VITRIFICATION OF BOVINE OOCYTES

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Vitrification of Bovine Oocytes

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ABSTRACT

Cryopreservation of oocytes is a challenge. Studies were conducted to vitrify mouse zygotes and cumulus-intact bovine oocytes from follicles of different diameters, small (≤4 mm) and medium (4 to 10 mm), using nylon mesh. The specific goals were to assess changes in apoptotic gene expression (Fas-FasL, Bax, Bcl-2, and survivin) in conjunction with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase assays. Mouse zygotes were exposed to increasing concentrations of ethylene glycol (EG), Ficoll-70 and sucrose in phosphate buffered saline (PBS) for vitrification on nylon mesh and plunged into liquid nitrogen. Warming resulted in 81.7% morphological survival. The rate of blastocyst formation was 59.9% for vitrified zygotes but, this was significantly lower than that of non vitrified embryos (66.2%). There was no difference in the hatching rates between groups. Both Fas and FasL mRNA were detected at the 4-cell and morula stages, suggesting Fas signaling was operational in early embryos. The level of expression of Bax mRNA tended to increase, while expression of survivin mRNA was not different for 2- and 4-cell embryos. Fragmented embryos showed an increase in Bax mRNA levels, while survivin mRNA level was reduced.

In the second experiment, vitrification of bovine oocytes was carried out. Pre-cooled cryovials resulted in 98.9% morphological survival. The oocytes from small and medium size follicles had a significant impact on cleavage (53.7 ± 1.6% vs. 43.8 ± 1.6%, respectively) and blastocyst rates were 16.9 ± 1.0% and 11 ± 1.2%, respectively. Follicle size for oocytes had no impact on the expression of apoptotic genes. The Fas-FasL and Bax-Bcl-2 mRNA showed increased expression after vitrification, but tended to decrease after 9 h of maturation. Yet,
results from TUNEL and caspase assays did not support the evidence of the downstream apoptotic signaling pathway in embryos. The semen utilized for in vitro fertilization in both vitrified and control oocytes responded differently in the 4 tested bulls than their field fertility record. The altered transcriptional activities of apoptotic genes, Fas-FasL and Bax-Bcl-2, and survivin were indicative of possible apoptotic activity in vitrified embryos and oocytes subjected to in vitro fertilization.
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CHAPTER ONE

REVIEW OF LITERATURE

1.1 Overview of Cryopreservation of Oocyte

Oocyte competence can be defined as the ability of an oocyte to be fertilized and develop into an embryo, establish a pregnancy, and produce healthy offspring (Anguita et al., 2007). The availability of viable, developmentally competent oocytes has been a major problem preventing progress in the development of in vitro fertilization (IVF), embryo culture and related reproductive technologies in many species. Additionally, the relatively short fertile life of mammalian oocytes like spermatozoa limits implementation of many in vitro methodologies. This limitation could be overcome by the availability of cryopreserved oocytes. However, the developmental potential of cryopreserved mammalian oocytes is typically highly compromised. Consequently, there is a growing interest in the possibility of obtaining blastocysts from cryopreserved oocytes for the application to new technologies and future commercial use in different species, so research on cryopreservation of oocytes has become a priority. Success in this area could have important practical implications both in cattle and in human assisted reproduction.

1.1.1 Applications of Oocyte Cryopreservation

Oocyte cryopreservation expands the potential of IVF. It is crucial in the human reproduction field due to legal and ethical issues associated with embryo cryopreservation. Cryopreservation is a useful treatment option for women who are at risk of losing their gonadal function because of pelvic diseases, surgery, or radio/chemotherapy. Better management of
livestock and laboratory animal species could be achieved by cryopreservation. Cryopreservation would improve the conservation of biodiversity including endangered species.

1.1.2 What makes the oocytes a difficult cell type to cryopreserve?

Fundamental knowledge of cryobiological characteristics of each cell type is needed to develop an effective cryopreservation protocol (Critser et al., 1997). As a single cell, the oocyte is likely more vulnerable to any environmental challenge than the multi-cellular preimplantation embryo, and each procedural step during cryopreservation contributes singly or jointly to the low developmental potential of frozen oocytes (Parks and Ruffing, 1992). Because oocytes are specialized cells that have zona pellucida, they have a reduced membrane permeability to both water and cryoprotective agents. The lower surface area to volume ratio of oocytes compared with other cells makes it more difficult for water and cryoprotectants to move across the cell plasma membrane (Leibo, 1981), which increases the probability of intracellular ice formation during freezing. The oocyte needs to maintain the integrity of several unique structural features to undergo fertilization and further development.

A problem with cryopreservation of mammalian oocytes lies at the organelle/sub cellular level. The organelles affected by low temperatures. The organelles affected include the plasma membrane, the zona pellucida, the cortical granules, the microtubular spindle, microfilaments, and condensed chromosomes. Oocytes are highly sensitive to cooling and can be injured even at room temperature. This problem is partly due to the delicacy of the spindle apparatus and to the high lipid content of the cells. The oocyte is unique in that the maternal DNA is held suspended in the cytoplasm on the meiotic spindle and not within the protective confines of the nuclear membrane. Damage to the DNA and/or microtubules could explain the limited success of oocyte cryopreservation. When bovine oocytes were chilled to room temperature their viability was
drastically reduced and the spindle apparatus anchoring the chromosomes disassembled (Saunders and Parks, 1999; Wu et al., 1999) and chromatid non-disjunctions possibly occurred during cooling, resulting in aneuploidy (Sathananthan et al., 1987) or polyploidy (Eroglu et al., 1998) after fertilization and termination of embryonic development.

The main biological alteration in the oocytes subsequent to vitrification as observed by Hyttel et al. (2000) was the lack of peripheral cortical granule distribution, typical of mature oocytes in vivo and to some degree in vitro. Instead, cortical granules were found in clusters and, moreover, the granules displayed varying degrees of degeneration, rendering the oocytes highly susceptible for polyspermic penetration. Cortical granules are the mediators for the polyspermy block occurring during fertilization.

Furthermore, it was observed that cryoprotectant (CPA) exposure may increase intracellular Ca++, and trigger premature exocytosis of cortical granular material leading to enzymatic modification of the zona pellucida and rendering it harder to bind spermatozoa (zona hardening; Larman et al., 2006), which significantly inhibited IVF. The premature release of cortical granules disrupted the timing of the polyspermy block and increased rates of polyspermy and subsequently polyploidy (Fuku et al., 1995).

Membranes are the primary site for structural and functional chilling injury in oocytes. Chilling injury results from thermotropic phase transitions in the membrane of the oocyte. As thermal energy is removed from the samples, molecular motion in the lipid bilayer decreases. This allows increased interaction between adjacent lipid molecules, resulting in a transition from the more fluid liquid crystal phase to a rigid gel phase (Arav et al., 1996).

Transmission electron microscopic study of vitrified bovine oocytes by Abe et al. (2005) showed that the cytoplasm of these oocytes contained many vacuoles and mitochondria with
ruptured membranes. This corresponded with low developmental rates. The shape and intracellular distributions of mitochondria are known to relate to the level of cell metabolism, proliferation, and differentiation for generation of the essential energy required in crucial periods of the cell cycle (Matsumoto et al., 1998).

The cumulus oocyte complex (COC) is normally cultured intact so that the physiological interactions between the oocyte and its somatic cell envelope are preserved (Downs et al., 2006). Although effects of cumulus cells on oocyte survival after freezing and thawing are controversial, beneficial effects were reported (Imoedemhe and Sigue, 1992; Im et al., 1997). According to Imoedemhe and Sigue (1992), the presence of the cumulus cells may offer some protection against osmotic changes and stresses induced by rapid influx and/or efflux of the cryoprotectant during the process of equilibration and removal of the cryoprotectant during post-thaw periods. However, cryopreservation of the intact cumulus oocyte complex is problematic because the optimal times for equilibration are likely different for the oocyte than its diminutive cumulus cells. Additionally, immersion in hypertonic CPA causes cell shrinkage, which may disrupt the granulosa cell processes and gap junction communication with the oocyte so that physiological interactions between the oocyte and its somatic cell envelope can not be preserved (Fuku et al., 1995; Gosden, 2005).

1.2 Slow Cooling vs. Vitrification

While the ability to freeze embryos has become a standard of practice in assisted reproductive technologies, cryopreservation of oocytes remains a challenge and has not been widely adapted despite significant research. The principles of cryopreservation are believed the same for all living cells; the most important consideration being the removal of most of the water from cells before they freeze intracellularly. The fundamental principles of cooling a particular
cell from a physiological state to subzero temperatures, re-warming, and finally achieving cellular viability/functionality have been established.

Traditionally, there have been two approaches to embryo/oocyte cryopreservation, slow and rapid cooling. The standard method used for the cryopreservation of mammalian oocytes and embryos is conventionally called “slow rate cooling (freezing)” or “equilibrium cooling” (Pegg, 2002), since the cooling rate is slow and it uses solutions containing low concentration of CPA. Slow cooling has been used in embryo cryopreservation since 1972 (Whittingham et al., 1972). Later, the feasibility of slow cooling of mammalian oocyte cryopreservation was first demonstrated in mice (Whittingham, 1977). Subsequently there were publications reporting success in the cryopreservation of oocytes and embryos from different species. The developmental potential of cryopreserved oocytes using traditional slow rate, equilibrium freezing is still low in many species.

As reviewed by Gordon (2003), Willadsen pioneered techniques by showing that slow freezing of embryos (0.3 °C/min) to low subzero temperatures (-80 °C) required slow thawing. Slow freezing of embryos to relatively high subzero temperatures (-25 to -35 °C), on the other hand, required rapid thawing (360 °C/min). These observations formed the basis of the cryopreservation techniques for embryos. Freezing includes the precipitation of water as ice, with the resulting separation of the water from the dissolved substances. Both ice crystal formation and the high concentration of dissolved substances pose problems. For human embryos the time taken to complete these freezing procedures ranges from 90 min to 5 h. Many studies have been undertaken to reduce the time of the freezing procedure and to try to eliminate the cost of expensive, programmable freezing equipment.
A round 70 yr ago Luyet (1937) reported he could achieve an ice free, structurally arrested state for cryopreservation of living cells and tissues. He indicated that cooling of living cells at ultra high speeds of freezing was possible by eliminating ice formation, due to creation of a glass like (vitreous) state. Hence the term vitrification refers to the solidification of a solution brought about not by crystallization, but by acute elevation in the viscosity of the medium during cooling. The solid called a glass, retains the normal molecular and ionic distributions of the liquid state and is described as an extremely viscous super cooled liquid (Fahy et al., 1984).

Successful ice free cryopreservation of murine embryos at -196°C by vitrification, as an alternative to the conventional freezing technique, was reported by Rall and Fahy (1985). They used a French mini straw (0.25 mL) and the procedure required about 35 min to allow a stepwise equilibration before embryos were plunged into liquid nitrogen (LN2). Although vitrification was reported in the mid 1980s, as an alternative to traditional slow rate freezing of cattle embryos, its application was generally confined to research studies.

The principle behind vitrification is based on the belief that chilling injury to oocytes is time-dependent, so freezing at a rate fast enough to solidify the intracellular water before it can crystallize will prevent injury (Martino et al., 1996a). The merit of vitrification is seen in terms of simplifying and speeding up the cryopreservation process, avoiding the need for a lengthy cooling period and expensive freezing equipment.

1.3 Variables in Vitrification

The effectiveness of cryopreservation involves a number of variables (Table 1-1): 1) exposure of the cells to varying concentration of cryoprotectants; 2) cooling rate of the cells to subzero temperature; 3) storage at the “glass” transition temperature of water below -130°C; 4)
thawing; and finally 5) dilution and removal of cryoprotectant and rehydration of the cells (Leibo, 1986, 2004; Lebio and Songsasen, 2002).

**Table 1-1. Variables of vitrification that can profoundly influence its effectiveness**

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Type and concentrations of cryoprotectant</td>
</tr>
<tr>
<td>2</td>
<td>Temperature of the vitrification solution at exposure</td>
</tr>
<tr>
<td>3</td>
<td>Length of the time cells/tissues are exposed to the final cryoprotectant before plunging into LN&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>4</td>
<td>Variability in the volume of cryoprotectant solution surrounding the cells/tissue</td>
</tr>
<tr>
<td>5</td>
<td>Devices used</td>
</tr>
<tr>
<td>6</td>
<td>Quality and developmental stage of the tested cells/tissue</td>
</tr>
<tr>
<td>7</td>
<td>Direct contact of the LN&lt;sub&gt;2&lt;/sub&gt; and vitrification solution containing the biological material can be a source of contamination; to eliminate this danger, using sterile LN&lt;sub&gt;2&lt;/sub&gt; for cooling and storage is essential</td>
</tr>
</tbody>
</table>

Adapted from Liebermann et al. (2002)

The cooling rate is one of the most important characteristics for the success of vitrification. If the rate of cooling is low, cytoplasmic water will flow out of the cell and freeze extracellularly and if the rate is too rapid, cytoplasm will not have sufficient time to dehydrate resulting in the super cooling and eventual freezing (Rall et al., 1984). In effect, low cooling rates may kill the cells by exposing them to concentrated solutions for long time periods and rapid cooling can kill cells by the formation of ice crystals. Mazur (1977) reported that cell survival was low at low cooling rates, and declined at high cooling rates as well. The optimal cooling rate was that which permitted the most water to move out of the cells and to vitrify extracellularly. Hence, the primary strategy of any vitrification protocol must be to pass rapidly through the critical temperature zone of 5 to -15°C to decrease chilling injuries (Liebermann et al., 2002).

