoproteins THPs. In another study, THPs were again isolated from fish and used for the cryopreservation of pig oocytes (Rubinsky et al., 1991). These authors reported that 80% of the oocytes in the THP group were membrane-intact and 27% of these oocytes went on to mature in vitro. This was significant since previous studies of porcine oocyte cryopreservation found that no oocytes survived vitrification upon warming (Arav et al., 1990).

Dehydration

Dehydration of cells is crucial in cryopreservation. Damage to cells most likely occurs between 15°C and –90°C. If cells are not properly dehydrated, intracellular ice formation occurs when temperature is lowered below 0ºC (Ruffing et al., 1993).

Slow-Cooling vs. Vitrification

There are two very different approaches to cryopreservation of mammalian cells, conventional slow-cooling and vitrification. Although these methods are drastically different, both can produce successful results in mammalian cell cryopreservation. Success depends on choosing the optimal method for each cell type.

Slow-cooling involves use of low concentrations (1-2 M) of CPAs added to the cell culture medium, and rather low cooling rates (0.1-1.0°C/min), achieved by use of a programmable freezing machine, in most cases. Cells are dehydrated during the slow-cooling process. Damage to cells during this process may occur due to osmotic shock, ice crystal formation, or toxicity of CPAs (Rall, 1992).

In 1937, Luyet first described the use of vitrification for the preservation of tissues (Luyet, 1937). The method of vitrification involves the use of high concentrations (5-7 M) of CPAs and ultra-rapid cooling rates (2,000-25,000°C/min). Rall (1987) noted that theoretically even water can be vitrified using a cooling rate of 107°C/sec. The cells are dehydrated by exposure to high concentrations of CPAs prior to cryopreservation which also increases the viscosity of the solution. Cells are suspended in a CPA are plunged directly into LN_2, forming a glass-like suspension of cells. This technique completely eliminates intracellular ice formation (Porcu, 2001). However, cells may be damaged by exposure to such high concentrations of

CPA. Nevertheless, vitrification has the advantage of being low-cost, since it eliminates the need for programmable freezing equipment, and it is quicker and easier to perform compared to slow-cooling.

CRYOPRESERVATION OF EMBRYOS

In 1953, Smith reported the effects of low temperatures on rabbit zygotes and their subsequent development. Since this early effort, much progress has been made (Smith, 1953). Since the birth of the first live animals from cryopreserved embryos (Whittingham et al., 1972), births have now been reported in >20 mammalian species. For example, live young of all of the common lab animal species (mouse, rat, rabbit, hamster, guinea pig) and domestic animal species (sheep, goat, horse, cat, dog) have been achieved. Exotic animals have also been successfully produced using previously cryopreserved embryos (antelope, baboon, marmoset). Table 2 lists the first reports of live young produced from the transfer of frozen-thawed embryos.

Whittingham et al. (1972) reported the first live births in the mouse following transfer of frozen-warmed embryos into recipient females. In that study, an implantation rate of 65% (n=501) of the embryos transferred was observed. Forty-three percent of those implanted embryos resulted in live births. It was also noted that an increase in cooling rate (above 0.3°C/min) resulted in a decrease in post-warmed embryo survival.

The first live calf born from a frozen-warmed embryo was reported by Wilmut and Rowson (1973). That study involved slow-cooling of d-10 to d-13 bovine hatched blastocysts in Me_2SO in glass ampules. Nine embryos were transferred to five recipients, resulting

Table 2. First Births Resulting from the Successful Cryopreservation of Mammalian Embryos

Species	References
Mouse	Whittingham et al. (1972)
Cow	Wilmut and Rowson (1973)
Rabbit	Bank and Maurer (1974)
Rat	Whittingham (1975)
Sheep	Willadsen et al. (1976)
Goat	Bilton and Moore (1976)
Horse	Yamamoto et al. (1982)
Human	Zeilmaker et al. (1984)
Antelope	Kramer et al. (1983)
Baboon	Pope et al. (1984)
Cynomolgus monkey	Balmaceda et al. (1986)
Marmoset monkey	Summers et al. (1987)
Cat	Dresser et al. (1988)
Pig	Hayashi et al. (1989)

Adapted from Leibo and Songsasen (2002)

in one healthy calf named "Frosty II". The first calf produced by artificial insemination with frozen-thawed spermatozoa in the U.S. had been named "Frosty I" (Polge and Rowson, 1952).

Vitrification has also been used for the cryopreservation of embryos. The first report was by Rall and Fahy (1985). Vitrification has been used widely to cryopreserve the embryos of mice (Kasai et al., 1990; Scheffen et al., 1986; Rall, 1987; Rall et al., 1987; Valdez et al., 1990; Van Der Zwalmen et al., 1988), cattle (Massip et al., 1986; Douchi et al., 1990; Kuwayama et al., 1992), rats (Kono et al., 1988), rabbits (Kasai et al., 1992; Smorag and Gajda, 1991; Smorag et al., 1994) and pigs (Rubinsky et al., 1991; Yoshino et al., 1993).

OOCYTE STRUCTURE AND CRYOPRESERVATION DAMAGE

Oocytes are more difficult to freeze than embryos due to the fact that they are large, delicate spherical cells with a low surface area to volume ratio and low hydraulic conductivity (Leibo, 1980). The oocyte is the largest cell in the body of most mammalian species (Wassarman, 1988). Approximately 80% of their volume is water; when they are cooled to subzero temperatures, they may undergo intracellular ice formation, the likelihood of which is dependent on the cooling rate (Leibo et al., 1978).

The mature oocyte of most mammalian species contains a spindle that is usually arrested in the metaphase stage of the second meiotic division. This spindle is made up of microtubules connected to maternal chromosomes (Aigner et al., 1992). The microtubules of oocytes are vulnerable to CPAs and changes in temperature (Pickering et al., 1990; Van Blerkom and Davis, 1994). Exposure of oocytes to CPAs and changes in temperature may cause depolymerization of tubulin within the oocyte (Aman and Parks, 1994). Damage to the meiotic spindle may change

15

the position of the chromosomes and thus limit the fertilization capabilities of the oocyte (Eroglu et al., 1998). Spindle damage is not only a concern with fertilizability, but also because of the potential for chromosomal abnormalities in the resultant embryo (Aman and Parks, 1994). Furthermore, as first described for mouse oocytes by Magistrini and Szöllösi (1980), the microtubules and meiotic spindles of oocytes of all mammalian species undergo disassembly and disaggregation when oocytes are cooled to temperatures near 0°C for just a few minutes (reviewed in Parks and Ruffing, 1992; Vincent and Johnson, 1992). Observations of chilling injury have been made on oocytes of cattle (Martino et al., 1996a), monkeys (Songsasen et al, 2002) and humans (Pickering et al., 1990; Zenzes et al, 2001).

Oocytes are also damaged when exposed to various CPAs. Johnson and Pickering (1987) showed that brief exposure of oocytes to Me_2SO resulted in the emergence of microtubular asters and longer exposure to Me_2SO resulted in disassembly of the spindle and chromosome dispersal. Vincent et al. (1990) also found that Me_2SO affects microtubules and spindles in oocytes. However, they concluded that this damage is temperature-dependent. They observed that at certain temperatures, Me_2SO can not permeate the cell very rapidly and therefore, less CPA is inside the cell.

Shaw and Trounson (1989) reported that propylene glycol causes parthenogenetic activation of mouse oocytes. Parthenogenic activation is the artificial activation of the oocyte. This activation occurs due to an increase in the concentration of Ca^{2+}, which normally comes from spermatozoa. The wave of Ca^{2+} causes the release of cortical granules within the oocyte and this in turn results in the resumption of meiosis and the formation of the second polar body (White and Yue, 1996). Gook et al. (1995) reported that human oocytes also undergo

parthenogenesis after cryopreservation. They observed that 27% of fresh and 29% of aged oocytes that had been cryopreserved were parthenogenetically activated after thawing.

Cortical granules are aligned under the oolemma of most mammalian oocytes. Zona hardening after fertilization or activation of the oocyte is caused by the release of these cortical granules (Wolf and Hamada, 1977; Gulyas and Yuan, 1985). Coritical granules are released to the outer edge of the cytoplasm during the zona reaction. This reaction is normally caused by exposure to sperm prior to fertilization and prevents polyspermy (Wassarman, 1988). Propylene glycol has been shown to cause premature cortical granule release (Schalkoff et al., 1989) and disruption of cortical microfilaments (Vincent et al., 1991). Dimethylsulphoxide has been shown to cause zona hardening and a reduction of cortical granules in mouse oocytes (Vincent et al., 1990). This latter study found that exposure of oocytes to Me_2SO between 20 and 37°C had negative effects on the zona pellucida, fertilization rate and spindle organization. This study also indicated that the oocyte itself caused the hardening of the zona and not the zona. Zonae were isolated from the oocytes, but the isolated zonae were not affected by exposure to Me_2SO, thus proving that the oocyte itself, not the zona was responsible for the hardening effect.

Caroll et al. (1990) reported that adverse effects to the zona pellucida due to cryopreservation can be bypassed by zona drilling. Zona drilling was first reported by Gordon and Talansky (1986) in which the mouse zona was drilled with acid Tyrode's solution through a fine micropipette. The zona can also be penetrated with enzymes such as trypsin or pronase (Gordon et al, 1986).

APPLICATIONS OF OOCYTE CRYOPRESERVATION

The inability to cryopreserve oocytes reliably poses major problems in the field of gamete and embryo biology of the large domestic species. Although full-term pregnancies and live calves have been derived by IVF of bovine oocytes that have been cryopreserved by being cooled slowly to low subzero temperatures, the rate of development of cryopreserved oocytes into blastocysts has been low, usually amounting to <5% of the cooled oocytes (see reviews in Hochi, 2003; Parks and Ruffing, 1992; Vajta, 2000). The ability to cryopreserve oocytes reliably would ease logistical problems associated with the use of abattoir-derived oocytes, since these must be collected from the ovaries and subjected to maturation and fertilization within a relatively short time.

Furthermore, long-term storage of oocytes would substantially alleviate constraints of time in the procedure of SCNT, since it would permit oocytes to be stored in LN_2 and warmed to physiological temperatures to be used as required. In the case of oocytes to be enucleated for use as cytoplasts, it is possible that slight damage to the germinal vesicle or chromosomes resulting from cooling or exposure to CPAs might be tolerated, since the nuclear material itself is to be removed. For example, the nuclei of mouse zygotes subjected to vitrification were found to be more damaged than the cytoplasm (Kono and Tsunoda, 1988).