In addition to cooling, thawing and re-warming processes after cryostorage have equally important roles for survival of the oocytes, because oocyte has to be rehydrated and the
cryoprotectant removed. The optimum warming rate for a cell is dependent on the optimum cooling rate that preceded it. Warming of vitrified oocytes is critical, as transient formation of ice crystals might occur during devitrification, if the speed of warming is not adequate (Rall, 1987). In most successful experiments, the method of warming of oocytes after they have been vitrified was direct and rapid. Libermann et al. (2002) reported a warming rate of 4,460°C/min when oocytes were directly plunging into warming solution at 37°C from -196°C. The rapid warming process allowed a very rapid dispersion of intracellular ice crystals, if any; the extracellular ice melted and permeated the cellular membrane in a liquid state to rehydrate the oocytes (Friedler et al., 1988). Sucrose has a beneficial influence during removal of cryoprotectant. As an osmotic buffer, sucrose restricts water permeation and prevents excessive swelling of the cells during warming (Niemann et al., 1982). Vajta et al. (1998) and Abe et al. (2005) employed a stepwise dilution to prevent excessive swelling and lysis of oocytes as the cryoprotectant leaves. The stepwise exposure starting from the low concentration of the cryoprotectant enhances intracellular vitrification, which in turn reduces the time needed for exposure to a high concentration of vitrification solution that could be toxic for oocytes. In support, Fuku et al. (1995) observed that the structural integrity of mitochondria as well as the zona pellucida was improved in vitrified oocytes when cryoprotectant was removed in stepwise dilution than a single step dilution. In contrast to stepwise vitrification, one step vitrification has the possibility of insufficient permeation of the cryoprotectant that may result in intracellular ice formation during cooling and warming (Chen et al., 2000).

1.4 **Cryoprotectants**

Cryoprotectants are divided into two types: membrane-permeating (e.g., glycerol, ethylene glycol (EG), dimethyl sulfoxide (DMSO), propanediol) and membrane non-permeating
(e.g., sucrose, glucose, Ficoll, proteins, lipoproteins). The membrane permeating cryoprotectants act through several mechanisms: 1) they decrease the freezing point of the solution and prevent oocyte damage from high electrolyte concentrations; 2) they interact with the membrane modifications occurring during cryopreservation process (from a relatively fluid state to a relatively rigid state); and 3) they prevent the exposure of oocytes to high concentration of both intra and extra cellular electrolytes by linking to the electrolytes and they may act as partial substitute to the water (Rall et al., 1984). For slow freezing procedures the concentrations of cryoprotectants are limited to 1 to 1.5 $M$, and hence the toxicity of the cryoprotectants is very low in contrast to vitrification where the concentration can be as high as 8 $M$ (Kasai, 2002). Low molecular weight glycerol and DMSO are permeating agents. In the past decade EG at a concentration of 1.5 to 1.8 $M$ has emerged as a useful cryoprotectant for cattle embryos.

The membrane non-permeating cryoprotectants exert their beneficial effects by increasing the concentration of solutes generating an osmotic gradient across the cell membrane. Adding sugars to the vitrification solution could enhance viscosity of the solution, whereby incubation of cells in this solution before vitrification helps withdraw more water from the cells and reduce exposure of cells to the toxic effects of the cryoprotectants (Orief et al., 2005). Polysaccharides like Ficoll could influence the viscosity of the vitrification solution and, reduce the toxicity of the cryoprotectant through lowered concentration to prevent cells from cryoinjury by reducing mechanical stress which occurs during cryopreservation (Dumoulin et al., 1994). The non-permeating cryoprotectant like bovine serum albumin (BSA) protects the cells in the immediate post-thaw phase by helping to stabilize the cell membranes.

The damaging effects of solutes can be lessened by using a mixture of two or more cryoprotectants or by stepwise equilibration (two or more steps) in solutions of intermediate
concentrations at room temperature or after cooling to refrigeration temperature. At present, the combined use of both types of cryoprotectants is the standard. It was reported that cryoprotectant mixtures have some advantages over solutions containing only one permeable cryoprotectant (Vajta et al., 1998).

Kasai (1996) described a typical low toxicity vitrification solution, EFS40, containing three cryoprotectants: 1) 40% EG-rapidly permeating, low toxicity agent; 2) Ficoll-70 at 18%, a macromolecule; and 3) sucrose-a non permeating hexose sugar. The cattle embryos were incubated in the EFS40 for 1 min before plunging into LN$_2$. A stepwise pre-equilibration procedure, in which the amount of penetrating cryoprotectant was gradually increased, was very effective for human oocytes (Kuleshova et al., 1999) and bovine oocytes (Vajta et al., 1998; Abe et al., 2005).

1.4.1 Ethylene Glycol as a Cryoprotectant

Ethylene glycol is currently the most commonly used permeating CPA for the vitrification of oocytes including human oocytes and embryos, because of its low molecular weight, high permeation ability, and low toxicity (Kuwayama et al., 2005). The molecular weight is 62.1, lower than that of glycerol (92.1), propylene glycol (76.1) and DMSO (78.1). Its low molecular mass permits fast influx and efflux into and out of embryos. Cocero et al. (2002) found that EG protected membrane and cytoplasmic structures of embryonic cells from cryoinjury better than glycerol and lack of success in the freeze-thawing of sheep embryos could be attributed to the lack of protection of inner cells. A cryoprotectant with less permeability (glycerol) had a protective action on the cytoplasmic membranes, whereas EG, which has more penetrating effect, protects all the membranes of intracellular structures, including lysosomes.
When there is lysosome destruction occurs in the freezing process, lytic enzymes are released with adverse effects on cell structures and breakdown of blastomeres (Isachenko et al., 1994a,b).

1.5 Stage of Oocyte Development

Many problems have been encountered with vitrification of oocytes at different meiotic stages of maturation. Oocytes at the metaphase-II (MII) stage have undergone both cytoplasmic and nuclear maturation, including extrusion of first polar body and alignment of chromosomes on the meiotic spindle. Like MII oocytes, MI-stage oocytes have a metaphase-I plate and a functional spindle. In contrast, chromatin of germinal vesicle (GV) oocytes is still in the diplotene phase of prophase-I without a meiotic spindle. At the MII stage, the DNA of the oocyte is condensed into chromosomes that are aligned along the equatorial region of the meiotic spindle and are susceptible to disruption. Parks and Ruffing (1992) reported that survival post-vitrification depended on the influence of stage of oocyte maturation from the GV stage to the MII stage. Hochi et al. (1998) investigated the effect of nuclear stages during in vitro maturation on the survival of vitrified-warmed oocytes and reported that in vitro embryonic development after vitrification of maturing oocytes was superior to matured and immature oocytes. The same authors (Hochi et al., 1997) observed more polyspermic fertilization after matured oocyte were vitrified (24 h maturation) in contrast to monospermic fertilization of maturing oocyte after 2-step freezing.

Nevertheless, attention has focused more widely on the cryopreservation of oocytes at the MII stage, but at this stage, the process induces chromosome scattering by microtubular disruption of the meiotic spindle (Aman and Parks, 1994). Furthermore, Luna et al. (2001) reported that oocytes vitrified before (0 h) or after 8 h of the onset of in vitro maturation (IVM) had a significantly higher incidence of diploid cells compared to those vitrified 12 or 22 h after
the onset of maturation. They reasoned that bovine oocytes vitrified 12 h after the onset of maturation had the formation of the first metaphase plate and avoided the retention of the first polar body providing evidence of reduced incidence of diploid oocytes.

Since no microtubular spindle (Wu et al., 1999) is present, GV oocyte is assumed to be less prone to microtubular and chromosomal damage. However, progress in the cryopreservation of GV bovine oocytes has been limited. One of the main disadvantages of immature oocyte cryopreservation is that in vitro maturation is required post-thaw. Freezing of immature oocytes at the GV stage was tried by Suzuki et al. (1996) and Küchenmeister and Kuwayama (1997) but with low survival results (42% cleavage rate with less than 5% blastocyst rates). Though in vitro embryonic development is still low, pregnancies (Otoi et al., 1995; Yang et al., 1998) and births (Vieira et al., 2002; Abe et al., 2005) were reported. Poor embryonic development was explained by some due to irreversible structural damage to the oocyte membrane (Arav et al., 1996) or impaired intercellular communication between the oocyte and the cumulus cells consequent to cryopreservation (Fuku et al., 1995). In an interesting study Barnes et al. (1997) reported evidence suggesting that oocytes cooled at the GV breakdown stage cleaved and developed to the blastocyst stage more so than oocytes cooled at the GV or MII stage.

1.6 Cryodevices

A variety of new techniques and types of holders/devices have been tested with the aim of improving the overall survival of oocytes/embryos post-cryopreservation. Several open-carrier systems and closed carrier methods for vitrification of oocytes were developed in the last 2 decades. Until recently, 0.25 mL standard insemination straws were used almost exclusively for vitrification of oocytes and embryo with a relative cooling speed of 2,500°C/min (Palasz and Mapletoft, 1996) by direct plunging into LN₂. Methods using cryodevices such as microscope
grids (Martino et al., 1996b), open pulled straws (OPS; Vajta et al., 1998), cryoloops (Lane et al., 1999) and cryotops (Kuwayama and Kato, 2000) allow oocyte vitrification in a volume as little as few microliters. Open pulled straws rendered a high cooling-warming rate of more than 20,000 °C/min. Dropping oocytes containing vitrification solution directly into LN2 (Papis et al., 1999) successfully eliminated the insulation effects of the container wall. Yet, plunging a warm object into LN2 resulted in boiling the liquid and creating an isolating layer of nitrogen vapor around the object. A modification by Dinnyes et al. (2000) used a pre-cooled metal surface, solid surface vitrification (SSV), which provided an efficient method of heat transfer in conjunction with container-less vitrification in microdrops. Another approach developed by Arav (1992) was the ‘minimum drop size (MDS)’ method, in which nanoliter (0.1 to 0.5 μL) volumes of vitrification solutions were cooled and warmed, and successfully used for bovine oocytes (Arav and Zeron, 1997). A vitrification device was developed by the same group, Arav et al. (2000), the VitMaster, which used LN2 slush by reducing the temperature of LN2 to -205 to -208 °C. These methods use very high cooling rates coupled with minimal volume of freezing medium containing high concentration of cryoprotectants that permits rapid traversal of temperature zones, corresponding to chilling injury. Most authors of these papers attributed improved developmental rates of oocytes to the increased rates of temperature changes both at cooling and warming. Nevertheless, most of these approaches have introduced a problem related to storage and danger of contamination due to direct contact of LN2. Therefore, the critical requirement is to combine a high cooling rate with a safe procedure.

1.7 Developmental Potential

Variations associated with success of cryopreservation of bovine oocytes include the source of oocytes, stage of maturation, degree of cumulus cell investment, osmotic stress, and
chilling sensitivity (Massip, 2003). The main sources of bovine oocytes for in vitro studies are from abattoir ovaries and then from ovum pick up procedures. Loss of developmental potential after cryopreservation makes mammalian oocytes probably one of the most difficult cell types to cryopreserve. Indeed, the survival and developmental capacity of the cryopreserved oocytes are greatly impaired, probably as a consequence of morphological and cytological damage induced by the cryopreservation process. According to Men et al. (2003) both slow cooling and vitrification in 0.25 mL straws could result in significant morphological damage to oocytes compared to vitrification by the OPS method. DNA may be damaged by cryopreservation whatever the method used.

Factors most likely to affect the success of bovine oocyte cryopreservation are the particular structural and functional characteristics of the oocyte, such as its size (Liebermann et al., 2002), COC, maturation status (Hochi et al., 1998; Gal and Massip, 1999; Men et al., 2002), and dynamics of subcellular organelles during meiosis (Shamsuddin et al., 1996). The maturation stage has a significant influence on the developmental competence of oocytes after cryopreservation. The ability of oocytes to complete all stages of development and to support freezing was related to follicle size (Lonergan et al., 1994) and consequently to the size of the oocyte (Fair et al., 1995). A relationship emerged between follicular size and development potential of oocytes in cattle (Pavlok et al., 1992). It was well recognized that oocytes from follicles with a diameter smaller than 2 to 3 mm develop in vitro to the blastocyst stage at much lower rates than do those from follicles greater than 4 or 5 mm (Donnison and Pfeffer, 2004).

1.8 Oocyte/Embryos Damage, an Affect of Vitrification

Exposure to hypertonic or hypotonic conditions has a significant effect on the developmental competence of bovine COC at both the GV and MII stages. Oocytes at the GV
stage are more sensitive to hypertonic or hypotonic stress than MII oocytes (Agca et al., 2000). Oocytes are exposed to a number of unnatural stressors during cryopreservation, i.e., changes in temperature, volume, and solute concentrations. Mazur and Schneider (1986) reported that the shrinkage and or swelling during cryoprotectant addition and removal, respectively, can cause damage and or even death of the cell.