Oocyte cryopreservation is also crucial in the human reproduction field due to legal and ethical issues associated with embryo cryopreservation. This technique could also be useful for women wanting to cryopreserve their genetic material for use later in life and also women undergoing radio- or chemotherapy for treatment of various types of cancer.

CRYOPRESERVATION OF OOCYTES AT VARIOUS DEVELOPMENTAL STAGES

Oocytes can be stored either as metaphase II (MII) oocytes or as germinal vesicle (GV) stage oocytes. Oocytes at the MII stage of development have undergone several developmental paths including both nuclear and cytoplasmic maturation, extrusion of the first polar body and the arranging of the chromosomes on the MII spindle. Younger, GV oocytes come from Graafian follicles. The chromatin of these immature oocytes is still in the diplotene phase of prophase I. At this stage of development, GV oocytes do not have a spindle (Shaw et al., 1999).

As previously mentioned, MII oocytes are vulnerable to cryoinjury due to the delicate spindle they possess. In contrast, GV oocytes seem to be less susceptible to cryoinjury than MII oocytes, because they are slightly smaller, lack a zona pellucida and cortical granules and are still in a quiescent stage of development. These immature oocytes also have a longer period to recover from cryoinjury because they have to mature in vitro prior to insemination or other manipulations (Shaw et al., 1999) (refer to Table 3).

CRYOPRESERVATION OF OOCYTES

In 1961, Smith reviewed the differences in cryopreservation survival between mammalian unfertilized oocytes and oocytes that had recently been fertilized noting that the fertilized ova had a much better chance of development in vitro than the unfertilized cells (Smith, 1961). Even though this difference between oocytes and embryos was recognized in the early days of oocyte cryopreservation research, the first mammalian oocyte cryopreservation protocols mimicked the equilibrium "slow-cooling" methods used for embryo cryopreservation (see Reviews by Rall, 1992; Fabbri et al., 1998, 2000; Paynter, 2000). Fabbri et al. (1998) described a commonly used protocol for slow-cooling and rapid thawing of oocytes

Table 3. Factors that Influence Sensitivity to Cryoinjury and Suitability for Cryostorage

Material	Primordial oocyte	GV stage oocyte	MII stage oocyte
Availability	Abundant, always present	Scarce, only from antral follicles	Scarce, only at mid-cycle
Ease of collection	Easy, e.g. biopsy	Oocyte retrieval	Oocyte retrieval
Size	<50 μm	80 to 300 μm	80 to 300 μm
Nuclear status	Resting prophase I, Nuclear membrane	GV, has nuclear membrane	Resting MII, temperature sensitive spindle, no nuclear membrane
Zona	No	Yes	Yes
Cortical granules	No	Central	Peripheral
Intracellular lipid	Little	May be abundant	May be abundant
Metabolic rate	Low	Low	Low
Surface:Volume ratio	High	Low	Low

Adapted from Shaw et al. (1999)

using Me_2SO and straws. Oocytes were frozen from 0ºC or 20ºC to -8ºC at a rate of 2ºC/min,

then at a rate of 0.3ºC/min to -30ºC and finally at a rate of 50ºC/min to -150ºC. The straws were

then transferred to LN_2. Thawing was performed at room temperature by washing oocytes

through a series of decreasing concentrations of CPA solutions, with the final dilution containing

sucrose. Live births have been reported using similar conventional slow-cooling methods in

several species including those of mice (Parkening et al., 1976; Whittingham, 1977), rabbits (Al-

Hasani et al., 1989), cattle (Fuku et al., 1992; Otoi et al., 1992) and humans (Chen, 1988). A list

of births resulting from oocyte cryopreservation is listed in Table 4.

The procedure of vitrification of embryos was first described by Rall and Fahy (Rall,

1987; Rall and Fahy, 1985) who showed that mouse embryos could be successfully

cryopreserved by suspending them in concentrated solutions of CPAs and cooling the embryos

rapidly in LN_2. Vitrification is defined as the solidification of a solution (glass formation) at

extremely low temperatures (-196°C) without any internal ice formation (Vajta, 2000). Vitrified

mouse embryos were subsequently shown to develop into normal offspring (Rall et al., 1987).

This procedure has become increasingly important as an alternative method of

cryopreservation, since many reports have suggested that vitrification, rather than conventional

slow-cooling, might be a better method to cryopreserve mammalian oocytes. Research has

found that slow-cooling of oocytes results in zona hardening, due to release of cortical granules,

disruption of the chromosomes, and the loss in the ability to be fertilized (Carroll et al., 1990;

Aigner et al., 1992).

Table 4. First Births Resulting from the Successful Cryopreservation of Mammailan Oocytes

Species	Method	Author
Mouse	Slow-cooling	Parkening et al. (1976)
	Vitrification	Kono et al. (1991)
Rabbit	Slow-cooling	Al-Hasani et al. (1989)
		Vincent et al. (1989)
Cattle	Slow-cooling	Fuku et al. (1992)
	Vitrification, OPS	Vajta et al. (1998)
Human	Slow-cooling	Chen (1986)
	Vitrification	Kuleshova et al. (1999)
Horse	Vitrification, Cryoloop	Maclellan et al. (2002)

Recently, studies have focused on vitrification as a method for cryopreservation of oocytes. Vitrification has also been used to cryopreserve oocytes of mice (Chen et al., 2000; Lane and Gardner, 2001; Nakagata, 1989), rats (Nakagata, 1992), goats (Begin et al, 2003), horses (Maclellan et al., 2002), humans (Kuleshova et al., 1999; Liebermann and Tucker, 2002; Yoon et al., 2000) and especially cattle (Dinnyes et al., 2000; Le Gal and Massip; 1999; Le Gal et al., 2000; Martino et al., 1996b; Matsumoto et al., 2001; Mavrides and Morroll, 2002; Otoi et al., 1998; Papis et al., 2000; Vajta et al 1998; Vieira et al., 2002). Recently, bovine blastocysts have been produced by transfer of nuclei from adult fibroblasts into oocytes or cytoplasts that had been vitrified (Booth et al., 1999).

Injury of oocytes resulting from exposure to temperatures near 0ºC is a time-dependent phenomenon (Martino et al., 1996a). Therefore, various methods have been derived to increase cooling and warming rates to "outrace" chilling injury. These include use of electron microscope grids as a carrier of oocytes (Martino et al., 1996b), placing oocytes into small-diameter open pulled straws (Vajta et al., 1998), dropping 1- to 2-µl volumes of medium containing oocytes directly onto a cold surface at -150ºC (Dinnyes et al., 2000), or onto films of CPA within small nylon cryoloops (Lane et al., 1999). The rationale of all of these methods is that the oocytes are suspended in very small volumes of medium so that they can be cooled at extremely high rates.

Martino et al. (1996b) found that bovine oocytes succumb to chilling damage very quickly. These researchers used electron microscope grids as a vitrification device, which allowed for faster cooling rates and compared this method to conventional straw freezing. After >20 h in culture post-warming, oocytes cryopreserved on grids had a 51% to 72% survival rate

compared to 34% of the oocytes cryopreserved in straws. After IVF, 40% of the oocytes that had been cryopreserved on grids cleaved when placed into culture and 15% developed into blastocysts. The straw method resulted in a 3% cleavage rate and <1% blastocyst development. Rates of blastocyst formation of oocytes cryopreserved on grids, although significantly lower than controls, were significantly higher compared to that of oocytes cryopreserved in conventional straws.

Other groups have used open pulled straws (OPS), which hold oocytes in small amounts of solution for vitrification inside extremely thin hand-pulled straws. Vajta et al. (1998) had improved pregnancy rates with the OPS when compared to conventional straws. Three (3/14; 21%) normal bull calves were born after nonsurgical transfer of embryos resulting from IVF of previously cryopreserved oocytes. Isachenko et al. (2001) also found that the OPS method of vitrification, when used to cryopreserve ovine GV-stage oocytes, resulted in a higher number of MII stage oocytes after culture than conventional straw freezing.

Dinnyes et al. (2000) developed a vitrification procedure that uses a precooled metal surface (solid-surface vitrification; SSV) and small amount of vitrification solutions dropped directly onto this metal surface. In their IVF experiment, they found a 58 to 62% cleavage rate of oocytes fertilized after IVF compared with 69% cleavage of controls. Of the vitrified group of oocytes, 11% to 19% of them developed into blastocysts on d 9 compared with 33% of the fresh control group.

The cryoloop, first described as a device for embryo cryopreservation by Lane et al. (1999), is a small nylon loop with a film of medium formed within it, onto which oocytes or embryos are carefully pipetted; the loop itself is then plunged directly into LN_2. The cryoloop

has been successfully used to cryopreserve oocytes and embryos of the mouse (Lane and Gardner, 2000), the hamster (Lane et al., 1999), cattle (Le Gal et al., 2000), human (Kuleshova et al, 1999) and more recently the horse (Maclellan et al., 2002).

Lane and Gardner (2001) compared the cryoloop method of vitrification with slow-cooling using sodium-free medium, replacing sodium with choline, in mouse oocytes. Lovelock (1954) hypothesized that an increase in electrolyte concentration can cause cellular damage. During cryopreservation, cells are exposed to an increase in electrolyte concentration. Virtually all cell culture media contain sodium saltsand, therefore, damage to cells can be attributed to solution effects during cryopreservation. Stachecki et al. (1998a,b) reported that sodium ions may contribute to osmotic shock in slow-freezing methods. They demonstrated that as the concentration of choline increased and sodium decreased in the freezing medium, higher survival and blastocyst rates were observed post-thaw.

Lane and Gardner (2001) used the cryoloop to vitrify mouse oocytes that they fertilized after puncturing the zona pellucida with a laser. They found a 99.3% survival rate using the cryoloop for vitrification compared to 80.9% survival rate using slow-freezing. The cryoloop resulted in 69.7% fertilization and 67.1% blastocyst rates after IVF post-warming. These results were significantly higher than the rates achieved with the slow-freezing methods, 39.7% and 25.9%, respectively. After transfer into foster recipient mice, the resultant embryos from IVF using vitrified-warmed oocytes developed into full-term fetuses and offspring. In the vitrified oocyte group, 52 of 92 (56.5%) blastocysts transferred resulted in viable fetuses, which was significantly higher than the slow-freezing group (11 of 42; 26.2%).