But the factors which determine the extent of injury include size and shape of the cells, permeability of the membranes, quality, and sensitivity of the oocytes (Vajta, and Kuwayama, 2006). Nevertheless, oocytes and embryos have the ability to repair this damage in many species. Unfortunately, in some species, such as bovine and human, meiotic spindle cannot completely reform after warming due the lack of pericentriolar materials (Vincent and Johnson, 1992). Chen et al. (2001) recommended that sufficient culture time for recovery of the meiotic spindle would be imperative for fertilization events of vitrified oocytes. They evaluated morphological survival, dynamics of meiotic spindles, and fertilization in vitro of vitrified mouse oocytes using closed pulled straws (CPS), conventional straws, OPS and copper grids. Surviving oocytes were stained for spindles and chromosomes after 1, 2, and 3 h incubations. At 1 h incubation vitrified oocytes of four groups had significantly fewer normal spindles, while after a 3 h incubation; recovery of vitrified oocytes with normal spindles was significantly improved in all groups. Accordingly, the percentage of fertilization and blastocyst formation of vitrified oocytes after 1 h incubation was lower than that after 2 or 3 h incubations.

DNA fragmentation without alteration of the oocyte morphology as a direct consequence of cryopreservation was observed by Men et al. (2002). The same authors and others (Schellander et al., 1994) observed that cryopreservation causes bovine oocytes to degenerate during subsequent culture. They showed that apoptosis was the reason for degeneration.
Apoptosis was observed in oocytes after vitrification by others as well (Fuku et al., 1995; Shaw et al., 2000). Systems associated with cellular injury and deaths are found in Table 1-2.

**Table 1-2.** Factors associated with cooling and cryopreservation that contribute to cellular injury and death in biological systems

<table>
<thead>
<tr>
<th>Cellular System</th>
<th>Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Entire cell</td>
<td>Intracellular ice formation, extracellular ice formation, apoptosis,</td>
</tr>
<tr>
<td></td>
<td>toxicity, calcium imbalance, free radical, general metabolism,</td>
</tr>
<tr>
<td></td>
<td>fertilization failure, cleavage failure</td>
</tr>
<tr>
<td>Membrane</td>
<td>Rupture, leakage, fusion, microvilli, phase transition</td>
</tr>
<tr>
<td>Chromosomes</td>
<td>Loss/gain, polyspermy, polygyny</td>
</tr>
<tr>
<td>DNA</td>
<td>Apoptosis, fusion</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>Microtubules dissolve</td>
</tr>
<tr>
<td>Proteins/enzymes</td>
<td>Dehydration, loss of function</td>
</tr>
<tr>
<td>Ultrastructure</td>
<td>Microvilli, mitochondria, vesicles, cortical granules, zona pellucida</td>
</tr>
<tr>
<td>Zona pellucida</td>
<td>Hardening, fracture</td>
</tr>
</tbody>
</table>

Adapted from Shaw et al. (2000)

### 1.8.1 Overview of Apoptosis

"Programmed" as opposed to "accidental" death of cells is an important mechanism in both development and homeostasis in adult tissues for the removal of either excess, infected, transformed or damaged cells by activation of an intrinsic suicide program (Kerr et al., 1972). One form of Programmed Cell Death (PCD) is apoptosis, which is characterized by maintenance of intact cell membranes during the suicide process so as to allow adjacent cells to engulf the dying cell so that it does not release its contents and trigger a local inflammatory reaction.

In vertebrates, apoptosis can be induced by a number of distinct death inducing stimuli, including the lack of extracellular survival factors, steroid hormones, activation of membrane bound death receptors, viral infection, heat shock, oxidative stress, excitotoxicity, ionizing radiation and various other cellular insults. In the ovary, apoptosis of oocytes or other cells occurred during both antral follicular atresia and luteal regression (Yuan and Giudice, 1997). These normal cyclic processes were attributed to decreasing gonadotrophic hormone...
concentration, but the molecular basis of apoptosis initiation in these tissues is not understood fully. Clearly oocytes from atretic follicles are more likely to exhibit apoptosis in vitro (Rubio-Pomar et al., 2004). In a recent study Ghafari et al. (2007) reported that the possible reasons for the prenatal and neonatal loss of oocytes was apoptosis. But, the role and control of PCD in oocytes are still unknown. Nonetheless, Bosco et al. (2005) opined that ovarian cellular death was an essential process for the maintenance of the homeostasis of the ovarian function, both in humans and in other mammalian species, since it guarantees the selection of a dominant follicle and the elimination of excessive follicles. This process ensured the development of few, but healthy embryos. Tilly (2001) showed that oocyte survival was controlled by a number of cell death regulatory molecules responsible for either activation or suppression of cell death. Cell survival molecules when inadequately accumulated during oogenesis (either too few suppressors or too many activators), or changed during the ageing of oocytes in vitro (Metcalf et al., 2003) or in vivo (Jurisicova and Acton, 2004) may lead to survival disequilibrium, resulting in zygote or early embryo loss. Levels of apoptosis are environmentally regulated (Spanos et al., 2000). Various pathological stimuli, such as radiation, chemotherapy, and environmental toxicants, can initiate oocyte apoptosis (Perez et al., 1997; Hu et al., 2001; Matikainen et al., 2001). Apoptosis involved actions of group II caspases in disruption of oocyte function caused by heat shock during maturation (Roth and Hansen, 2004). Cryopreservation caused a significant increase in the rate of degeneration in oocytes and embryos derived from cryopreserved oocytes during culture (Lim et al., 1992; Gal and Massip, 1999).

1.8.2 Gene Regulation of Apoptosis

Apoptosis requires internal machinery of the cell. Most cells have programs for apoptosis and the protein components of the programs are already expressed and are in association with
inhibitors. Blockage of inhibitor synthesis induces apoptosis. Once the cell receives inducing signals, it expresses genes either promoting or inhibiting the cell death machinery (Vinatier et al., 1996). In other words, external factors regulate the expression of these regulator genes. Genes, normally silent or negatively regulated, are activated by inductive stimuli leading to the production of proteins that mediate or act directly in the death process.

1.8.3 Genes and Signaling

According to Donnison and Pfeffer (2004), molecular changes that underlie the increase in oocyte competence depend on follicular size and involve mRNA and protein synthesis, degradation, and modification driving development from meiotic maturation and fertilization to the time point of embryonic genome activation at the 8- to 16-cell stage. Altered function of a frame work of genes and signaling pathways determine whether or not ovarian germ cells and somatic cells will die in response to either developmental cues or pathological insults. Extracellular stimuli, such as cytokines (Fas ligand or FasL), survival factors, stress, and genotoxicants utilize a number of signal transduction molecules, including sphingolipids (ceramide, sphingosine-1-phosphate or S1P) and protein kinases (phosphatidylinositol 3’ kinase), to relay information to a central programmed cell death rheostat governed by Bcl-2 family members (Figure 1-1). Several studies suggested a possible role of Fas and FasL in ovarian apoptosis. The Fas antigen belongs to a conserved family of membrane receptors known as the tumor necrosis factor receptor or (TNFR) family. Fas ligand exists as a soluble and cell associated molecule that engages the Fas antigen and initiates programmed cell death (Suda et al., 1993) or Fas can be activated by some anti Fas antibodies. Fas and FasL are expressed in the ovary, and human, mouse, rat, and bovine ovarian cells are sensitive to FasL induced apoptosis under certain conditions in vitro (Kim et al., 1998; Vickers et al., 2000). Dharma et al. (2003)
demonstrated that denuded mice oocytes from atretic, but not normal follicles showed localization of Fas and expression of Fas protein as well as the Fas gene. In the bovine ovary, Fas and FasL expression was reported in theca and granulosa cells (Vickers et al., 2000; Porter et al., 2001) and the levels of mRNA and proteins were higher in atretic follicles than in healthy dominant follicles (Porter et al., 2001). Fas mRNA and protein were expressed in bovine cumulus cells and oocytes. Fas ligand mRNA was found both in cumulus cells and oocytes, but its protein was found only in cumulus cells (Rubio-Pomar et al., 2004).

**Figure 1-1.** Schematic model of the PCD machinery in cells.

In the case of FasL initiated death, two distinct intracellular pathways are believed to exist, one that can utilize mitochondria and one that can proceed directly to the caspase cascade.

Caspases are a family of evolutionarily conserved cysteine proteases that constitutes the effector arm of the apoptotic machinery. Each caspase is synthesized as an inactive precursor which is activated only after a cell has received a death signal. Upon receipt of a death stimulus, caspase zymogens are processed to generate a large subunit and a small subunit, two heterodimers which...
compose the active enzyme. The primary role of caspase-1 and its closest relatives (caspase-4, -5, and -11) is the processing of proinflammatory cytokines. Caspases involved in apoptosis were grouped into two classes. Class I caspases (caspase-2, -8, -9, and -10), known as upstream or initiator caspases, contain long amino terminal prodomains that possess protein-protein interaction domains required for their association with specific death adaptor molecules. This association was required for proximity induced activation of caspases. Class II caspases (caspase-3, -6, and -7), known as downstream or effector caspases, lack long prodomains, and require proteolytic activation by class I caspases and target cellular proteins for proteolytic cleavage (Linda et al., 1999). Caspase-8 initiates apoptosis by death receptor-ligand interactions, whereas caspase-9 initiates apoptosis in response to a variety of signals including hypoxia, UV light, and certain drugs. Apoptosis is induced in Fas bearing cells when FasL binds to Fas leading to the activation of the Fas associated death domain. The death domain, in turn, forms a docking site for the autocatalytic activation of procaspase-8 (Chinnaiyan et al., 1995; Ashkenazi and Dixit, 1999). Once activated, caspase-8 can stimulate downstream proteases such as caspase-3. These proteolytic effectors of cell death cleave key cellular substrates, thereby disorganizing the cellular assembly and executing apoptosis (Thornberry et al., 1997). In other words, activated upstream caspases activate downstream caspase, which cleave proteins important for cell survival and integrity, and endonucleases which cleave DNA (Nagata, 2000). It was proposed that the inclination to apoptosis is continuously counterbalanced in the cell by genes stimulating cell survival and proliferation. Upon induction by an appropriate trigger, the cell activates or stops the repression of gene products responsible for control of the suicidal mechanism (Ellis et al., 1991). The Bcl-2 family, which is involved in the regulation of caspase activity, is subdivided into proapoptotic (e.g., Bax and Bad) and antiapoptotic members (e.g., Bcl-2), which
share one or more similar regions (Adams and Cory, 2001). The cell’s decision to carry out apoptotic responses is thought to depend on the ratio between pro- and anti-apoptotic proteins (Wyllie, 1995; Exley et al., 1999).

Bax, a pro-apoptotic member of the Bcl-2 family of apoptotic regulators causes apoptosis by inducing permeabilization of mitochondrial membranes and opening of mitochondrial porin channels (Shimizu et al., 1999). Supporting the crucial role of this protein family in the regulation of apoptosis in the ovary, bax-deficient mice have abnormal follicles with an excessive number of granulosa cells (Perez et al., 1999). Based on a number of reports (Perez et al., 1999; Matikainen et al., 2002), it was found that Bax was required for the initiation of oocyte death under diverse conditions like heat stress and chemical agents. When Bax is overexpressed in cells, apoptotic death is accelerated. Bcl-2 protein prevented apoptosis induced by a variety of stimuli and maintained cell survival by influencing the release of cytochrome c from mitochondria rather than by altering proliferation (Yang and Rajamahendran, 2002). Hence, cell survival is dependent on the expression of ratio of Bax to Bcl-2 proteins.

1.8.4 Caspase (cysteine-dependant aspartate specific proteases)

Apoptosis is typically accompanied by the activation of a class of death proteases (caspases) and widespread biochemical and morphological changes to the cell (Nicholson et al., 1995). These changes invariably involve extensive double stranded DNA fragmentation (Wyllie, 1980), in addition to other morphological changes to cells like shrinkage and blebbing. Whatever the apoptotic stimuli are, a set of caspases seems to be activated to cleave cellular substrates to kill the cells (Samali et al., 1999).
Caspases are not indiscriminant proteases; however, they search for a specific sequence in their target proteins, and typically cleaving solely next to aspartate amino acids. Caspases were identified as effectors of the apoptotic process (Nicholson et al., 1995) and DNA fragmentation was placed downstream of caspase activity (Janicke et al., 1998).

Caspase expression was studied in different species (Jurisicova et al., 1998; Spanos et al., 2000; Hinck et al., 2001; Roth and Hansen, 2004). Expression of mRNA for caspases was reported throughout mouse preimplantation development (Exley et al., 1999). Spanos et al. (2000) reported caspase activity appears only after compaction, at morula and blastocyst stages both in fresh and frozen thawed human embryos. They further observed that active caspase positive cells were correlated with nuclear fragmentation. In contrast, Martinez et al. (2002) reported that caspases in preimplantation human embryos were involved in developmental processes after fertilization and unrelated to embryo morphology and cell death.