Very few groups have cryopreserved equine oocytes (Hochi et al., 1994, 1995, 1996; Hurtt et al., 2000; Maclellan et al., 2001, 2002). Maclellan et al. (2002) used the cryoloop method for vitrification of equine oocytes. CPAs were loaded in a three-step method and warming was also performed in three steps. Mares were inseminated with 2 x 10^9 progressively motile sperm 15 to 18 h prior to oocyte transfer. Two healthy foals were born after transfer of vitrified-warmed oocytes into inseminated mares.

A small number of groups of investigators have cryopreserved oocytes to alleviate logistical problems encountered in SCNT methods. Using equilibrium cooling, Kubota et al. (1998) cryopreserved bovine oocytes to serve as cytoplasts for SCNT, ultimately producing a normal calf from the 206 oocytes that had been frozen.

Using an alternative method, Booth et al. (1999) first enucleated bovine oocytes and then vitrified them by the open-pulled straw method; the cryopreserved cytoplasts were then used for SCNT. Fusion rates of vitrified (83.7%±9.2) and control cytoplasts (79.8%±4.6) were not significantly different. However, cleavage and blastocyst development of the vitrified cytoplast group (55.7%±2.9; 7.2%±5.0) was significantly different than the control group (92.8%±3.9; 32.6%±7.8). One SCNT blastocyst transferred into a recipient divided in utero, giving rise to twin calves. Unfortunately, both were dead at birth.

Bovine blastocysts have also been produced by SCNT into oocytes that had been vitrified as microdrops on a metal surface at -150°C (Dinnyes et al, 2000). After SCNT, the vitrified-warmed oocyte group had an 85% cleavage rate and 27% d 9 blastocyst rate. This was not significantly different when compared to a 90% cleavage rate and a 29% d 9 blastocyst rate in the control group.

REVERSAL OF MICROTUBULE DAMAGE AND POST-WARMING INCUBATION PERIODS

As noted above, Magistrini and Szöllösi (1980) found that the spindles of mouse oocytes are damaged by being exposed to low temperatures. However, they also reported that this damage may in fact be reversible. After step-wise rewarming, a significant number of the oocytes contained normal spindles. Another study by Pickering et al. (1990) found that 25 to 50% of the disassembled spindles in human oocytes reverted back to normal appearance after 30 min incubation post-warming.

Chen et al. (2000) developed a way to judge oocyte intracellular freezing damage using fluorescent stains in the mouse. Using this method they were able to decide which method of vitrification and warming worked best based on morphological changes of the meiotic spindle and chromosomes of the oocyte. They hypothesized that after being warmed, longer incubation times of oocytes prior to their being inseminated or undergoing SCNT may increase cleavage and blastocyst rates (Chen et al., 2000; 2001). That study found that oocytes contained severe microtubule damage immediately after warming. After 1, 2 and 3 h of culture, the percentage of normal oocytes increased significantly. Leaving post-warmed oocytes in the incubator for 1 to 3 h prior to fertilization may promote spindle and chromosomal recovery. Also, the CPAs are still inside the cells post-warming. Allowing longer post-warm culture periods may permit complete expulsion of these CPAs.

CRYOPRESERVATION OF OVARIAN TISSUES

As has been described, oocytes are extremely sensitive to freezing damage. Therefore, as an alternative, other investigators have sought to cryopreserve oocytes in situ, that is, while the

oocytes are still embedded within the ovarian tissue. There are several variables to consider in ovarian tissue cryopreservation, such as the type of CPA being used, the cooling rates, the size of the follicles in the tissue the type of animal being used. In the early 1950s, Parkes and Smith were the first to report successful cryopreservation of ovarian tissue slices of rats. Their study showed that ovarian tissue that had been cooled slowly and frozen in LN_2 would produce steroid hormones after autotransplantation (Parkes and Smith, 1953). Since then, slow cooling has been used to freeze ovarian tissue of several species, including that of mice (Gunasena et al., 1997), rats (Deanesly, 1954), rabbits (Daniel et al., 1983), cattle (Daniel and Juneja, 1987), hamsters (Parkes and Smith, 1953), sheep (Gosden et al., 1994), elephants (Gunasena et al, 1998), marmosets (Candy et al., 1995) and humans (Newton et al., 1996).

Many studies have also shown the success of using different CPAs to cryopreserve ovarian tissue such as glycerol, Me_2SO, EG and 1,2 propanediol. Candy et al. (1997) reported remarkable results regarding exposure time to CPA and the type of CPAs used for ovarian cryopreservation and their effects on tissue and follicle survival. That study showed that EG, ME_2SO and 1,2 propanediol all produce somewhat similar survival rates when used as CPAs. However, glycerol produced significantly lower survival rates when used to cryopreserve ovarian tissue.

Vitrification can also be used to preserve ovarian tissue. Vitrification is a much simpler method of cryopreservation because it requires fewer steps than slow-cooling. Ovarian tissue slices are plunged directly into LN_2 after CPA exposure. A recent study showed that transplanted ovarian tissue that had been vitrified had endocrine function and development of antral follicles similar to that of fresh transplants occurred (Sugimoto et al., 2000). However,

28

few studies have been done using vitrification of tissue, and some researchers believe that slow-cooling is a much more effective method (Shaw et al., 2000b).

Survival of oocytes frozen in situ within ovarian tissue is largely dependent on their stage of maturity, as previously described in Table 3. Oocytes within primordial follicles appear to survive the best due to their smaller size, cell-cycle stage, absence of zona and cortical granules and finally less intracellular lipids. In contrast, oocytes within the larger and more mature follicles appear to suffer the most damage from cryopreservation. Mature MII oocytes are susceptible to cryoinjury because the spindle holding the chromosomes on the metaphase plate depolymerizes as the temperature decreases (Magistrini and Szöllösi , 1980; Eroglu et al., 1998; Zenzes et al., 2001).

Successful cryopreservation is also dependent on the size of the tissue to be preserved. This is not really a problem in the mouse or the rat, but does present a problem for larger species. In order to cryopreserve larger ovaries, the tissue must be cut into small pieces, 1 to 3 mm^3, prior to cryopreservation. This also means that the tissue must be returned to the animal in small sections. Obviously this creates another problem in that the small tissue slices have a more difficult time to become revascularized. It is crucial that the tissue regains vascularity as soon as possible so that the ovary can recover and to avoid damage to the follicles.

CONCLUSION

Although the term cryobiology only began to be used a little over 40 years ago, the science of low temperature biology has been a subject of study for over 300 years. Reproductive physiology has radically changed with the development of cryopreservation methods for gametes and embryos. Developing the correct protocol for each cell type is a tedious and long process.

Cooling rate, warming rate and choice of CPA are the key parameters that can be adapted to fit a certain cell type. Successful cryopreservation protocols have been established for mammalian sperm and embryos, and hundreds of thousands of live offspring have been produced from cryopreserved sperm and embryos. In contrast, there are still difficult problems to be solved to cryopreserve oocytes of all mammalian species.

CHAPTER II.

VITRIFICATION OF BOVINE OOCYTES FOR USE IN IVF

INTRODUCTION

As described above, mammalian oocytes, unlike embryos, are extremely difficult to cryopreserve successfully. Two approaches to cryopreservation were discussed above, equilibrium or standard slow-cooling and non-equilibrium or vitrification. There are innumerable variations in the specific methods used within both approaches. Utilizing cryopreserved oocytes for use in in IVF has been the focus of much research.

Brackett et al. (1980) were the first to report IVF of bovine oocytes. As summarized by Gordon (1994), in vitro production of embryos involves several critical steps. Oocytes must be obtained either by aspirations from in situ ovarian follicles, harvested from ovaries collected at the abattoir, or a commercial oocyte source. In all cases, the oocytes undergo in vitro maturation (IVM). After IVM, mature oocytes are placed into droplets of medium containing spermatozoa for IVF. Two years later, Brackett and his colleagues reported the first calf born from IVF embryos that were transferred into the oviduct of a recipient cow (Brackett et al., 1982). The first calf to be born from transfer of IVM-IVF bovine embryos was reported by Critser et al. (1986). These embryos had been cultured in the oviduct of a sheep for 5 d prior to being transferred into the recipient cow. After IVF, subsequent embryos are often subjected to in vitro culture (IVC). The first calves to be produced from IVM, IVF and IVC were reported in 1987 (Fukuda et al., 1990).

As noted above, oocytes can be obtained by in situ ovarian follicle aspiration or from excised ovaries. Transvaginal ultrasound-guided oocyte aspiration (TUGA) was first described

for cattle by Pieterse et al. (1988). This method of oocyte collection is widely used and is often referred to as ovum pick-up (OPU). Excised ovaries can be obtained from cows that are sent to abattoirs. In fact, there are now commercial oocyte sources that collect ovaries at an abattoir, collect the oocytes and ship them *via* overnight courier to laboratories all over the country (Hasler, 1998; Galli et al., 2003, Merton et al., 2003).

Two features are notable about in vitro maturation of bovine oocytes. First, immature oocytes at many different stages of development aspirated from the ovarian follicle can all be forced into synchronized maturity within ~24 h. Second, a wide variety of media can cause oocytes to mature (Hasler, 1998). A mature oocyte is covered with expanded cumulus cells. These cells aid in sperm capacitation and, therefore, successful fertilization can only occur when oocytes are mature at the time of insemination (Gordon, 1994).

In vitro fertilization of bovine oocytes has shown that it is much different than in vivo fertilization. Sperm used for IVF is most often obtained from frozen-thawed semen samples. Sperm concentration in the insemination droplet has a profound effect on cleavage and embryo development in that polyspermy can result if the sperm concentration is too high. Sperm samples are usually washed or put into a Percoll density gradient media prior to insemination. These techniques increase the percent of viable, motile and morphologically normal spermatozoa in a semen sample (Gordon, 1994).

In vitro culture media can be labeled as either "defined", semi-defined" or "non-defined", however, there is much diversity among culture systems. Defined media are composed only of discrete chemical quantities and do not include supplements such as serum or BSA. Semi-defined media are defined media that contain additives such as serum or BSA. Non-defined

media also include sera and BSA, but may be also contain various amounts of hormones or other biological compounds that can not be repeated in another laboratory. Non-defined media may also include co-culture systems, since they make use of various types of cells as "feeder" layers to the embryos (Hasler, 1998). Each step of IVM-IVF-IVC (IVMFC) is independent of the other steps. Therefore, the optimization of each of these steps was a long and tedious process. However, today IVMFC is repeated on a day-to-day basis in bovine embryo production for both commercial and research purposes.

The overall objectives of this study were to reliably and successfully vitrify bovine oocytes. Successful vitrification was tested by subjecting vitrified-warmed oocytes to IVF and comparing fertilization and subsequent embryo development to those of fresh oocytes.

MATERIALS AND METHODS

Oocyte Preparation

Starting during the mid-fall season of 2001, weekly batches of bovine oocytes were obtained from a commercial source (BoMed, Inc., Madison, WI) over a period of 12 wk. The oocytes were shipped by overnight courier to the laboratory at a temperature of ~35ºC, while undergoing in vitro maturation in transit. Although the specific breeds of the donor cows were unknown, the majority of the oocytes provided by this commercial source were from mature Holstein cows. After a 20 h maturation period, oocytes were recovered from the maturation medium, partially denuded of cumulus cells by exposure to 0.1% hyaluronidase (Sigma Chemical Co., St. Louis, MO) in Tissue Culture Medium-199 (TCM-199, Gibco, Grand Island,

NY) leaving two to three layers of cumulus cells attached to the zona pellucida. All of the media were purchased from Sigma or Gibco, unless otherwise specified.

Vitrification and Warming Procedure

The vitrification method used in this experiment is a modified version of a protocol previously reported by Maclellan et al. (2002) to vitrify equine oocytes. Vitrification and warming procedures are illustrated diagrammatically in Figure 1. Oocytes were prepared for vitrification by being exposed sequentially and very briefly to three CPA solutions. These solutions were prepared in TCM-199 medium supplemented with 10% FBS (HyClone, Logan, UT). The three solutions were:

CPA-1: 0.7 M Me_2SO + 0.9 M EG

CPA-2: 1.4 M Me_2SO + 1.8 M EG

CPA-3: 2.8 M Me_2SO + 3.6 M EG + 0.65 M trehalose

To prepare for vitrification, plastic cryovials (Hampton Research, Laguna Niguel, CA) without lids were inserted into a small rack that was placed into a Styrofoam box partially filled with LN_2 and the vials were filled with LN_2. To perform the actual vitrification at room temperature of ~22°C, oocytes were pipetted for 30 sec each into CPA-1 and CPA-2 and were then placed into CPA-3 for 20 sec. During this 20-sec exposure, groups of five oocytes each were picked up with a fine pipette from the CPA-3 solution and quickly loaded onto a 20 μm cryoloop (Hampton Research, Laguna Niguel, CA) that had been inserted into the lid of a plastic cryovial (Figure 2). Without pause, the cryoloop was quickly placed directly into a LN_2-filled cryovial and the lid was screwed into the threaded bottom.
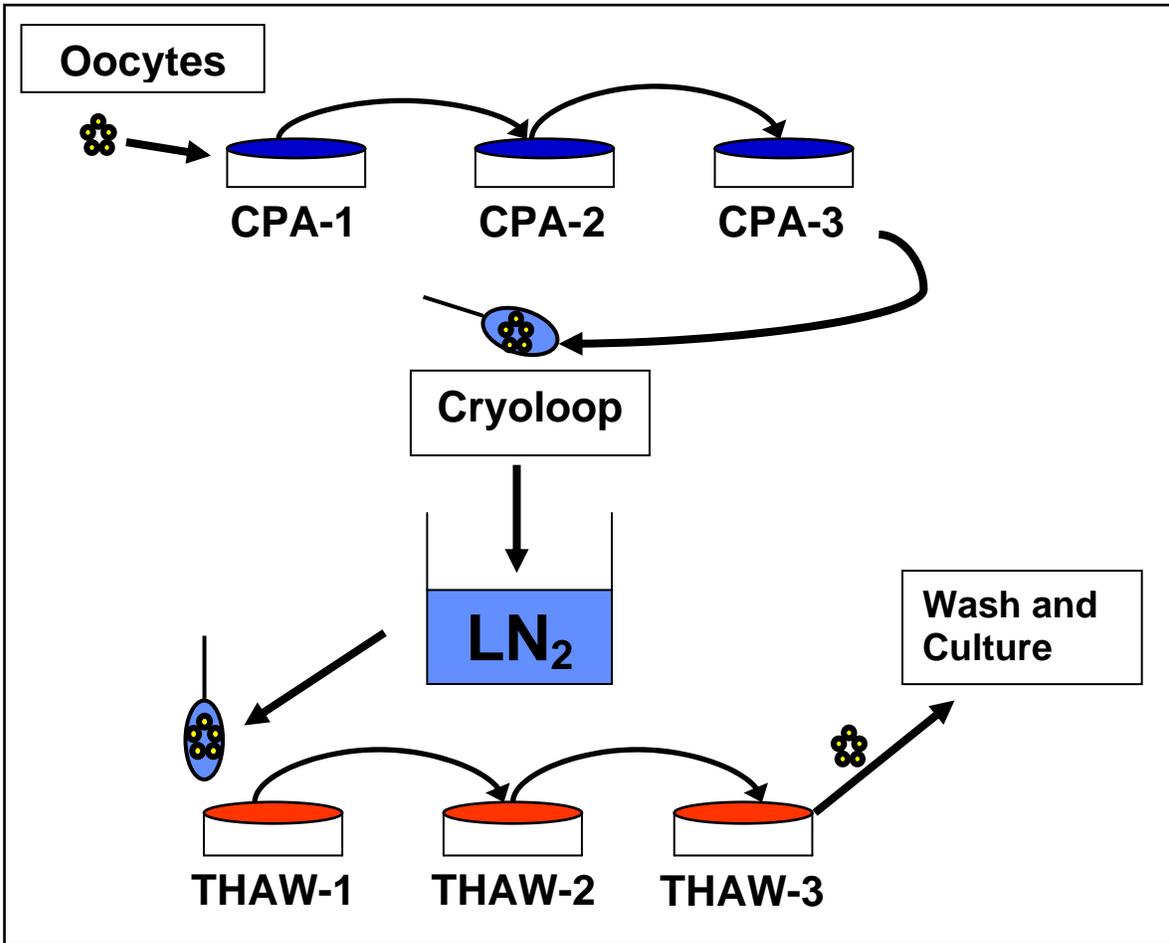
34

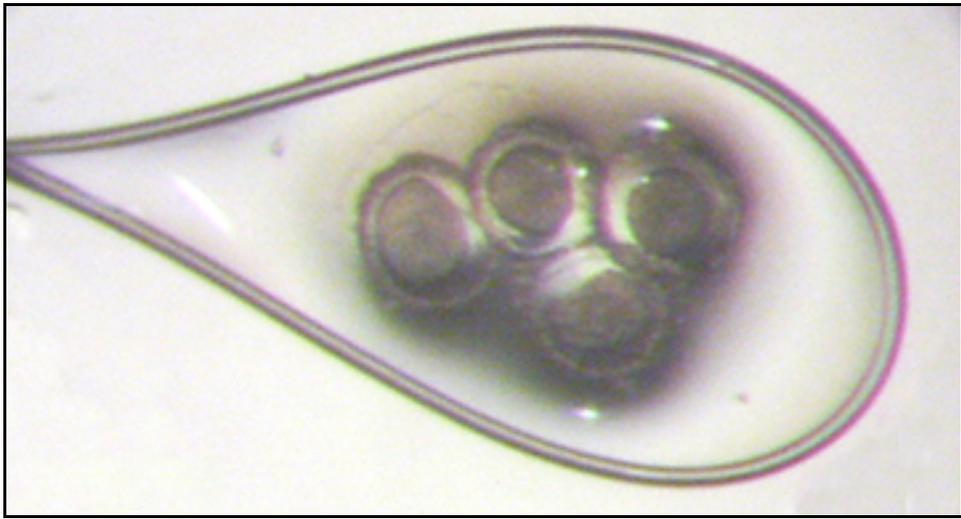Figure 1. Cryoloop vitrification and warming procedure

Figure 2. Bovine oocytes loaded onto nylon cryoloop

After the vitrified oocytes had been held in LN_2 for at least 1 h, the lid was unscrewed from its cryovial, and the vitrified oocytes solidified on the cryoloop were immersed directly into a Petri dish containing ~3 ml of 0.25 M trehalose in TCM-199 supplemented with 10% FBS at 37°C. When several cryoloops had been warmed and liquefied to yield a group of 20 to 40 oocytes, the entire group was then washed by step-wise dilution in 0.19 M and then in 0.125 M trehalose in TCM-199 and finally in TCM-199 alone. Oocytes that had been vitrified were cultured at 38°C in a humidified atmosphere of 5% CO_2 in air for 1 h prior to being subjected to intro fertilization.

<u>In Vitro Fertilization</u>

In vitro fertilization was performed using spermatozoa from a single batch of semen from one Holstein bull that had been frozen in straws. One 0.25 ml straw of frozen semen was thawed in a 37°C water bath for 1 min. Semen was washed twice in Brackett-Oliphant (BO) medium (Brackett et al., 1975) supplemented with 5 mM caffeine by centrifugation at 500 x g for 6 min at room temperature. The sperm pellet was resuspended in BO medium supplemented with 0.06% BSA, 0.036% heparin (Elkins-Sinn, Cherry Hill, NJ) and 2.5 mM caffeine. Then 1 h after vitrified oocytes had been warmed, control and vitrified oocytes were placed into 100 μl insemination droplets under mineral oil with spermatozoa at a concentration of 1 x 10^6 sperm/ml.

<u>Embryo Development</u>

After 5 h of co-incubation, all oocytes were then washed and cultured in CR1aa culture medium (Rosenkrans et al., 1994) at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Embryos were observed for cleavage and the medium was changed (CR1aa plus 10% FBS) at 72 h post-insemination. At 192 and 216 h post-insemination, cleaved embryos were

observed for blastocyst development and formation of blastomeres were confirmed by staining with 1μg/ml Hoescht 33342 and observed with an epi-flourescent microscope equipped with UV excitation and appropriate barrier filters.