Exley et al. (1999) showed that both normal and apoptotic preimplantation embryos express various members of the caspase families of genes and proteins. In an embryo toxicity study using high glucose, Hinck et al. (2001) demonstrated that caspase-3 was expressed in rat blastocysts in all the cells and that their activity was important for chromatin degradation, but not for nuclear fragmentation. Transcripts for caspase-2 and -3 were detected at all cleavage states during preimplantation development. Though the levels of caspase-2 mRNA were not altered during fragmentation, increased accumulation of caspase-3 mRNA was detected in human fragmented embryos (Jurisicova et al., 2003). In a heat shock study using bovine embryos, Paula-Lopes and Hansen (2002) reported that group II caspase mediated heat induced apoptosis. They further suggested that inhibition of these caspases had a detrimental effect on embryonic resistance to heat shock corroborating that apoptosis was an adaptive mechanism to allow
embryonic survival and further development following stress. Men et al. (2003) reported that in cryopreserved oocytes both oocytes having apoptotic morphology and those without any apparent apoptotic morphology showed the activation of caspase signals. Further, they observed the presence of increased caspase activity in degenerating embryos derived from cryopreserved oocytes.

1.8.5 DNA Fragmentation

Apoptosis is accompanied by the degradation of chromosomal DNA in most cases. One of the distinct features that differentiate apoptosis from necrosis is the pattern of DNA fragmentation. Nuclear DNA, but not mitochondrial DNA in apoptotic cells undergoes a unique, step-wise pattern of fragmentation over time (Wyllie, 1980; Sun and Cohen, 1994). During the first step, fragmentation starts as early as 5 min after the onset of apoptosis with the help of Mg\(^{++}\) ions. Later chromosomal DNA is cut into 600 to 750 kb fragments and then into smaller 50 to 300 kb fragments (Brown et al., 1993; Oberhammer et al., 1993; Cohen et al., 1994; Sun and Cohen, 1994). During the second step further degradation continues over 2 to 24 h to much smaller fragments to form an oligonucleosomal ladder of \(~ 200\) bp increments that requires both Ca\(^{++}\) and Mg\(^{++}\) ions (Arends et al., 1990; Sun and Cohen, 1994).

DNA fragmentation, a hallmark of the biochemical changes seen in apoptotic cell death can result because of cryopreservation protocols like slow cooling. Men et al. (2003) reported the evidence of different degrees of DNA damage in vitrification of oocytes using mini-straws and OPS. Generation of DNA fragments of \(~ 180\) to \(200\) bp, a characteristic of apoptosis, was a result of caspase-activated endonucleases (Wyllie, 1997). The damage can be to DNA or to the chromatin structure. Defects in genetic materials of oocytes resulting from cryopreservation were
one of the leading causes for the compromised developmental competence of the cryopreserved oocytes (Aman and Parks, 1994; Wu et al., 1999).

1.8.5.1 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

In preimplantation embryos it is impossible to use electrophoretic techniques to look for DNA laddering that is typical of apoptotic nuclei due to the small number of cells in the embryos. Nevertheless, the development of terminal deoxynucleotidyl-transferase (TdT) mediated deoxy uridine tri phosphate (dUTP) nick end labeling (TUNEL; Gavrieli et al., 1992) enabled assessment of nuclear DNA fragmentation in situ. The TUNEL reaction is the most frequently used method to detect apoptotic cells, by labeling DNA fragmentation generated by endogenous DNase activity during the apoptotic process. In the TUNEL procedure, free 3’ OH ends of DNA are labeled with fluorescein isothiocyanate-conjugated dUTP by means of the enzyme TdT. This technique can be used effectively on specimens with few numbers of cells. Additionally, Gavrieli et al. (1992) reported that TUNEL had the advantages of showing both localization and quantification of the percentage of nuclei with DNA fragmentation.

Use of the TUNEL assay to detect DNA fragmentation revealed the presence of apoptotic cells in preimplantation embryos in different species (Brison and Schultz, 1997; Byrne et al., 1999; Hardy, 1999; Hao et al., 2003; Walters et al., 2005). Spontaneous apoptosis was observed in normal embryos at the 8- to 16-cell stage, a time coincident with embryonic genome activation in the bovine (Byrne at al., 1999). Hardy (1999) reported that a majority of human blastocysts have one or more dying cells in both the inner cell mass and the trophectoderm. In support, others (Munne et al., 1994; Ruangvutilert et al., 2000) found that 40 to 60% human embryos consist of a mixture of healthy and abnormal cells. Hardy et al. (1999) showed the evidence of
correlation of cell death to embryo quality and the total dead cell index ranged from <10 to 27% in d 6 blastocyst of good morphology to those of poor morphology. They showed that blastocysts with fewer cells had a range of TUNEL positive cells from 0 to 30%, whereas the ones with more cells had less than 10% TUNEL positive cells. Paula-Lopes and Hansen (2002) reported that heat shock induces apoptosis only in a fraction of cells (15 to 29%), apoptosis may ensure that damaged cells are removed from the affected embryos to facilitate further development. Jurisicova et al. (1996) suggested that PCD was triggered in human embryos at a stage prior to blastocyst formation and further opined that embryo fragmentation was a result of activated PCD in some blastomeres. Fugino et al. (1996) showed that the rate of DNA fragmentation, interpreted as apoptotic changes, was significantly higher for oocytes from aged mice than that from mature and young mice. Accordingly, DNA fragmentation of oocytes might be one of the reasons for low oocyte quality and lower fertility in aged group. A higher percentage of TUNEL-positive oocytes was noted at the end of maturation at 40 and 41°C than for those at 38.5°C (Roth and Hansen, 2004).

In a study to investigate the relationship between cytoplasmic and DNA fragmentation, Hao et al. (2003) showed that cytoplasmic fragmentation was observed in 37.7% of the embryos on d 3 of in vitro culture. Although they found fragmented embryos before d 5 of in vitro culture, no apoptotic signal was detected. Earliest positive TUNEL signals were detected in embryos at d 5 of culture.

1.9 Sire Effect on Fertilization and In Vitro Development of Vitrified Oocytes

Male fertility can be defined as the ability of sperm to fertilize oocytes and of the resulting zygotes continuing through embryonic and fetal development until birth. The standard
procedure for evaluating the fertility status of young bulls entering the artificial insemination (AI) program is to record pregnancy data of a certain number of females following AI with approved frozen-thawed semen. Though this procedure is reliable and accurate; the disadvantage is it is time consuming and expensive. Hence, there is great interest in finding an in vitro method for evaluating semen which can provide valuable information about the likely performance of a particular bull when used in AI. This enables identification of sires with potentially low fertility to be removed from the breeding program. Zhang et al. (1997, 1998) found significantly low correlations between in vitro assessed characteristics including the concentration of motile spermatozoa after swim-up separation, in vitro ability to fertilize, and the field fertility of the same batches of frozen bull semen. It is well documented that bulls used in AI may differ in their fertility by as much as 10% or more and similar evidence was produced by those working in cattle IVF (Gordon, 2003).

The male gamete influences fertility both by affecting fertilization rates, and imparting characteristics to the embryo that influence its ability to proceed through embryonic development. Lowered sperm quality resulted not only in the reduction of embryo quality, but in the failure of implantation and the induction of maternal recognition of pregnancy (Thundathil et al., 1999; Saacke et al., 2000). Yet, the functional integrity of both the sperm and the oocytes are required for successful fertilization and embryo development. In most species IVF has become a valuable tool for assessing sperm functionality and for studying the success or failure of the gamete interaction. In vitro fertilization was used to evaluate bull fertility (Gordon and Liu, 1990). In cattle it was proved that the donor of the semen largely influences the outcome of both IVF and in vitro culture (Eyestone and First 1989; Shamsuddin and Larson, 1993). The bulls of high in vivo fertility sired embryos that cleaved to the 2-cell stage earlier and had a higher
chance of developing to the morula/blastocyst stage in culture than those sired by lower fertility bulls. This suggested a relationship between in vivo bull fertility and in vitro sire effects on embryonic development (Eid et al., 1994). The same authors further reported that the time of first oocyte cleavage varied between different bulls and that the kinetics of early cleavage could be used to differentiate between bulls of high and low fertility; part of the explanation was due to a delay in sperm penetrating the oocyte.

Though there is difference in fertility among fertile bulls (DeJarnette et al., 1992), the causes of these differences remain unclear (Saacke et al., 1988, 1994). Additionally, individual bulls differ in their ability to fertilize oocytes following IVF procedures (Hillery et al., 1990; Shi et al., 1990) and even after intracytoplasmic sperm injection (Wei and Fukui, 2000). In support, Byme et al. (2000) concluded that IVF had the potential to assess the fertilizing capacity of frozen thawed semen. The bull can have a major effect on the outcome of IVF (Hillery et al., 1990). Correlations between the non-return rate and cleavage or blastocyst rate (IVF outcome) to the fertility of bull semen were reported (Hillery et al., 1990; Marquant Le Guienne et al., 1992; Shamsuddin and Larson 1993). Zhang et al. (1997) reported a higher correlation between field fertility and cleavage rate than field fertility and blastocyst rate. Janny and Menezo (1994) reported a strong paternal influence on human preimplantation embryo development in in vitro studies.
1.10 OBJECTIVES

We utilized a modified method of vitrification using nylon mesh as described (Abe et al., 2005) for our studies. Our goal was to cryopreserve large numbers of oocytes within a short period of time, demonstrate ease of manipulation during cryopreservation and recovery, determine beneficial effects of cumulus cells on oocyte survival, and eliminate potential hazard of contamination associated with this method. Hence, the overall objective was to test a technique to vitrify large numbers of cumulus-intact bovine oocytes after 15 h of maturation. Success of vitrification was determined by subjecting vitrified-warmed oocytes to IVF and comparing fertilization and subsequent embryo development to control fresh oocytes. Specific objectives were to: 1) determine if the mechanism of cell death in vitrified oocytes involved the Fas signaling pathway (through measurement of the abundance of Fas and FasL mRNA); 2) compare cryopreserved oocytes isolated from small (≤4 mm) and medium (4 to 10 mm) follicles; 3) determine if vitrification affected differences in relative mRNA abundance between the two groups of vitrified oocytes for genes related to apoptosis, Bax and Bcl-2; and 4) determine if vitrification induced changes in caspase activity and DNA fragmentation parallel patterns of apoptosis. Another objective was to determine whether a relationship exists between post IVF outcome and field fertility using estimated relative conception rate (ERCR) based on 70 d non-return rate after AI using semen selected from bulls of known fertility, based on the laboratory assessment of IVF fertility (to fertilize cryopreserved oocytes vs. fresh oocytes of varying populations and to pursue subsequent embryonic development in vitro).
1.11 LITERATURE CITED


and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J. 12:3679-3684.


EFFECT OF VITRIFICATION OF ZYGOTES IN NYLON MESH ON THE
DIFFERENTIAL EXPRESSION OF APOPTOTIC GENES

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2.1 ABSTRACT

Vitrification offers a rapid and simple alternative to cryopreservation compared with slow cooling methods. The objective was to test a technique to vitrify large numbers of cumulus-intact mouse zygotes using nylon mesh. Vitrified mouse embryos were assayed for the expression of apoptosis related genes at various stages of pre-implantation development. For vitrification, zygotes were transferred to a cryoprotectant solution A, composed of 10% (v/v) ethylene glycol (EG), 4.5% (w/v) Ficoll-70 and 0.075 M sucrose in modified Dulbecco’s Phosphate Buffered Saline (PBS) for 7 min followed by solution B consisting of 20% (v/v) EG, 9.0% (w/v) Ficoll-70 and 0.15 M sucrose in PBS for 2 min, and finally in solution C, composed of 40% (v/v) EG, 18% (w/v) Ficoll-70 and 0.3 M sucrose in PBS for 1 min. After equilibration, 30 to 40 cumulus enclosed zygotes were loaded onto the nylon mesh, immediately transferred to 2 mL cryovials, and directly plunged into liquid nitrogen within 40 to 60 sec. Post warming of vitrified zygotes resulted in 81.7% morphological survival. There were high rates of blastocyst development (59.9%), but the development was lower than controls (66.2%; $P < 0.05$). There was no difference in the hatching rates between groups. Both Fas and FasL mRNA were detected at the 4-cell and morula stages of development, suggesting that Fas-FasL system might be operational in the early embryos. The level of expression of Bax mRNA tended to increase, while expression of survivin mRNA was not different for 2- and 4-cell embryos. Fragmented embryos showed an increase in Bax mRNA levels, while survivin mRNA level was reduced. Our data demonstrated that vitrification of cumulus-intact zygotes using nylon mesh resulted in successful survival and development to the blastocyst stage similar to previous literatures.
2.2 INTRODUCTION

The mouse embryo was the first mammalian embryo to be frozen successfully (Whittingham et al., 1972) and the first successful vitrification of mammalian embryos was reported (Rall and Fahy, 1985) in the mouse. Since this initial effort, embryos of many mammalian and a few non-mammalian species were successfully cryopreserved (Leibo, 1986). Cryopreservation of mouse embryos is a cost-effective approach for the maintenance of scientifically important stocks, strains, and lines (Glenister et al., 1999). The use of cryopreserved mouse zygotes would provide a more efficient approach to the production of transgenic mice using pronuclear injection by reducing or eliminating the need for on-site maintenance of animals serving as embryo donors and thereby, reduce the costs associated with animal housing (Leibo et al., 1991; Tada et al., 1995). Furthermore, mouse embryo banking is an important technical supportive tool for genome preservation purposes. The efficiency of embryo banking for mouse models of human disease and normal biological processes depends on the ease of obtaining embryos.