Statistical Analysis

This experiment consisted of five replicates. Assessment of oocyte viability based on morphology, the fertilization rate as indicated by cleavage into 2-cell embryos and the blastocyst development rate were compared within respective parameters using Chi-square analyses. Means with a probability value of $P<0.05$ were considered significantly different in this study.

RESULTS

Development of In Vitro Fertilized Embryos

Prior to in vitro insemination, oocytes were evaluated and assigned either a positive or a negative rating based on their morphology. Embryos were assessed for morphological development on d 3, 7 and 8 after IVF. In this study, 98% of both the control and vitrified oocytes were judged to be normal based on their overall morphology when first placed into culture. After in vitro insemination and 72 h of in vitro culture, the respective cleavage rates for 137 control and 327 vitrified oocytes were 84% and 82%, respectively (Table 5). However, after 192 h and 216 h of in vitro culture, significantly fewer expanded and hatched blastocysts developed from the vitrified oocytes compared with the control oocytes. Nevertheless, early and hatched blastocysts derived from both treatment groups were similar in size (diameter) and in embryo quality (Figure 3).

Table 5. Development of Bovine Embryos Derived from Vitrified In Vitro Fertilized Oocytes

Treatment	Oocytes n	Cleaved at 72 h n (%)	BLST* at 192 h n (%)	Hatched BLST at 216 h n (%)
Control	137	115 (83.9)	44 (32.1)[a]	32 (23.4)[a]
Vitrified	327	267 (81.7)	32 (9.8)[b]	17 (5.2)[b]

The values shown are the totals from five replicate experiments
* BLST = blastocysts
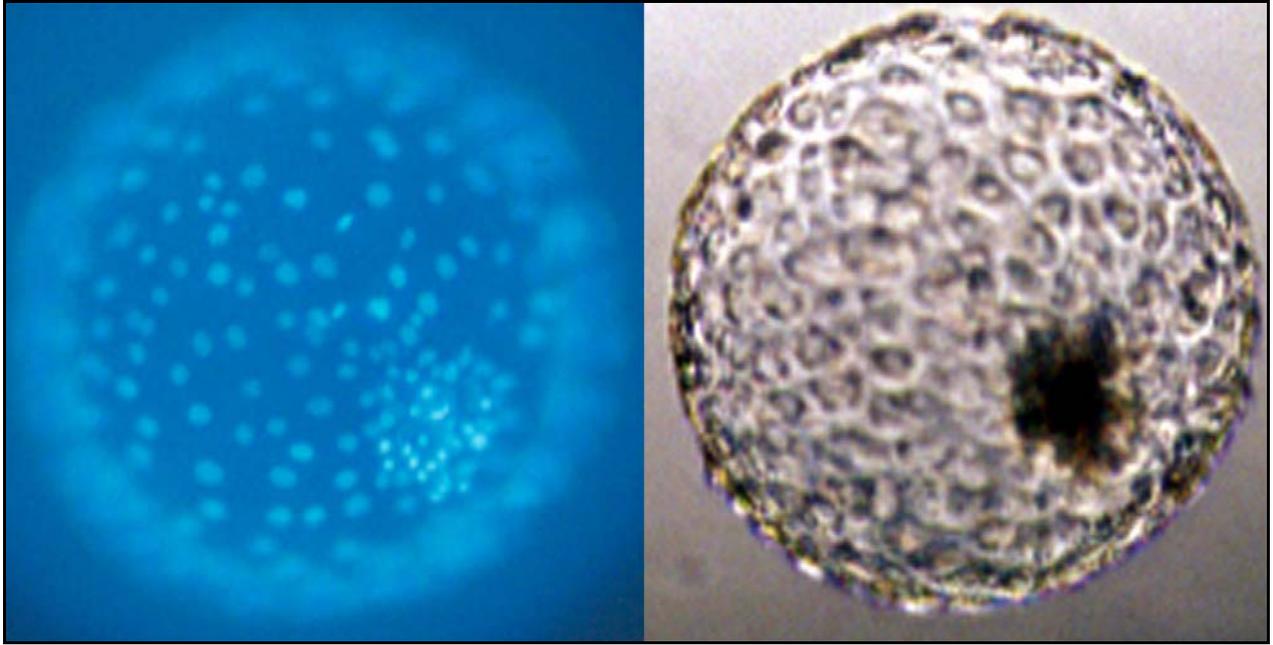[a, b] Means in the same column with different superscripts are significantly different (P<0.05)

Figure 3. Hatched blastocyst derived from IVF using vitrified-warmed bovine oocytes

DISCUSSION

The first experiment of this study verified the observations of others that bovine oocytes can be vitrified using a nylon cryoloop, resulting in a high rate of cleavage after IVF, equivalent to that of control oocytes. However, only 10% of 327 vitrified oocytes developed into blastocysts, significantly fewer than the 32% of control oocytes.

Mammalian oocytes of various species have been shown to be sensitive to chilling in a time-dependent fashion (Magistrini and Szollosi, 1980; Martino et al., 1996a; Otoi et al., 1992; Pickering et al., 1990; Songsasen et al., 2002; Suzuki et al., 1996; Wu et al., 1999; Zenzes et al., 2001). Therefore, since standard methods of equilibrium cooling for cryopreservation inevitably expose oocytes to temperatures near 0°C for several min, it is not surprising that the overall success rates of oocyte cryopreservation have been low, often amounting to less than 3% of cryopreserved oocytes developing into blastocysts after IVF (see reviews in Parks and Ruffing, 1992; Vincent and Johnson, 1992; Leibo, 2004b).

However, recent evidence suggests that the composition of the solution used to prepare the CPA in which oocytes are frozen may also significantly influence their survival and developmental capability (Stachecki et al., 2002). Nevertheless, in general, vitrification has proven to be more effective than standard slow-cooling methods to cryopreserve mammalian oocytes (Begin et al., 2003; Chen et al., 2000; Dinnyes et al., 2000; Kuleshova et al., 1999; Lane and Gardner, 2001; Lane et al., 1999; Le Gal et al., 1999, 2000; Liebermann and Tucker, 2002; Maclellan et al., 2002; Martino et al., 1996b; Matsumoto et al., 2001; Mavrides and Morroll, 2002; Nakagata, 1989, 1992; Otoi et al., 1998; Papis et al., 2000; Vajta, 2000; Vajta et al., 1998; Vierira et al., 2002; Yoon et al., 2000).

41

Vitrification involves the use of concentrated solutions of CPAs and high rates of cooling and warming (Rall and Fahy, 1985; Vajta, 2000). Exposure of oocytes to high concentrations of CPAs cause the oocytes to undergo osmotic dehydration prior to cooling; that treatment coupled with extremely high cooling rates prevents the formation of intracellular ice crystals within oocytes, thus reducing disruption and damage to the cellular architecture. The cryoloop as a device to perform vitrification of embryos was first described by Lane et al. (1999). This approach has proven to be efficacious because it is a rapid and simple technique that can be easily executed reproducibly and requires little specialized equipment or time. As previously illustrated by Figure 1, the cryoloop is formed from a very fine nylon loop that allows minute quantities of medium to be held as a film, onto which oocytes can be "suspended". These very small volumes of medium allow oocytes to pass through the damaging temperature zone of +10° to -10°C at a very high rate.

In the present study, rather high concentrations of CPAs [2.8 M Me_2SO + 3.6 M EG + 0.65 M trehalose] were used with the cryoloop. The vitrification treatment did not affect the proportion of the oocytes with intact morphology after warming, although it has been reported that mammalian oocytes are very sensitive to high concentrations of CPAs (Johnson and Pickering, 1987). As in the original vitrification procedure of Rall and Fahy (1985), oocytes were exposed to the CPAs in a step-wise, sequential fashion prior to vitrification. After warming and liquefaction, the CPAs were also diluted in a step-wise fashion with trehalose. This may have allowed the oocytes to undergo volume excursions at a somewhat lower rate and may have reduced the potential cytotoxic effects of the CPAs. Otoi et al. (1998) reported that step-wise equilibration prior to vitrification is beneficial in bovine oocyte vitrification. They noted that the

42

survival of oocytes vitrified using a one-step method of dilution was significantly lower than that using a two- or three-step procedure. In that study, no oocytes in the one-step dilution group exhibited any cleavage or developed to blastocysts during in vitro culture.

In this study, the 10% rate of blastocyst development in the vitrification treatment group was significantly lower than the 32% of the nonvitrified control group. Similarly, the 5% hatching rate of blastocysts derived from vitrified oocytes was significantly lower than the 23% rate of the control group. It has been proposed that cryopreservation of oocytes causes the zona pellucida to undergo hardening due to early release of ooplasmic cortical granules (Carroll et al., 1990). Lane and Gardner (2001) used a focused laser to make a small hole in the zona pellucida of mouse oocytes prior to fertilization, as did Stachecki et al. (Stachecki et al., 2002) with oocytes frozen at low rates. Compared with control oocytes, lower fertilization rates of vitrified oocytes have been reported when some method, such as a laser or needle, was not used to enhance fertilization by making an artificial opening in the zona prior to being placed into insemination droplets. In this study, we did not open the zona artificially and did not observe a difference between control and vitrified oocytes with respect to fertilization and cleavage, although we did find a significant difference between the two groups as to their ultimate development into blastocysts. Vieira et al. (2002) have noted that there appears to be a low correlation between cleavage and further embryonic development for vitrified oocytes.

In this study, development of vitrified oocytes into blastocysts after IVF was significantly lower than that of the corresponding control oocytes. Therefore, our results suggest that the cytoplasm of bovine oocytes may be damaged by the entire process of vitrification in some subtle, but significant manner that affects the developmental capability of the embryos. Clearly,

further experiments are needed to improve blastocyst and pregnancy rates from IVF using vitrified-warmed oocytes. Furthermore, transfer of blastocysts derived from IVF using vitrified-warmed oocytes would verify the nuclear competency of these oocytes.

CHAPTER III.