Nevertheless, the developmental stage at which cryopreservation is performed varies among different species. This is determined by the practicality of recovery techniques, culture methods, and by embryo survival rates after cryopreservation (Pfaff et al., 1988). Mouse embryos can be readily recovered from the oviductal ampulla as zygotes, but are routinely cryopreserved at the 8-cell stage because of higher survival rates (Takeda et al., 1987). Although cryopreservation of zygotes is usually more difficult than later embryonic stages, these embryos can be successfully cryopreserved either by conventional slow freezing or vitrification methods (Leibo et al., 1991; Tada et al., 1995; Takahashi et al., 1999). But, the time consuming and laborious process of slow cooling has made vitrification an attractive alternative for freezing
embryos. New methods based on the immersion of very small amounts of solutions into liquid nitrogen were developed to increase speed (>10,000°C/min) of freezing and warming (Vajta et al., 1998; Dinnyes et al., 2000; Lane and Gardner 2001). A simple and inexpensive vitrification method utilizing nylon mesh was described recently to vitrify large numbers of bovine germinal vesicle oocytes with high rates of survival (Abe et al., 2005). However, the effectiveness of nylon mesh vitrification methods for cryopreservation of mouse embryos has not been tested. The pore size of the nylon mesh is ~60 μm, making it questionable for use as a support structure for cryopreservation of mouse embryos because the diameter of mouse zygotes is about the same size.

Regardless of the method of freezing employed, the freezing and thawing processes decrease embryo viability, an effect attributed to physical and chemical damage induced during cryopreservation (Overstrom, 1996; Bagusi et al., 2000). This can trigger a cell stress response that can activate the apoptotic cascade or lead to necrosis (Sonna et al., 2002). During development of mouse embryos, different anti- and pro-apoptotic genes are expressed (Exley et al., 1999; Hinck et al., 2001). Apoptosis, also known as programmed cell death, is a coordinated event dependent upon the actions and interactions of a number of gene products that either suppress or activate the process of cellular self-destruction (Adams and Cory, 1998). Transcript analysis of apoptosis related and/or stress related genes are useful for examining the quality variation of embryos (Gutierrex-Adan et al., 2004). Yet, there is lack of information about vitrification induced changes in apoptotic genes during early development in mouse embryos.

The fate of the cell is determined by the ability of the cell death suppressors to sequester and neutralize the actions of the cell death inducers. Hence, the relative concentration of pro- and anti-apoptotic genes determines whether a cell lives or dies (Antonsson and Martinou, 2000). Fas
ligand (CD95L), a cell surface molecule belonging to the tumor necrosis factor family, binds to its receptor Fas (CD95), inducing apoptosis of Fas-bearing cells. Bax, a proapoptotic mRNA transcript, appears consistently expressed throughout preimplantation development, with only a slight increase in accumulation of the protein in low quality embryos (Spanos et al., 2002). The pro-survival Bcl-2 proteins are localized to intracellular membranes, including mitochondria, endoplasmic reticulum and nuclear envelope. Over expression of Bcl-2 prevents cells from undergoing apoptosis in response to a variety of stimuli. This is brought about by the prevention of an efflux of cytochrome c, which is necessary for the initiation of apoptotic program from the mitochondria and the initiation of apoptosis (Yang et al., 1997). An increased transcription of apoptosis related genes, Fas and Survivin, reduces the developmental capacity of frozen thawed bovine blastocysts (Park et al., 2006). Survivin is a mammalian protein that acts as an inhibitor of apoptosis proteins.

The overall objective of this study was to evaluate a procedure to cryopreserve large numbers of mouse pronuclear stage embryos successfully for later use. Specific objectives were: 1) to complete step-wise vitrification of large numbers of cumulus-intact mouse zygotes using nylon mesh and compare their developmental capacity compared to fresh (control) embryos; and 2) determine relationship between stage-dependent development and the differences in the expression of mRNA for Fas, FasL, Bax, Bcl-2, and survivin.

2.3 METHODS

2.3.1 Embryo Collection

Female ICR mice were superovulated by an intraperitoneal injection of 5 IU eCG (Sigma Chemical Co., St. Louis, MO, USA) followed 48 h later by 5 IU hCG (Sigma). Females were
placed with males immediately after hCG administration, and presumptive zygotes were collected from the oviducts approximately 16 h after hCG injection. Animal usage was approved by the Institutional Animal Care and Use Committee (05-0169). Cumulus enclosed zygotes were released into M2 medium (Sigma). Approximately 2/3 of embryos selected for cryopreservation were washed 3 times in M2. The remaining 1/3 were released into M2 medium containing 80 IU/mL hyaluronidase to remove the cumulus cells. The denuded embryos were washed 3 times with M2 medium and 3 times with K+ modified simplex optimized medium (KSOM) (Specialty Media, Lavellette, NJ, USA) before they were transferred into a 10 µL drop of KSOM under mineral oil.

2.3.2 Vitrification Protocol

Vitrification of cumulus-enclosed zygotes was conducted using a nylon mesh holder (pore size 60 µm; Sefar America Inc., USA) as described (Matsumoto et al., 2001). Cumulus-enclosed zygotes were used to reduce loss through the nylon mesh. A stepwise vitrification procedure (Figure 2-1) was adopted using an ethylene glycol-Ficoll-sucrose vitrification solution (Abe et al., 2005). The set up for the vitrification protocol is shown in Figures (2-2 and 2-3). In this method, the cumulus enclosed zygotes were exposed for 7 min to 100 µL droplets of solution A, composed of 10% (v/v) ethylene glycol (EG), 4.5% (w/v) Ficoll-70 (F70) and 0.075 M sucrose (S) in modified Dulbecco’s Phosphate Buffered Saline (PBS). Next, zygotes were exposed for 2 min to 100 µL droplets of solution B, consisting of 20% (v/v) EG, 9.0% (w/v) F70 and 0.15 M S in PBS, and finally they were immersed for 1 min in 100 µL droplets of solution C, composed of 40% (v/v) EG, 18% (w/v) F70 and 0.3 M S (EFS40) in PBS in 35 mm culture dishes. After equilibration, 30 to 40 cumulus enclosed zygotes at room temperature were loaded
onto the nylon mesh holder, which was placed on a single fold towel (Wausau paper, 22.9 cm x 25 cm, Harrodsburg Plant, Harrodsburg, KY) to remove excessive solution, immediately transferred to 2 mL cryovials using a forceps and directly plunged into liquid N<sub>2</sub> (LN<sub>2</sub>) within 40 to 60 sec. Embryos were stored for 2 h in LN<sub>2</sub>. After storage, the embryos were ultra rapidly thawed, by maintaining in air for 5 sec and then cryoprotectants were removed in a stepwise manner at 37°C. The nylon mesh was transferred from LN<sub>2</sub> into 2.7 mL of warm PBS (37°C) with a sequential series of 0.5, 0.25 and 0.125 M S dilutions by placing in each solution for 1, 2, and 3 min, respectively, and finally transferring into PBS for 3 min in culture dishes. After warming, the nylon mesh was transferred into M2 medium containing 80 IU/mL hyaluronidase to remove the cumulus cells. The denuded zygotes were washed 3 times with M2 medium and 3 times with KSOM medium before they were transferred into a 10 µL drop of KSOM under mineral oil.

**Figure 2-1.** Three-step vitrification and thawing procedure adopted for mouse embryo cryopreservation using nylon mesh. CPA-1 is EG-10%, Ficoll-4.5%, sucrose-0.075 M, CPA-2 is EG-20%, Ficoll-9%, sucrose-0.15 M, and CPA-3 is EG-40%, Ficoll-18%, sucrose-0.3 M. Thaw-1 is sucrose 0.5 M, Thaw-2 is sucrose 0.25 M, and Thaw-3 is sucrose 0.125 M.
2.3.3 Assessment of Embryo Survival

Viability of warmed embryos was determined based on visual examination of the integrity of embryo membrane, zona pellucida and the normality of the cytoplasm immediately
after warming. During in vitro culture, embryo development was evaluated every 24 h, starting on d 2 (48 h post-hCG). The numbers of 2- to 8-cells, morulae, early, expanded and hatched blastocysts stages were recorded.

2.3.4 Expression of Cell Death Regulatory Genes during Preimplantation Development

Pools of pronuclear stage embryos which are not exposed to vitrification protocol, embryos that succumbed to the vitrification process, and 2-cell to blastocyst stage embryos from both control and vitrified groups were analyzed for quantitative expression of Fas, FasL, Bax, Bcl-2 and survivn genes associated with apoptosis. The patterns of expression of the above genes were determined by reverse transcription polymerase chain reaction (RT-PCR).

2.3.5 Isolation of RNA

Embryos (n = 435) were collected, washed 3 times in PBS and then transferred to a 0.5 mL tube in as small a volume as possible. Thirty zygotes, 15 normally developed embryos of each stage (2-cell, 4-cell, 8-cell, morula, and blastocyst) and 40 fragmented embryos after freeze thaw were put into separate tubes. The embryos were snap frozen in LN₂, and stored at -80°C before analysis. Total RNA was isolated from embryos using Absolutely RNA Microprep Isolation Kit (Strategene, La Jolla, CA, USA) according to the manufacturer’s instruction. Briefly, embryos were resuspended in 100 μL of the Lysis Buffer on ice and vortexed or pipetted repeatedly to facilitate the lysis of the embryos and release of RNA. An equal volume of 70% ethanol was used to precipitate and recover total nucleic acid after mixing thoroughly.
2.3.6 *Reverse Transcription-Polymerase Chain Reaction*

RNA was immediately converted to cDNA as described below. The reverse transcriptase reaction was performed with random primers using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA). A 20 μL reverse transcription reaction was prepared by mixing 2 μL of 10 x RT Buffer, 0.8 μL of 25 x dNTPs, 2 μL of 10 x Random Primers, 1 μL of Multiscribe Reverse Transcriptase (50 U/μL), and 14.2 μL of RNA sample at 25°C for 10 min, followed by 37°C for 120 min. Each reverse transcription reaction contained 40 ng RNA.

Real-time PCR of the above cDNA was carried out in an ABI Prism 7300 sequence detector using Power SYBR Green (Applied Biosystems) as the detector, essentially following the manufacturer’s instructions. All primer sequences (Table 2-1) were designed using the Primer Express 3.0 software (Applied Biosystems). The thermal cycling profile used was as follows: holding for 10 min at 95°C followed by 40 cycles of PCR for 15 sec at 95°C and final extension at 60°C for 60 sec. Dissociation curve analysis was performed for each gene to ensure the specificity of PCR products. Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Each analysis was performed on groups of 15 to 40 embryos and results are representative of 3 replicates on separate pools of embryos.
Table 2-1. Primers used for real time PCR

<table>
<thead>
<tr>
<th>Genes</th>
<th>Gene Bank ID</th>
<th>Tm (°C)</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>NM_007393.1</td>
<td>59</td>
<td>5’CCAGTTCCATGGATGAC3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’GCTGTTGAGGGCCGTA3’</td>
</tr>
<tr>
<td>Fas</td>
<td>NM_007987.1</td>
<td>59</td>
<td>5’CCGAAAGGCCTGGAAATCA3’</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>5’ACTTTTACTCTCTGTTACAGACC T3’</td>
</tr>
<tr>
<td>FasL</td>
<td>NM_010177.2</td>
<td>59</td>
<td>5’AAGAAGGACCACACAAATCTGT3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ACCAAGACCACGAGACC AA3’</td>
</tr>
<tr>
<td>Bax</td>
<td>NM_007527</td>
<td>59</td>
<td>5’GGCCTTTTTGCTACAGGGTTT3’</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>5’TCTACCCGCCCTCTG G3’</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>NM_009741</td>
<td>59</td>
<td>5’AAGGGCTTACACCCAAATCT3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’AGTTTCGGTCGTCTGCATCTT3’</td>
</tr>
<tr>
<td>Survivin</td>
<td>NM_009689</td>
<td>58</td>
<td>5’GGAGGCTGCTTCATCCA3’</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5’ACTAAACGAGTGATC AAAAAA3’</td>
</tr>
</tbody>
</table>

2.3.7 Statistical Analysis

The zygote survival subsequent to post vitrification-thawing, number of morphologically damaged embryos, fragmented embryos during culture, and the developmental rates of embryos at different stages based on the cleaved embryos subjected to culture were determined. Development score and percent of blastocyst that hatched were analyzed using mixed model (PROC MIXED in SAS 9.1). The proportion of cultured embryos that fragmented, the proportion that progressed to 2-cells, the proportion of 2-cell embryos that progressed to 4-cell, 8-cell, morula, and blastocyst, and the proportion of blastocysts that hatched for the two treatments were tested by Chi Square. The relative abundance of selected mRNA was subjected to one-way ANOVA using the general linear model (PROC-GLM in SAS 9.1). The significance was assigned at $P < 0.05$.

2.4 RESULTS

The morphological survival of vitrified pronuclear stage embryos is shown in Table 2-2. Recovery rate of zygotes after thawing was 93.1%. Immediately after thawing, 81.7% of the
zygotes survived the process of vitrification and warming based on morphological examination. A degeneration rate of 18.3% was observed subsequent to the thawing process. Degenerated zygotes disintegrated into a poorly defined mass of debris; none of them cleaved during in vitro culture. The embryos that survived the process of thawing and returned to culture showed significantly ($P < 0.05$) higher rate of fragmentation (10%) than that in the control (4%). The rate of development of zygotes to the blastocyst stage in the non-vitrified group was significantly ($P < 0.05$) higher than the corresponding rates in the vitrified group (Table 2-3).

### Table 2-2. Mean (± SE) recovery, morphological survival rate, and fragmentation of mouse zygotes after vitrification and thawing

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Embryos collected</th>
<th>Recovery from vitrification medium</th>
<th>Recovery from mesh</th>
<th>Post thaw Survival</th>
<th>Fragmentation in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>N</td>
<td>%</td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>Nylon Mesh</td>
<td>1469</td>
<td>1381</td>
<td>94.0</td>
<td>1286</td>
<td>93.1</td>
</tr>
<tr>
<td>Control</td>
<td>733</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a-b Values with different superscripts within column are significantly different at ($P < 0.05$).

### Table 2-3. Post vitrification development (mean ± SE) of mouse zygotes using nylon mesh after 5 d of culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Embryo cultured</th>
<th>1-cell</th>
<th>2-cell*</th>
<th>4-cell</th>
<th>8-cell</th>
<th>Morula</th>
<th>Blastocyst</th>
<th>Hatched blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Nylon mesh</td>
<td>1051</td>
<td>135</td>
<td>12.8 a</td>
<td>811</td>
<td>77.2 a</td>
<td>642</td>
<td>79.2 a</td>
<td>585</td>
</tr>
<tr>
<td>Control</td>
<td>773</td>
<td>82</td>
<td>10.6 b</td>
<td>662</td>
<td>85.6 b</td>
<td>593</td>
<td>89.6 b</td>
<td>561</td>
</tr>
</tbody>
</table>

a-b Values with different superscripts within each column are significantly different at ($P < 0.05$).

*Further cleavage from 4-cell stage to blastocyst stage is based on the number of 2-cells cleaved; hatched blastocyst rates are based on the total blastocysts.
2.4.1 Differential Expression of Apoptotic Genes in Vitrified Embryos

One purpose of the experiment was to quantify the differences in the expression of gene transcripts involved in apoptosis at different stages of development of vitrified embryos. Fas and FasL were inconsistently expressed among the tested samples, while Bax and survivin were most consistently expressed (Figure 2-4). In contrast, Bcl-2 mRNA could not be detected in any sample. The efficiency of Bcl-2 primers was tested using samples positive for Bcl-2 expression and it was expressed. Fas ligand mRNA was expressed in 4-cell, morula and blastocyst stages, whereas Fas mRNA was detected in both 4-cell and morula stage embryos. The expression of Fas and FasL in the 4-cell embryos was 2.3- and 3.9-fold relative to control, respectively. A 1.1-fold change in Fas and FasL mRNA was detected in the morulae. A 2.2-fold increase in the FasL alone was found in the blastocysts.

The relative abundance of Bax mRNA increased \((P < 0.05)\) in the vitrified embryos compared to the untreated control. There was a significant 3.9 \((P < 0.05)\) and 2.8 \((P < 0.05)\) fold increase in Bax mRNA levels in the 2-cell and 4-cell embryos relative to control embryos, respectively. Bax mRNA levels did not change significantly \((P > 0.05)\) in the morula and blastocyst stages compared to untreated controls. The relative abundance of survivin did not differ significantly \((P > 0.05)\) among the different stages of embryonic development in the vitrified group compared with controls; transcript levels varied slightly among the different stages of embryos.
Figure 2-4. Differential expression of apoptotic genes at various pre-implantation stages of development of vitrified mouse zygotes. Data are shown as mean ± SEM. For each gene means with different superscripts differ at $P < 0.05$.

2.4.2 Expression of Apoptotic Genes in Embryos Undergoing Fragmentation

Transcript analysis of fragmented embryos was carried out to test whether any change in apoptosis related genes occurred in embryos subjected to environmental changes consequent to vitrification and thawing. The gene expression of the embryos that had undergone fragmentation after vitrification and thawing was compared to the untreated zygotes. As expected, there was a robust increase in Bax mRNA levels in the embryos after vitrification and thawing; a typical 6.6-fold increase was found. Our results indicated that vitrification and thawing resulted in 95% decrease in the survivin mRNA level.
2.5 DISCUSSION

The results demonstrate that nylon mesh vitrification can result in high developmental rates for mouse zygotes. A simple and inexpensive technique for vitrification and post-thaw recovery of large numbers of cumulus-intact pronuclear stage embryos was developed using nylon mesh. This is the first report of vitrification of cumulus-intact mouse zygotes. The results for cleavage (77.2%) and blastocyst (59.9%) development of vitrified zygotes in this study were similar to those of other investigators (Keskintepe et al., 2001; Bagis et al., 2004) using different systems. Conversely, the results obtained with vitrified germinal vesicle stage cumulus oocyte complexes (Aono et al., 2003, 2005) were much lower than the present report, possibly because the presence of the meiotic spindle in oocytes is usually thought to make them more susceptible to damage compared with that of zygotes (Moisan, 2006).

Furthermore, potential hazards of contamination can be completely avoided by this method since there is no direct contact between the biological sample and LN₂ since the plastic wall of the cryovial represents an isolating layer between the LN₂ and the nylon mesh containing the embryos. Recently Kuwayama et al. (2005) described a similar beneficial effect with Cryo Tip method for vitrification of human embryos.

Successful cryopreservation involves an adequate approach to minimize toxic, osmotic, and other injuries during cryopreservation. Vitrification is a non-equilibrium cryopreservation method that needs relatively high concentration of cryoprotectants. Stepwise additions of cryoprotectants are beneficial for alleviating the toxic or major cell volume expansion effects exerted by cryoprotectants at high concentration (Rall, 1987). Our high survival rates using murine zygotes and limited stepwise additions of cryoprotectants mirror the high survival rates
of bovine blastocysts after vitrification using a 16-step method with permeable cryoprotectants (Kuwayama et al., 1994).

The large mounting area of nylon mesh used simplified handling for mounting and recovery of embryos and was effective for the prevention of cumulus-intact zygote damage (Matsumoto et al., 2001). Supplementation of saccharides such as Ficoll and sucrose in the vitrification medium could reduce the toxicity to the embryos by reducing the extra-cellular concentration of the cryoprotectant (Kasai et al., 1990).

The present study demonstrated the co-expression of Fas and FasL genes in the 4-cell and morula stages. The simultaneous expression of Fas and FasL indicate that Fas-FasL signaling pathway of apoptosis may be operational in early embryos. A similar co-expression result for Fas and FasL mRNA in 4-cell embryos was reported (Kawamura et al., 2001; Kelkar et al., 2003) for mouse and human embryos, respectively. We demonstrated the expression of two major regulatory apoptotic genes, Bax and survivin, through all developmental stages. None of the samples tested showed the presence of Bcl-2 gene expression. A similar failure of detection of Bcl-2 gene expression in viable human embryos was reported by Liu et al. (2000), suggesting the failure of detection of Bcl-2 may be due to the lack of sensitivity of the detection method or mRNA for Bcl-2 might have undergone degradation, thereby becoming virtually undetectable. We observed that transcription of the Bax gene was higher in the 2- and 4-cell embryos, while the levels were lower in the later stages of development. Yet, the relative abundance of survivin was not altered in 2- and 4-cell stages, but the values were higher in the later stages of development. It is possible that proapoptotic activity of Bax could be negated by increased levels of survivin after the 4-cell stage as suggested by Exley et al. (1999) for Bax and Bcl-2. To our
knowledge this is the first report of stage dependant developmental alterations in the expression of apoptotic genes following vitrification.

Based on the expression observed in Bax and survivin in embryos that survived cryopreservation and thawing, we analyzed transcript accumulation in the embryos that had undergone fragmentation after thawing. Expression of large amounts of Bax mRNA, but barely detectable amounts of survivin mRNA in these classes of embryos together with their total failure to progress in culture suggests that the balance of expression of these two genes may play a role in the survivability of pre-implantation embryos. In a similar study Jurisicova et al. (1998) proposed that the balance of expression of cell death promoter and protector genes might regulate cell death, and that embryo fragmentation might be attributable to a failure to achieve a balance of expression that favors embryo survival. The fate of the cell is determined by the ability of the cell death suppressors to sequester and hence neutralize the actions of the cell death inducers. Hence, the relative concentration of pro- and anti-apoptotic genes determines whether a cell lives or dies (Antonsson and Martinou, 2000).

In conclusion, nylon mesh provides an efficient and functional method for vitrification of large numbers of cumulus-intact murine zygotes. We provide strong evidence that fragmented embryos consequent to vitrification degenerate through an apoptotic cell death mechanism after warming and there is increase in the relative abundance of apoptosis related genes in the vitrified pre-implantation embryos. The nylon mesh vitrification technique is promising and may be efficiently used for cryopreservation of large number of pronuclear stage embryos.
2.6 LITERATURE CITED


CHAPTER THREE

NYLON MESH VITRIFICATION OF BOVINE OOCYTES

3.1 ABSTRACT

The study was conducted to vitrify cumulus-intact bovine oocytes at first meiotic stage (MI) from follicles of different diameters, small (≤4 mm) and medium (4 to 10 mm) using nylon mesh. Oocytes were exposed to the cryoprotectant composed of 40% (v/v) ethylene glycol, 18% (w/v) Ficoll-70, and 0.3 M sucrose (EFS40) in three stepwise dilutions. Thawing was conducted with a series of 0.5, 0.25 and 0.125 M sucrose dilutions in 20% fetal bovine serum (FBS). Thawing resulted in 98.9% morphological survival with intact cumulus cells in both populations of oocytes. There was a significant difference ($P < 0.05$) in the embryonic developmental rates between vitrified and control oocytes. The cleavage rates were significantly lower ($P < 0.05$) for the vitrified oocytes (39.1% vs. 58.5%). The rates of embryo development to the morula (13.1% vs. 35.1%) and blastocyst (5.1% vs. 22.9%) stages were also significantly lower ($P < 0.05$) in vitrified oocytes. Follicle size had a significant ($P < 0.05$) impact on the developmental rates of embryos from cleavage to blastocyst stage. The oocytes from medium sized follicles had a 9.9% increase in the cleavage rate compared to oocytes from small follicles. The increase in the blastocyst rate was only 5.9% between the oocytes from medium and small-sized follicles. The objective of a second experiment was to determine whether a relationship exists between post in vitro fertilization (IVF) outcome and field fertility based on 70 d non-return rate after artificial insemination (AI) with the frozen thawed semen of 4 AI dairy bulls based on estimated relative conception rate (ERCR). The sires showed significant ($P < 0.05$) effects on embryonic developmental rates. Similarly, sire by follicle size interaction was significant for blastocyst rates.
and sire by treatment interaction was significant on cleavage and blastocyst rates. There were deviations in the results obtained among bulls in the in vivo and in vitro studies. Fertilized oocytes using semen from Bulls I, II, III, and IV showed cleavage rates of 49.3, 55.0, 50.0, and 40.9%, respectively ($P < 0.05$). Both morula and blastocyst stages of development were significantly different ($P < 0.05$) among the 4 tested bulls. While the developmental rates to morula were 26.4, 29.9, 23.4 and 16.7% for Bull I, II, III, and IV, respectively, and the blastocyst developmental rates in the above bulls were 18.1, 17.9, 10.0, and 10.0%, respectively. Bull I had the highest blastocyst development for vitrified oocytes (10.9%; $P < 0.05$) and the corresponding rate for control oocytes was 25.3%. The blastocyst rates for the vitrified oocytes were 6.0, 2.5, and 1.0% respectively, for Bull II, III and IV, the corresponding rates in the control oocytes were 29.9, 17.4, and 18.9% respectively, for the above bulls. Our results show that differences in development exist in source of oocytes and sire used for IVF after vitrification. No correlation exists between post-IVF outcome and field fertility based on ERCR.

3.2 INTRODUCTION

Cryopreservation of oocytes is difficult in most animal species because of their large volume, high sensitivity to cooling, low surface area to volume ratio, high water content and low hydraulic conductivity (Leibo, 1980). Various protocols for cryopreservation involved slow cooling, rapid cooling, and ultra rapid cooling. Equilibrium freezing is not satisfactory, because oocytes are damaged by exposure for several minutes to temperatures near 0°C and thus, the time consuming and laborious process of slow cooling has made vitrification an attractive alternative for freezing of oocytes and embryos. Progress in the development of protocols for cryopreservation indicated that vitrification at the ultra rapid cooling rate is a promising approach for oocyte cryopreservation (Men et al., 2002). Therefore, cryopreservation of oocytes
by vitrification, especially with the use of very high cooling rates for oocytes suspended in extremely small volumes of various additives seems the most appropriate method (Vajta et al., 1998). Several open-carrier systems and closed carrier methods for vitrification of oocytes were developed in the last 2 decades. Yet, the most widely emphasized concerns of the vitrification procedures are toxicity effects due to high concentration of cryoprotectants and the danger of contamination due to direct contact with liquid nitrogen (Kuwayama, 2007).