VITRIFICATION OF BOVINE OOCYTES
FOR USE IN SOMATIC CELL NUCLEAR TRANSFER

INTRODUCTION

In 1986, Willadsen (1986) introduced the use of micromanipulation of mammalian oocytes and embryos that has evolved into methods to clone domestic animals when he successfully cloned a sheep using nuclear transfer of blastomeres. The birth of "Dolly" the sheep by Wilmut and his colleagues (Wilmut et al., 1997) set the stage for an entire new technology to be used for the production of domestic species. Dolly was cloned by use of a technique called somatic cell nuclear transfer (SCNT). She was the first domestic animal to be produced by this procedure. Today, cloning of domestic animals is widely used in multiple species (Reggio et al., 2001; Boquest et al., 2002; Dinnyes et al., 2002; Forsberg et al., 2002; Heyman et al, 2002; Keefer et al, 2002).

Somatic cell nuclear transfer is an important tool not only for basic domestic animal production research, but also in the production of transgenic animals. Donor cells that have been geneticically manipulated by either adding or "knocking out" specific genes by homologous recombination can be used for SCNT to produce innumerable copies of the gene of interest, aiding in the research of both agriculture and human medicine (see reviews, Zuelke, 1999; Niemann and Kues, 2000; Prather et al, 2003).

Although extensive research has been conducted in the area of SCNT, the efficiency of this procedure is still extremely low, with <10% of embryos transferred be born as live young (Greve and Callesen, 2004). As previously mentioned above, cryobiology has several

applications in reproductive biology. Cryopreservation of oocytes would substantially alleviate constraints of time in the procedure of SCNT, since it would permit oocytes to be stored in LN_2 and warmed to physiological temperatures to be used as required. This would eliminate seasonal obstacles associated with research animals, such as the goat and horse. In the case of oocytes to be enucleated for use as cytoplasts, it is possible that slight damage to the germinal vesicle or chromosomes resulting from cooling or exposure to CPAs might be tolerated, since the nuclear material itself is to be removed. For example, the nuclei of mouse zygotes subjected to vitrification were found to be more damaged than the cytoplasm (Kono and Tsunoda, 1988). Therefore, when used for SCNT oocytes may not need to be of the same quality as oocytes used for IVF.

Several groups of investigators have cryopreserved oocytes to alleviate logistical problems encountered in SCNT methods. Using a standard method of slow, equilibrium cooling, Kubota et al. (1998) cryopreserved bovine oocytes to serve as cytoplasts for SCNT, ultimately producing a normal calf from the 206 oocytes that had been frozen. Using an alternative method, Booth et al. (1999) first enucleated bovine oocytes and then vitrified them by the OPS method; the cryopreserved cytoplasts were then used for SCNT. One SCNT blastocyst transferred into a recipient divided in utero, giving rise to twin calves. Unfortunately, both were dead at birth (Booth et al., 1999). Bovine blastocysts have also been produced by SCNT into oocytes that had been vitrified as microdrops on a metal surface at -150°C (Dinnyes et al., 2000).

The objective of this study was to determine the possibility of using oocytes vitrified by the cryoloop method to serve as cytoplasts for SCNT.

MATERIALS AND METHODS

Oocyte Preparation

Starting during early spring of 2002, batches of bovine oocytes were obtained weekly from a commercial source (BoMed, Inc., Madison, WI) over a period of 12 wk. As previously described in Chapter II, oocytes were purchased from this commercial source and arrived at the laboratory after undergoing IVM in transit. Oocytes were denuded in the same manner as previously described in Chapter II and placed into culture awaiting enucleation.

Experimental Design

Oocytes were randomly assigned to one of two treatment groups, to be enucleated either as fresh oocytes (controls) or after being vitrified (vitrified). After enucleation, both groups were then used as cytoplasts to which somatic cells were fused. The somatic cells, which had been derived from a skin biopsy of a purebred Brahman cow, had been sub-cultured through two to eight passages.

Vitrification and Warming Procedure.

Vitrification and warming procedures were completed as described in Chapter II. Oocytes were prepared for vitrification by being exposed sequentially and very briefly to three CPA solutions. After exposure to the three CPA solutions, oocytes were quickly loaded onto the cryoloop and placed directly into a LN_2-filled cryovial and the lid was screwed into the threaded bottom. Warming procedures were again the same as those described in Chapter II. After being warmed in the three solutions of trehalose (0.25, 0.19, 0.125 M), oocytes that had been vitrified were cultured at 38°C in a humidified atmosphere of 5% CO_2 in air for 1 h prior to enucleation.

Nuclear Transfer Procedure

After control and vitrified oocytes had been allowed to mature in vitro for 1 h, oocytes were denuded of most cumulus cells by being repeatedly pipetted through a fine pipette. Morphological viability was assessed at this time and only good quality mature oocytes that had extruded the first polar body were selected for SCNT using a procedure slightly modified from that previously described (Shiga et al., 1999). Briefly, the oocytes were stained with Hoechst 33342 stain (5 μg/ml) and a small rent was made in the zona pellucida using a flexible glass needle. After a very brief (<10 sec) observation with fluorescence optics to determine the location of the metaphase plate, the first polar body and the metaphase plate were removed from each oocyte using a Nikon micromanipulation unit.

The donor cells, originally isolated from the skin biopsy of a mature, fertile Brahman cow, consisted of adult skin fibroblasts that had been sub-cultured two to eight times. A single donor cell was introduced into the perivitelline space of the enucleated oocytes to construct a cytoplast-karyoplast couplet. The couplets were induced to fuse in buffer (0.3 M mannitol, 0.05 mM calcium, 0.1 mM magnesium) with two direct current-pulses of 2.25 kV/cm for 15 μsec delivered by an electrofusion unit (BTX Model 200, San Diego, CA). Fused couplets were further activated by culturing in 10 μg/ml of cyclohexamide for 4 h and then cultured in CR1aa medium (d = 0) at 38°C under mineral oil in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Embryo Development and Transfer

Embryos were evaluated on d 3, 6, 7 and 8 following SCNT. Embryos were washed with fresh CR1aa medium supplemented with 5% FBS on d 3 and 7 of in vitro culture. As beef cows (mixed breed crosses) in the research herd became available to serve as recipients, individual embryos derived from SCNT of somatic cells into vitrified oocytes were nonsurgically transferred to females on d 6 of their estrous cycle (estrus = d-0). Pregnancies were detected using ultrasonography on d 30 of gestation and again verified by detecting fetal heart beats on d 60 and subsequently on d 90 of gestation.

Statistical Analysis

This experiment was composed of six replicates. The rates of cytoplast fusion and of embryo development of control and vitrified oocytes were evaluated using a post hoc, Fisher Protected-Least-Significant-Difference test (PLSD test). Means with a probability value of $P \leq 0.05$ were considered significantly different in this study.

RESULTS

Development of Nuclear Transfer Embryos.

After the cumulus cells were stripped from the oocytes, no significant differences were observed in the percentages of oocytes that appeared morphologically normal: 94.5% of 109 control oocytes; 90.8% of 293 vitrified oocytes. Normal morphologically were those oocytes that contained both an intact zona pellucida and vitelline membrane. These morphologically normal oocytes were then enucleated.

49

Development in vitro of the nuclear transfer embryos derived from vitrified oocytes and from control oocytes is shown in Table 6. After enucleation, the percentages of couplets (cytoplast + karyoplast) produced, of fused embryos and of embryos that cleaved to the 2-cell stage were similar for both vitrified and nonvitrified control oocytes. However, the percentage of embryos that developed from vitrified oocytes into blastocysts by d 7 of in vitro culture (13%) was significantly lower (P<0.05) compared with those in the nonvitrified Control group (33%).

Embryo Transfer and Pregnancies.

As this experiment proceeded, a total of 31 embryos derived from vitrified oocytes developed into blastocysts during 192 h of in vitro culture. In the second replicate of this experiment, as recipient females became available, 20 of these nuclear-transfer embryos were nonsurgically transferred individually into beef cattle recipients on d 6 of their estrous cycle. Three of the 20 females (15%) were diagnosed as being pregnant by ultrasonography as evidenced by heart beats from singleton fetuses on d 30 of gestation. Of the three SCNT pregnancies, two were lost prior to 90 d of gestation. However, one cow delivered a live healthy SCNT Brahman calf by Caesarean section on d 284 of gestation. The female calf weighed 42 kg at birth (Figure 4) and was fed an artificial milk supplemented diet while in the nursery quarters. At over one year of age, the heifer is alive and gaining weight on a normal growth curve.

DISCUSSION

This experiment showed that SCNT embryos can be produced by fusion of somatic cells to oocytes cryopreserved by ultra-rapid cooling and warming. Although significantly fewer of the SCNT embryos derived from vitrified oocytes developed into blastocysts compared with

Table 6. Development of Recontructed Bovine Oocytes after Somatic Cell Nuclear Transfer

Treatment	Oocytes* n	Couplets n (%)	Fused n (%)	Cleaved at 72 h n (%)	BLST** at 192 h n (%)[§]
Control	103	91 (88.3)	68 (74.7)	55 (60.4)	30 (33.0)[a]
Vitrified	266[‡]	248 (93.2)	195 (78.7)	152 (61.2)	31 (12.5)[b]

The values shown are the total of six replicate experiments.
* Number of mature oocytes enucleated.
** BLST = blastocyst.
[‡]A total of 293 oocytes were vitrified and warmed; 266 (90.8%) were judged to be morphologically normal and were enucleated.

[§] Blastocysts as a percentage of the number of couplets formed after fusion.

[a,b] Means in the same column with different superscripts are significantly different (P<0.05).

control oocytes, some of the former embryos were also capable of developing in utero into fetuses. One even developed into a live calf after transfer into a recipient cow.

As previously stated, successful cryopreservation of mammalian oocytes has been shown numerous times to be more effective by implementing vitrification rather than standard slow-cooling methods (Begin et al., 2003; Chen et al., 2000; Dinnyes et al., 2000; Kuleshova et al., 1999; Lane and Gardner, 2001; Lane et al., 1999; Le Gal et al., 1999, 2000; Liebermann and Tucker, 2002; Maclellan et al., 2002; Martino et al., 1996b; Matsumoto et al., 2001; Mavrides and Morroll, 2002; Nakagata, 1989, 1992; Otoi et al., 1998; Papis et al., 2000; Vajta, 2000; Vajta et al., 1998; Vierira et al., 2002; Yoon et al., 2000). Vitrification involves the use of concentrated solutions of CPAs and high rates of cooling and warming (Rall and Fahy, 1985; Rall, 1987; Vajta, 2000) and as previously reviewed, several different protocols and devices have been used to vitrify these delicate cells. As illustrated by Figure 2 (Chapter II), the cryoloop is formed from a very fine nylon loop that allows small quantities of medium to be held as a film. Oocytes are placed on this film of medium and vitrified at extremely high cooling rates, thus reducing the amount of time that oocytes are exposed to the known fatal temperature zone of 10ºC to -10ºC.