Problems currently associated with cryopreservation of bovine oocytes are source of oocytes, stage of maturation, cumulus cell investment, osmotic stress, and chilling sensitivity (Massip, 2003). The main source of bovine oocytes is from abattoir ovaries and the abattoir material is very heterogeneous, since oocytes are punctured from follicles of different sizes. Follicle diameter was widely used as a selection criteria and a relationship between follicle diameter and oocyte developmental competence was established in cattle (Furher et al., 1989). The ability of oocytes to complete all stages of development and to support freezing was related to follicle size (Lonergan et al., 1994a) and consequently to the size of the oocyte (Fair et al., 1995).

Many problems have been encountered with vitrification of oocytes at different meiotic stages of maturation. The degree of oocyte maturation, from the germinal vesicle stage to the MII stage, affects survival post-vitrification (Parks and Ruffing, 1992). A significant increase in the diploid cells after maturation was reported (Luna et al., 2001) when oocytes were vitrified before (0 h) or after 8 h of the onset of in vitro maturation compared to those vitrified 12 or 22 h after the onset of maturation.

With the objectives to cryopreserve large number of oocytes within a short period of time, provide ease of manipulation during cryopreservation and recovery, determine possible
beneficial effects of cumulus cells on oocyte survival, and eliminate potential hazards of contamination, we undertook assessment of a modified method of vitrification using nylon mesh as described (Matsumoto et al., 2001). Hence, the overall objectives were to test a technique to vitrify large number of cumulus-intact bovine oocytes after 15 h of maturation. The success of vitrification was determined by subjecting vitrified-warmed oocytes to in vitro fertilization (IVF) and comparing fertilization and subsequent embryo development to those of fresh oocytes.

In vitro fertilization can be used to evaluate bull fertility (Gordon and Liu, 1990). Correlations of cleavage and embryo production rates (IVF outcome) to the fertility of bull semen have been reported (Marquant-Le Guienne, 1990; Shamsuddin and Larson, 1993; Lonergan et al., 1994b). The estimated relative conception rate (ERCR) is a measure of the fertility of an individual sire and is predictable and repeatable over the productive life of an artificial insemination (AI) sire, if sufficient data have been collected (Clay and McDaniel, 2001). The ERCR predicted fertility value is a 70-d non return rate of an AI service relative to the service sires of herd mates. A high ERCR value for a sire has economic benefit to a dairy manager because of a probable conception rate advantage at AI (Pecsok et al., 1994). However, a high deviation in the ERCR values and the repeatability from time to time was reported (Cornwell et al., 2006). The purpose of this series of experiments was to determine whether a correlation exists between post IVF outcome and field fertility based on 70 d non-return rate after AI with the frozen thawed semen of AI dairy bulls using ERCR estimate.

3.3 METHODS

USDA calculated the ERCR and assigned the score to the bulls on the basis of the field fertility, non return rate at 70 d of AI. The ERCR scores calculated for the bulls under study are shown (Table 3-1).
Table 3-1. Estimated Relative Conception rate (ERCR)*, repeatability, and the number of breeding of the 4 bulls as on February 2007

<table>
<thead>
<tr>
<th>Bull No.</th>
<th>ECR</th>
<th>Repeatability</th>
<th>No. of breeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bull I</td>
<td>1</td>
<td>76</td>
<td>800</td>
</tr>
<tr>
<td>Bull II</td>
<td>-3</td>
<td>97</td>
<td>8,490</td>
</tr>
<tr>
<td>Bull III</td>
<td>2</td>
<td>99</td>
<td>18,040</td>
</tr>
<tr>
<td>Bull IV</td>
<td>-3</td>
<td>89</td>
<td>2,004</td>
</tr>
</tbody>
</table>

*Dairy Records Management Systems (Raleigh, NC).

3.3.1 Collection of Germinal Vesicle -Cumulus Oocyte Complexes (GV-COC)

Two to 10 mm sized follicles were aspirated from slaughterhouse ovaries (Brown Packing Co Inc., Gaffney, SC) from cows of unknown reproductive status with a 10 cc syringe and 20 gauge needle separately into 2 labeled 50 mL conical tubes. The oocytes with compact multilayered cumulus investment and homogenous ooplasm from each class were used for mRNA extraction. Oocytes, n = 25, were denuded of adherent cumulus cells by treatment with 0.3% hyaluronidase (Sigma- Aldrich, St. Louis, MO), followed by 5 min of constant gentle vortexing at room temperature. Denuded oocytes were rinsed in 0.1% polyvinyl pyrolindone (Sigma-Aldrich) in PBS and were lysed in 22.5 µL of lysis buffer containing DNase (1:100; Ambion, Austin, TX) and stored at -20°C. The remaining oocytes were used for in vitro maturation.

3.3.2 In Vitro Production of Embryos

Maturation of oocytes was performed in tissue culture medium (TCM-199, Gibco Life Technologies, Inc; Grand Island, NY) supplemented with 0.02 U/mL Follicle Stimulating Hormone (FSH; Sigma-Aldrich), 0.01 U/mL Luteinizing Hormone (LH; Sigma), 1 µg/mL 17 β-estradiol (E2; Sigma), and 10% fetal calf serum (FCS; Gibco) in a humidified atmosphere of 5% CO₂ in air at 38.5°C for 24 h (Walters et al., 2005). After 15 h of maturation 25 oocytes from
both sizes were denuded of cumulus cells and cell lysate was prepared as described for germinal vesicle (GV) oocytes, which served as control oocytes for RT-PCR.

For IVF, frozen semen from 4 bulls classified according to their fertility calculated on the basis of non-return rates was used. Two straws of semen were thawed in a water bath at $37^\circ$ C for 1 min. Motile spermatozoa were separated by a modified swim-up technique (Parish et al., 1986) in synthetic oviduct fluid (SOF) supplemented with 25 mM HEPES (SOF-H), fetal bovine serum (FBS, 2%), and sodium pyruvate (0.1 mg/mL; Sigma-Aldrich). After washing and centrifugation (700 x g for 10 min), spermatozoa were resuspended to a final concentration of $1 \times 10^6$/mL. Following maturation, oocytes were washed in SOFH medium and placed into 47 µL SOF for IVF (SOF-IVF) medium drop (10 oocytes in 1 µL SOF-IVF per drop) supplemented with 10 µg/mL heparin sodium sulfate (Sigma-Aldrich) and BSA (8 mg/mL; Sigma-Aldrich) and 2 µL of the sperm cell suspension was added to each microdrop of SOF-IVF medium (final volume of 50 µL and final concentration of $1 \times 10^6$ spermatozoa/mL; Parish et al., 1995).

After 18 hr of the oocyte and sperm cell incubation in a humidified atmosphere (5% CO$_2$ in air at 38.5°C) presumptive zygotes were denuded of cumulus cells by vortexing in 2.0 mL SOFH medium for 4 min. Embryos were washed three times in SOFH, and twice in SOF for IVC (SOF-IVC) and transferred to 50 µL SOF-IVC culture drops supplemented with 1 mg/mL BSA under mineral oil (Specialty Media, Lavellette, NJ) and cultured at 100% humidity in a gas phase of 5% CO$_2$, 5% O$_2$ and 90% N$_2$ at 38.5°C (Ward et al., 2001). The cultured drops were supplemented with 4% FCS at 96 h post culture.
3.3.3 Vitrification Protocol

Vitrification of oocytes was conducted using a nylon mesh holder (pore size 60 µm; Sefar America Inc., NY) as described (Matsumoto et al., 2001). A stepwise vitrification procedure (Abe et al., 2005) was adopted using an ethylene glycol, Ficoll, and sucrose (EFS40) vitrification solution. (Video Clip 3-1). In this method, oocytes after 15 h of maturation were washed three times with SOFH and exposed for 7 min to 100 µL droplets of solution A, composed of 10% (v/v) ethylene glycol, 4.5% (w/v) Ficoll-70 and 0.075 M sucrose in Ca++ free modified Dulbecco’s phosphate buffered saline (PBS). Next, oocytes were exposed for 2 min to 100 µL droplets of solution B, consisting of 20% (v/v) ethylene glycol, 9.0 % (w/v) Ficoll-70 and 0.15 M sucrose in PBS, and finally they were immersed for 1 min in 100 µL droplets of solution C, composed of 40% (v/v) ethylene glycol, 18% (w/v) Ficoll-70 and 0.3 M sucrose in PBS in 35 mm culture dishes. After equilibration, 20 to 25 oocytes were loaded onto the nylon mesh holder, which was placed on a single fold towel (Wausau paper, 22.9 cm x 25 cm, Harrodsburg Plant, Harrodsburg, KY) to remove excessive solution, immediately transferred to 2 mL pre-cooled cryovials and directly plunged into liquid nitrogen (LN₂) within 40 to 60 s. After 24 h of storage in LN₂, the oocytes were ultra rapidly warmed, by maintaining in air for 5 s and then cryoprotectants were removed in a stepwise manner. The nylon mesh was transferred from LN₂ into 2.7 mL of the warm Ca++ free PBS (37°C) in 20% fetal bovine serum (FBS) with a sequential series of 0.5, 0.25 and 0.125 M sucrose dilutions and by placing in each solution for 1, 2, and 3 min, respectively, and finally transferring into PBS (37°C) for 3 min in culture dishes. After warming, the nylon mesh was transferred into SOFH medium and the adhered oocytes were separated from the mesh by gentle flushing of the oocytes with SOFH drawn out of the pipette repeatedly and were washed three times before they were transferred into TCM-199 for
further maturation. After warming, 25 oocytes from both size classes were denuded of cumulus cells and cell lysates were prepared as described above for GV oocytes. Similarly, after 9 h of maturation 25 oocytes from both size classes were denuded of cumulus cells and cell lysates were prepared as described for GV oocytes for RT-PCR.

3.3.4 Assessment of Oocyte Survival

Oocytes with homogenous ooplasm, intact membranes, and zona pellucida post-thaw were recorded as living oocytes (Men et al., 2003). Survival of vitrified-warmed oocytes was evaluated after 18 h in fertilization medium. Cleavage and blastocyst formation rates were used to assess their developmental competence (Men et al., 2002).

3.3.5 Statistical Analysis

For experiments on the effect of vitrification on the developmental capacity of bovine oocytes and paternal influence on the post IVF outcome, data were analyzed by using the PROC MIXED in SAS (9.1). Cleavage and development rates were determined in this experiment. The effects included in the model were treatment (fixed effect), bull (fixed effect), follicle size (fixed effect), and the interactions between treatment and follicle size, sire and follicle size, sire and treatment, and sire by treatment by follicle size. Dependent variables were percentage of oocytes cleaved, percentage of morula, and blastocyst (Table 3-2). Percentage data were analyzed with and without the arcsine transformation to correct for problems of nonnormality associated with analysis of percentage data. Data are presented as least squares means ± SEM, and differences between treatments were considered significant at $P<0.05$. Pearson and Rank correlations were conducted between ERCR estimates for the 4 bulls and the significant cleavage (vitrified and control means). Similarly, correlations were conducted between ERCR estimates and the
significant blastocyst vitrified and control means, and the significant blastocyst means for small and medium follicle oocytes using the PROC CORR procedure of SAS.

**Table 3-2.** Test of significance for the effect of vitrification and paternal influence using PROC MIXED

<table>
<thead>
<tr>
<th>Effects</th>
<th>df*</th>
<th>Cleavage</th>
<th>df</th>
<th>Morulae</th>
<th>df</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sire</td>
<td>3</td>
<td>0.0023</td>
<td>3</td>
<td>0.0115</td>
<td>3</td>
<td>0.0022</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>&lt;.0001</td>
<td>1</td>
<td>&lt;.0001</td>
<td>1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Size</td>
<td>1</td>
<td>&lt;.0001</td>
<td>1</td>
<td>&lt;.0001</td>
<td>1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Treatmentx Size</td>
<td>1</td>
<td>0.1134</td>
<td>1</td>
<td>0.0068</td>
<td>1</td>
<td>0.0214</td>
</tr>
<tr>
<td>Sirex Size</td>
<td>3</td>
<td>0.3276</td>
<td>3</td>
<td>0.0694</td>
<td>3</td>
<td>0.0398</td>
</tr>
<tr>
<td>Sirex Treatment</td>
<td>3</td>
<td>0.002</td>
<td>3</td>
<td>0.096</td>
<td>3</td>
<td>0.0018</td>
</tr>
<tr>
<td>SirexTreatmentxSize</td>
<td>3</td>
<td>0.1214</td>
<td>3</td>
<td>0.966</td>
<td>3</td>
<td>0.5994</td>
</tr>
</tbody>
</table>

*degrees of freedom.

### 3.4 RESULTS

The main effects included in the study were treatment, bull, follicle size, and the interactions between treatment and follicle size, sire and follicle size, sire and treatment, and sire by treatment by follicle size. The dependent variables included were percentage of oocytes cleaved, percentages of morulae and blastocyst development (Table 3-2).

#### 3.4.1 Experiment I

**Morphological Survival of Oocytes and Embryos.** The survival of oocytes post-thaw and embryos after IVF was evaluated by morphological examination. In the 3-step vitrification procedure, the recovery of both small and medium oocytes together from the vitrification media was 99.3% of those initially equilibrated in the vitrification media. The post thaw recovery from the nylon mesh was 98.7%. The results indicated that the vitrification treatment did not affect the proportion of the oocytes with intact morphology after warming. The morphological survival rates jointly for the small and medium oocytes after warming were 98.9% (Table 3-3).