In this study, rather high concentrations of CPAs [2.8 M Me$_2$SO + 3.6 M EG + 0.65 M trehalose] were used as the vitrification solution with the cryoloop. The vitrification treatment did not affect the proportion of the oocytes with intact morphology (both intact zonae and vitelline membranes) after warming in this experiment, as was true in the previous experiment, although it has been reported that mammalian oocytes are very sensitive to high concentrations of CPAs (Johnson and Pickering, 1987).

Figure 4. Calf produced by SCNT using vitrified-warmed oocytes as cytoplasts

Embryo development from the vitrified group into blastocysts was significantly lower (13%) compared to that for the control group (33%). In mice, the blastocyst development rate was higher when vitrified oocytes were enucleated and fused with pronuclei from fresh oocytes compared with the rate when fresh oocytes were reconstructed using pronuclei from vitrified oocytes (Kono and Tsunoda, 1988). Those authors suggested that nuclei were more likely to be damaged than the cytoplasm during the vitrification process. In another report, Dinnyes et al. (2000) found that blastocyst development of vitrified bovine oocytes was reduced following either IVF (11%) or parthenogenetic activation (17%) compared with the rates using fresh oocytes. However, in that study, rates of cleavage and blastocyst development using vitrified oocytes (85% and 27%) were similar to those with fresh oocytes (90% and 29%) following SCNT. Nevertheless, development into blastocysts in culture was slower with vitrified oocytes than with embryos derived from fresh oocytes. The authors suggested that both nuclear and cytoplasmic components were damaged during the cryopreservation process.

In the present study, three of 20 (15%) cows into which SCNT embryos derived from vitrified oocytes had been transferred were pregnant on d 30 of gestation, a rate that was lower than the average pregnancy rate of 38% achieved with nuclear transfer embryos derived from fresh bovine oocytes in our laboratory. These observations and those of others further indicate that there is an effect of vitrification on bovine oocytes. Development from vitrified oocytes into blastocysts after SCNT into enucleated oocytes was significantly lower than that of the corresponding control oocytes. Therefore, our results suggest that the cytoplasm of bovine oocytes is damaged by the entire process of vitrification in some subtle but significant manner that affects the developmental capability of nuclear-transfer, just as we found in our IVF studies.

Clearly, further experiments are needed to improve blastocyst and pregnancy rates from SCNT using vitrified oocytes. Nevertheless, we have confirmed observations of others that live offspring can be produced by fusion of somatic cells with oocytes enucleated after vitrification.

MICROTUBULE STAINING OF VITRIFIED-WARMED CAPRINE OOCYTES

INTRODUCTION

The mature metaphase II oocyte of most mammalian species contains a spindle that is usually arrested at the metaphase stage of the second meiotic division. This spindle is made up of microtubules connected to maternal chromosomes (Aigner et al., 1992). The microtubules of oocytes are vulnerable to CPAs and changes in temperature (Pickering et al., 1990; Van Blerkom and Davis, 1994). Damage to the meiotic spindle may change the position of the chromosomes and thus limit the fertilization capabilities of the oocyte (Eroglu et al., 1998). Furthermore, as first described for mouse oocytes by Magistrini and Szöllösi (1980), the microtubules and meiotic spindles of oocytes of most mammalian species undergo disassembly and disaggregation when oocytes are cooled for 45 to 60 min to 0°C (reviewed in Parks and Ruffing, 1992; Vincent and Johnson, 1992). Observations of chilling injury have been made on oocytes of cattle (Martino et al., 1996a), monkeys (Songsasen et al, 2002) and humans (Pickering et al., 1990; Zenzes et al, 2001). Vincent et al. (1990) found that hardening of the zona occurs when oocytes are exposed to temperatures around 4°C without CPA.

Magistrini and Szöllösi (1980) found that damage to the spindle of mouse oocytes caused by cooling to low temperatures may in fact be reversible. After step-wise warming, a significant number of the oocytes were found to contain normal spindles. Another study by Pickering et al. (1990) reported that 25 to 50% of human oocytes that contained dissociated spindles caused by chilling appeared to contain normal spindles after 30 min incubation.

In a study by Chen et al. (2000), mouse oocytes were examined for microtubule damage by fluorescent staining of the microtubules. Using this method they were able to decide which method of vitrification and warming worked best based on morphological changes of the meiotic spindle and chromosomes of the oocyte. They also observed whether cryoinjury is in fact reversible. After warming oocytes, longer incubation times prior to insemination or SCNT may increase cleavage and blastocyst rates. In yet another study by Chen et al. (2001), it was noted that oocytes contained severe microtubule damage immediately after warming. However, after 1, 2 and 3 h of culture, the percentage of normal oocytes increased significantly. Incubating oocytes that have been cooled to 0ºC or below for 1 to 3 h prior to fertilization may promote spindle and chromosomal recovery. Also, the CPAs are still inside the cells postwarming. Allowing longer post-warm culture periods may permit complete expulsion of these CPAs.

MATERIALS AND METHODS

Oocyte Collection

The following procedures used to maintain and treat the research animals in this study were reviewed and approved in advance by the Institutional Animal Care and Use Committee (IACUC) of the LSU AgCenter, according to regulations of the U.S. Department of Agriculture. Crossbred Spanish-type caprine does were selected for follicle stimulating hormone (FSH) treatment during the luteal phase, approximately 7 to 12 d post estrus as determined by visual observation of standing estrus (d 0) using teaser bucks. Does were subjected to FSH treatment 4 d prior to oocyte recovery and received 1.32 mg ovine FSH (Ovagen) by twice daily intramuscular (im) injections for 4 d. Oocyte recovery occurred at ~18 h after the final FSH injection.

Does were anesthetized (Halothane; Fort Dodge Animal Health, Fort Dodge, IA) and placed on an operating table in dorsal recumbency. Ovaries were exteriorized using a mid-ventral laparatomy and visible follicles were aspirated into a sterile 50 ml centrifuge tube by use of a 20-gauge needle attached with polyethylene tubing to a vacuum pump (Cook Veterinary Products, Eight Mile Plains, Australia). Oocytes were recovered from the centrifuge tube containing PBS supplemented with 1 % calf serum (Gibco Laboratories, Grand Island, NY), 100 IU/ml of penicillin G potassium and 10 units/ml of heparin. Good-quality oocytes with compact cumulus were washed in maturation medium (TCM-199 supplemented with 10% goat serum (Sigma Chemical Co., St. Louis, Missouri), 10 μg/ml of LH, 5 μg/ml of FSH and 1 μg/ml of estradiol-17β) and allowed to mature for 20 h (39°C and 5% CO_2) in humidified air, in groups of 10 to 20 in 50 μl microdrops of maturation medium overlaid with mineral oil (Sigma). Oocytes were denuded in the same manner as previously described in Chapters II and III and placed into culture until 20 h mature.

Vitrification and Warming Procedure

Vitrification and warming procedures were performed in the same way as described in Chapter II. Oocytes were prepared for vitrification by being exposed sequentially and very briefly to three CPA solutions. After exposure to the three CPA ($Me_2SO + EG$) solutions, oocytes were quickly loaded onto the cryoloop and placed directly into a LN_2-filled cryovial and the lid was screwed into the threaded bottom. Warming procedures were again the same as those described in Chapter II. After being warmed in the three solutions of trehalose, oocytes that had been vitrified were cultured at 38°C in a humidified atmosphere of 5% CO_2 in air for 1 h prior to fixation.

Oocyte Fixation

Once oocytes were warmed, they were completely denuded of their cumulus cells using 0.3% hyaluronidase in TCM-199. The oocytes were rinsed through three washes in TCM-199 medium alone. After being washed, oocytes were exposed to microtubule-stabilizing buffer containing 25 mM HEPES, 1 mM DTT, 25 μM PMSF, 5 mM MgCl, 10 mM EGTA, 1% Triton 100X and 2% paraformaldahide for 1 h. After 1 h, oocytes were briefly washed in PBS.

Immunocytochemical Staining

Oocytes were incubated in a 1:100 dilution of rat monoclonal anti yeast tubulin antibody (Sigma) in PBS with 0.5% glycine and 0.1% BSA for 1 h. After being incubated in primary antibody, oocytes were washed in PBS with 0.1% BSA. Tubulin staining was amplified by incubating the oocytes in fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Sigma) in PBS with 0.1% BSA at a dilution of 1:40 for 2 h. Oocytes were washed three times each for 15 min in solutions of PBS with 0.1% BSA to remove excess antibody and label.

Observation of Spindles

Fluorescence was observed using a Leica model sp1 laser scanning confocal microscope equipped with a 20x objective. Labeled cells were excited with a 488 nm line from an argon laser and scanned images were captured with an adjusted barrier filter of 520-560 nm. Images were recorded onto CD-R disks and contrast enhanced before being printed with Adobe Photoshop 4.0 (Adobe System, Mountain View, CA).

Assessment of Spindle Morphology

Spindles were classified as either normal, abnormal or absent. Normal spindles were barrel-shaped. Those spindles that were amorphous in shape and size and lacking a bipolar organization were considered to be abnormal.

RESULTS

Survival of Oocytes

The morphological survival of caprine oocytes following vitrification using the cryoloop method are summarized in Table 7. The control group consisted of one replicate and the vitrified group is a total of three replicates. Oocytes with both intact zonae and vitelline membranes were considered morphologically normal. Oocyte survival as determined by morphological criteria was 100% (26/26) for the vitrified oocyte treatment group. All oocytes in the control group were also judged to be morphologically normal (100%; 10/10).

Spindle Morphology

The percentage of normal barrel-shaped spindles was 100% (10/10) for the control group. The vitrified group had 85% (22/26) normal barrel-shaped spindles. Figure 5 illustrates a representative picture of a normal barrel-shaped spindle in the vitrified oocyte group. Abnormalities were found in only 12% (3/26) of the vitrified-warmed oocytes and one of the 26 vitrified oocytes was devoid of any spindle. Figure 6 shows one of the three disrupted spindles observed in the vitrified oocyte group.

Table 7. Morphological Observations of Vitrified-Warmed Caprine Oocytes

Treatment	Oocytes n	Morphological observations		
		Normal spindle (%)	Disrupted spindle (%)	Absent spindle (%)
Fresh[1]	10	10 (100)	0 (0)	0 (0)
Vitrified[2]	26	22 (84)	3 (12)	1 (4)

[1]Total of 1 replicate
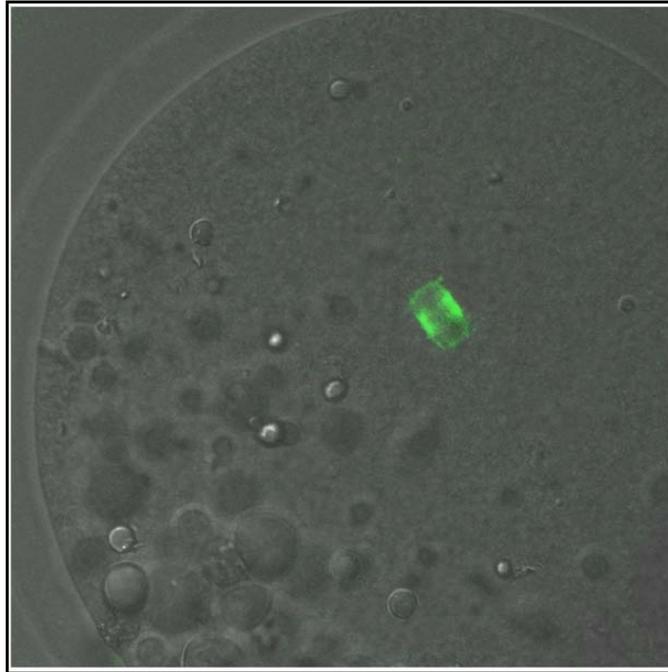[2]Total of 3 replicates

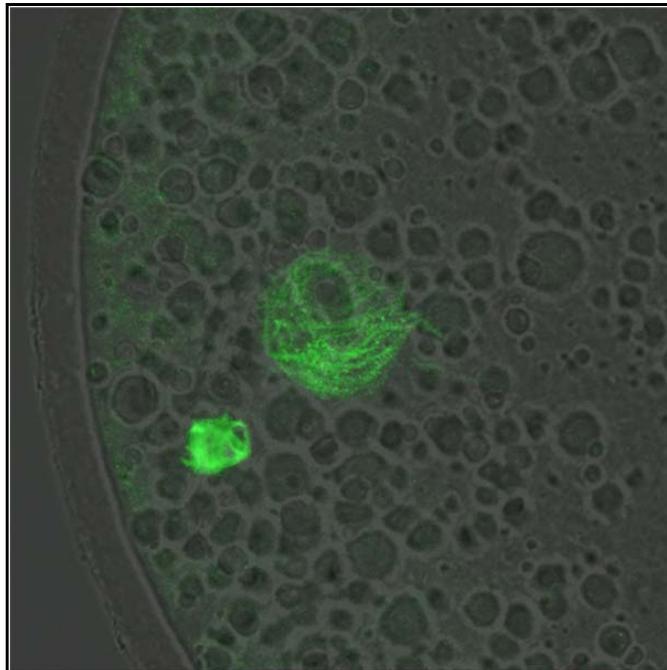Figure 5. Normal barrel-shaped spindle within a vitrified-warmed caprine oocyte

Figure 6. Abnormal-shaped spindle within a vitrified-warmed caprine oocyte

DISCUSSION

This study was conducted to determine the efficiency of a proven bovine oocyte vitrification protocol for use as a method to cryopreserve caprine oocytes. The objective was to test whether a protocol derived for bovine oocytes would be applicable for caprine oocytes, as judged by spindle morphology.

Most mammalian oocytes are ovulated at metaphase II of meiosis. The chromosomes at this time are aligned on the equatorial plate of the meiotic spindle. Normal, intact microtubules of the spindle are required for meiosis to be completed and for the chromatids to separate. Disruption of the spindle can cause disorder of the chromatids which increases the incidence of decreased fertilization rates or abnormal fetuses.

The immediate survival of the vitrified caprine oocytes was 100% and was also 100% for both treatment groups. Cryopreservation of caprine oocytes using the cryoloop method was reported by Begin et al. (2003). He and his colleagues reported that 92% of 73 vitrified oocytes were morphologically normal immediately following warming. After being incubated at 37°C, 89% of the 73 oocytes that had been vitrified were observed to be normal.

As noted above, cooling and exposure to CPAs can cause spindle and chromosomal abnormalities in oocytes (Pickering and Johnson, 1987; Johnson and Pickering, 1987). In those studies, mouse oocytes were cooled to 4°C for 60 min and then warmed to 37°C for 60 min. After the 60 min incubation period, 89% of the oocytes exhibited a normal spindle morphology compared to 0% of the oocytes that were examined without having been cultured. In contrast, human oocytes (Pickering et al., 1990; Zenzes et al., 2001) and bovine oocytes (Aman and Parks, 1994) are more sensitive to cooling than mouse oocytes, based on observation of their spindles.

63

In this study, I observed normal spindle morphology in the vitrified group (85%) similar to that of the mouse oocyte studies.

In conclusion, the results have met the original objectives for this experiment. The data show that the cryoloop method for vitrification previously used to vitrify bovine oocytes is an effective protocol for caprine oocytes as well. In addition, an effective and easily executable microtubule staining procedure was developed for caprine oocytes. More observations are needed to verify and improve the results. Experiments involving a negative control of oocytes cryopreserved using slow-cooling methods and double staining for chromosomal abnormalities could further develop this research. Furthermore, IVF and SCNT experiments using vitrified-warmed caprine oocytes would further refine this procedure for the goat.

SUMMARY

Three experiments were conducted to derive and test a method to cryopreserve bovine and caprine oocytes by vitrification. Vitrification and warming of oocytes were carried out in the same manner in all three experiments. Oocytes that had been in maturation media for at least 20 h were partially denuded and then exposed to ascending concentrations of an EG and Me_2SO mixture. Vitrification solutions were CPA-1: 0.7 M Me_2SO + 0.9 M EG; CPA-2: 1.4 M Me_2SO + 1.8 M EG; CPA-3: 2.8 M Me_2SO + 3.6 M EG + 0.65 M trehalose. Oocytes were exposed for 30 sec each to CPA-1 and CPA-2 and were then placed into CPA-3 for 20 sec. After exposure to CPA-3, oocytes were loaded onto a 20 μm cryoloop and plunged directly into LN_2. Oocytes were warmed by being placed directly into a Petri dish containing 0.25 M trehalose in TCM-199 supplemented with 10% FBS at 37°C. After being rinsed in solutions of 0.19 M and 0.125 M trehalose, oocytes were washed and cultured and were then subjected to IVF, SCNT or staining.

In Experiment I, bovine oocytes were vitrified and then subjected to IVF. Although cleavage rates for control and vitrified oocytes were not significantly different, vitrified oocytes had significantly different blastocyst development rates of 10% compared to 32% of the control oocytes. Even though fewer vitrified oocytes developed into blastocysts, these results are promising since they do show that oocytes can develop in vitro after vitrification and IVF. As hypothesized, these results also suggest that the nucleus of the oocyte may be damaged during vitrification and warming.

In Experiment II, bovine oocytes were vitrified using the cryopreservation method that had been derived in Experiment I. These oocytes were then enucleated for use in SCNT post-

warming. A 61.2% cleavage rate and a 12.5% blastocyst development rate resulted in the vitrified oocyte group compared with 60.4 and 33.0% in the control oocyte group respectively. Again, blastocyst development of vitrified oocytes was significantly different than control oocytes. However, three pregnancies and one live birth were established from SCNT embryos derived from vitrified oocytes. These experiments suggest that the cytoplasm of bovine oocytes may also be damaged during the vitrification procedure, as well as the nucleus of the oocyte.

Experiment III was a cytological analysis of vitrified caprine oocytes. These oocytes were evaluated for cell damage and then stained to assess post-warming microtubule damage. Although relatively few oocytes were examined, this experiment showed that caprine oocytes can be successfully vitrified and warmed using the same protocol derived in Experiment I and II for bovine oocytes. This study also defined a protocol for microtubule staining and observation of caprine oocytes that can also be used in other mammalian species. Further study is required using caprine oocytes and SCNT to verify the findings for bovine oocytes in Experiment II.

These three experiments demonstrate that both bovine and caprine oocytes can be successfully vitrified. Vitrified-warmed bovine oocytes also can result in pregnancies and live births when used either in SCNT experiments or for IVF. Additional research is needed using caprine oocytes for SCNT to confirm the bovine oocyte vitrification experiments. The protocol used in all three experiments could also be tested to assess it applicability for other large animal species to further confirm its efficiency.

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70

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APPENDIX

COMPOSITION OF TISSUE CULTURE MEDIUM - 199

Item	g/l
NaCl	8.000
KCl	0.400
$CaCl_2$	0.140
$MgSO_4 \cdot 7H_2O$	0.200
$NaHCO_3$	0.350
$NaHPO_4$	0.048
KH_2PO_4	0.060
Glucose	1.000
Ribose	0.0005
Deoxyribose	0.0005
Amino acids	Contains 21
Vitamins	Contains 16
Nucleic acids and precursors	Contains 8
Trace elements	Contains 1
Cholesterol	0.002
Na acetate	0.050
Tween 80	0.020
Glutathione	0.00005
Atocopherol PO_4 (Na)	0.00001
	(GIBCO, Grand Island, NY)

VITA

Sabrina Marie Luster was born to Richard and Gloria Luster on July 7, 1977, in Decatur, Illinois. She received her elementary education at Zion Lutheran School (1980-1991) and her secondary education from Mount Pulaski High School (1991-1995), both located in Mount Pulaski, Illinois.

She received her Bachelor of Science degree in animal science from the University of Illinois-Urbana in 1999. In October of 1999, she moved to Miami, Florida, to work as an andrologist for the South Florida Institute for reproductive medicine. In August of 2001, she entered graduate school at Louisiana State University and is currently working toward the Master of Science degree in Reproductive Physiology under Dr. Robert A. Godke in the Department of Animal Science.