Strikingly, the intactness of cumulus cells post vitrification and warming was a remarkable
feature of nylon mesh vitrification (Figure 3-1). In this study after in vitro fertilization there was no significant \( P > 0.05 \) difference observed in the fragmentation of embryos formed from the vitrified versus control oocytes based on their morphology when placed into culture (Table 3-3).

**Table 3-3.** Morphological survival of vitrified bovine oocytes using nylon mesh

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified</td>
<td>1968</td>
<td>1956</td>
<td>1931</td>
<td>1911</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>99.3</td>
<td>98.7</td>
<td>98.9</td>
<td>9.7( ^a )</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1685</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9.3( ^a )</td>
<td></td>
</tr>
</tbody>
</table>

Values with the same superscripts within the column are not significantly different \( P > 0.05 \).

**Figure 3-1.** Morphology of oocytes at 15 h maturation. a) Without vitrification c) Vitrified (presence of intact cumulus cells). Left (100 x), and right (40 x) magnification.
Embryonic Development. The developmental rates of embryos based on the total number of oocytes fertilized was significantly \((P < 0.05)\) affected by treatment (vitrified vs. control), and size \((≤4 \text{ mm vs. } 4 \text{ to } 10 \text{ mm})\) of follicles. The overall cleavage rates were significantly different \((P < 0.05)\) for the vitrified and control oocytes (39.1% vs. 58.5%). The rates of embryo development to the morula (13.1% vs. 35.1%) and blastocyst (5.1% vs. 22.9%) were significantly different \((P < 0.05)\) between the vitrified and control groups, respectively (Figure 3-2).

![Figure 3-2.](image-url) Embryonic developmental rate ± SE (%) of vitrified vs. control oocytes. (Means with different superscripts differ at \(P < 0.05\)).

Follicle size for the oocytes had a significant \((P < 0.05)\) impact on the developmental rates of embryos from cleavage to blastocyst stage. There was a 9.9% increase in the cleavage rate for medium sized follicle oocytes compared to small follicle oocytes. While medium sized follicle oocytes had an 11.8% increase in the developmental rates to morula, the increase in the blastocyst rate was only 5.9% between the medium and small sized follicle oocytes (Table 3-4).
However, there was no interaction between treatment and follicle size of oocytes for cleavage rate; but, morula and blastocyst rates were significantly ($P < 0.05$) different. The cleavage rate for vitrified small sized follicle oocytes was 35.4% vs. 42.8% for medium sized follicle oocytes in contrast to 52.3% vs. 64.7% for control small and medium sized follicle oocytes, respectively. The rate of development to the morula was 9.6% for vitrified small sized follicle oocytes and 16.6% for vitrified medium sized follicle oocytes in contrast to 26.9%, and 43.4% respectively, for control small and medium sized follicle oocytes. The vitrified small and medium sized follicle oocytes resulted in 3.2 and 7.0% blastocyst rates respectively, and the corresponding rates in control small and medium sized follicle oocytes were 18.9 vs. 26.9% (Table 3-4).

**Table 3-4.** Least squares means for embryonic developmental rate ± SE (%) of small and medium oocytes, and interactions with vitrified vs. control oocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cleavage</th>
<th>Morula</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>Small</td>
<td>Difference</td>
</tr>
<tr>
<td>Size</td>
<td>53.7&lt;sup&gt;a&lt;/sup&gt; ±1.56</td>
<td>43.8&lt;sup&gt;b&lt;/sup&gt; ±1.59</td>
<td>9.9</td>
</tr>
<tr>
<td>Vitrified</td>
<td>42.8 ±1.56</td>
<td>35.4 ±1.61</td>
<td>7.4</td>
</tr>
<tr>
<td>Control</td>
<td>64.7 ±1.56</td>
<td>52.3 ±1.56</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Means with different superscripts within the row are different at $P < 0.05$.
*(treatment by follicle size interaction) for cleavage was not significant.

The rate of development of morula was significantly different between treatment, follicle size and treatment by follicle size interaction. Similarly, sire had a significant impact on the rate of development of embryos to the stage of morula (Table 3-5).
Table 3-5. Least squares means ($P < 0.05$) for morula development ± SE (%) for treatment, follicle size, treatment by follicle size and sire

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Size</th>
<th>Treatment*Size</th>
<th>Sire</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitrified</td>
<td>Control</td>
<td>Medium</td>
<td>Small</td>
</tr>
<tr>
<td>13.1</td>
<td>35.1</td>
<td>30</td>
<td>18.2</td>
</tr>
<tr>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.02</td>
</tr>
</tbody>
</table>

*treatment by follicle size interaction

3.4.2 Experiment II

Paternal Influence on the In Vitro Embryonic Development of Vitrified Oocytes.

Treatment by sire interaction was significant ($P < 0.05$) on the cleavage and blastocyst rate. Nonetheless, there was no significant interaction ($P > 0.05$) noticed between sire and follicle size of oocytes for cleavage, but there was significant interaction ($P < 0.05$) on blastocyst development. The sire by treatment by follicle size interaction had no significant impact ($P > 0.05$) on the development of the embryos (Table 3-2).

The cleavage and subsequent in vitro embryonic developmental rates showed variations among individual bulls (Figure 3-3). The cleavage rates for Bull I, II, III, and IV were 49.3, 55.0, 50.0, and 40.9%, respectively ($P < 0.05$). Both morula and blastocyst stages of development were significantly different ($P < 0.05$) among the 4 tested bulls. The developmental rates to morula were 26.4, 29.9, 23.4 and 16.7% for Bull I, II, III, and IV, respectively. The blastocyst developmental rates in the above bulls were 18.1, 17.9, 10.0, and 10.0%, respectively.
Figure 3-3. Paternal influence on the in vitro development of embryos, developmental rate ± SE (%).

A comparative study was performed to evaluate the influence of 4 bulls on vitrified oocytes. The study showed significant ($P < 0.05$) variation among the cleavage and blastocyst rates of embryos fertilized by Bull I, II, III, and IV in both vitrified and the control groups (Table 3-6).

Treatments had a significant influence on the cleavage and blastocyst developmental rates for Bull I. The cleavage rates were 39.9 and 58.7%, respectively, for the vitrified and control oocytes (Table 3-6). The Bull I had the highest blastocyst development for vitrified oocytes (10.9%) and the corresponding rate for the control oocytes was 25.3%. There was a significant influence of the size of the follicle for the blastocyst development. The oocytes derived from small follicles developed to 15.2% blastocyst in contrast to 20.9% for those derived from medium follicle.
The Bull II had the highest cleavage rates for the control oocytes (69.3%). On the other hand, the cleavage rate of the vitrified oocytes using Bull II for IVF showed a pattern of development almost similar to that of Bull I, 40.8%. But, the blastocyst development rate for the vitrified oocytes showed a decreased developmental pattern (6%). The Bull II showed highest blastocyst (23.1%) development for the oocytes derived from medium follicles, but the development for those derived from smaller follicles was lower (12.8%) than the Bull I.

The influence of Bulls III and IV for the first division following fertilization in both vitrified and control oocytes was very similar to that of Bulls I and II, but further development was reduced in both bulls for both vitrified and control oocytes. The cleavage rate for the Bull III was 39.4 vs. 60.5 %, respectively, for the vitrified and control oocytes in contrast to 36.2 vs. 45.5%, respectively, for the Bull IV (Table 3-6).

A reduction in the blastocyst rates was observed in both Bull III and Bull IV for the vitrified and control oocytes. Bull III had 2.5 vs. 17.4% blastocysts in vitrified and control oocytes, respectively, in contrast to 1 vs. 18.9% obtained in Bull IV. The significant impact of follicle size on blastocyst development in the Bull III and Bull IV are almost similar for both sizes. Oocytes from small follicle showed blastocyst rates of 8.3 and 7.9% in contrast to 11.7 and 12.0 % respectively, for the medium sized follicle oocytes.

<table>
<thead>
<tr>
<th>Sire</th>
<th>Cleavage</th>
<th>Blastocyst</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitrified</td>
<td>Control</td>
<td>Control</td>
</tr>
<tr>
<td>No.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>39.9 ± 2.2</td>
<td>58.7 ± 2.0</td>
<td>10.9 ± 1.5</td>
</tr>
<tr>
<td>2</td>
<td>40.8 ± 2.4</td>
<td>69.3 ± 2.4</td>
<td>6.0 ± 1.7</td>
</tr>
<tr>
<td>3</td>
<td>39.4 ± 3.9</td>
<td>60.4 ± 2.4</td>
<td>2.5 ± 1.7</td>
</tr>
<tr>
<td>4</td>
<td>36.2 ± 2.36</td>
<td>45.5 ± 2.4</td>
<td>1.0 ± 1.7</td>
</tr>
</tbody>
</table>
3.5 DISCUSSION

3.5.1 Effect of Vitrification on Bovine Oocytes

In this study, oocyte cryopreservation using nylon mesh vitrification resulted in high recovery, post warming survival, fertilization and blastocyst rates. Although numerous studies of vitrification of bovine oocytes have been reported using open and closed carrier methods, the majority have been with open carrier systems.

Vitrification and recovery of large numbers of cumulus-intact bovine oocytes in a closed method was successful (Table 3-3). Only 2 studies have used nylon mesh for vitrification of germinal vesicle bovine oocytes (Matsumoto et al., 2001; Abe et al., 2005). In our study using Bull I for IVF resulted in the vitrified oocytes cleaving (39.9%) and developing into morula (18.4%) and blastocysts (10.9%) at a higher rate than other bulls. Still, cleavage rate and morulae and blastocysts yields were below that of control oocytes (58.7, 34.4, and 25.3 %, respectively). These results showed increased developmental rates of embryos in contrast to those reported by Matsumoto et al. (2001) and Abe et al. (2005) using nylon mesh. The increased cooling rate using the pre-cooled cryovial coupled with the membrane stability due to 20% FBS in the thawing solution might have had beneficial effects on the post thaw survival and ensuing embryonic development. Similar blastocyst rates were reported with matured oocytes (MII) that were vitrified with the cryoloop (10%; Luster, 2004; Checura and Siedel, 2007), but a lower rate was reported for OPS (5 %; Checura and Siedel, 2007). Similarly, using conventional straws, Hochi et al. (1998) reported a lower blastocyst (4.8%) using MI oocytes, the only study in the literature that used maturing (MI) bovine oocytes for vitrification. Another study using CryoTop reported blastocyst development of 4% in the vitrified oocytes, which was lower than that reported here (Moisan, 2006).
Although we have not determined the cooling and warming rates, both are assumed to be high, because of the usage of pre-cooled cryovials, easy handling of nylon mesh for mounting and location of the sample, and recovery of normal oocytes with less damage (Figure 3-1; Table 3-3). The presence of cumulus cells surrounding the oocytes (Figure 3-1) is crucial for ensuring complete oocyte maturation and for developmental competence (Fukui and Sakuma 1980; Critser et al., 1986; Fukui, 1990). Their role in fertilization can not be disregarded (Zhang et al., 1995). Park et al. (2001) reported that cumulus cells are beneficial for protecting the oocytes against osmotic shock caused by rapid concentration or dilution of cryoprotectants and by providing a rigid structure around oocytes that prevents its morphological damage. Our data support these findings.

A striking feature of our study was the retention of the cumulus cells after vitrification rewarming (Figure 3-1). This might have created beneficial effects for oocytes during cooling and aided fertilization. Furthermore, 6 to 8 h of maturation (inductive phase, Figure 3-4) is the stage at which the cumulus cells act in a particularly crucial supportive and a regulatory role in RNA synthesis (Gordon, 2003). In our study oocytes were vitrified after 15 h of maturation and even if there were alterations in the morphology of cumulus cell, they continued in a crucial supportive role.
Many problems have been encountered for vitrification of oocytes at different meiotic stages of maturation. The degree of oocyte maturation, from the germinal vesicle stage to the MII stage, was shown to effect survival post-vitrification (Parks and Ruffing, 1992). A significant increase in the diploid oocytes was noticed in oocytes vitrified before or 8 h of maturation in contrast to those vitrified 12 or 22 h of maturation (Luna et al., 2001). In the present study oocytes were vitrified at 15 h of maturation (MI stage). Microtubules were well organized near the spindles in the MI and MII stages. Though the temporary loss or clumping of microtubules might have been induced by vitrification on MI oocytes, they may be able to repair such changes during 9 h of further incubation (Hochi et al., (1997).

Oocytes from small follicles (≤4 mm) developed at lower rates than oocytes from medium (4 to 10 mm) sized follicles whether undergoing vitrification or acting as controls. Follicle diameter and atresia, cumulus oocyte complex (COC) morphology, and oocyte diameter are the important non invasive quality markers for oocyte competence. A clear correlation between follicle diameter and oocyte developmental competence was established in cattle (Fuhrer et al., 1989). In cattle, follicles <3 mm diameter contain oocytes with a diameter under
110 µm, which are considered developmentally incompetent (Gandolfi and Gandolfi, 2001) and the proportion of competent oocytes increases greatly in follicles >8 mm diameter (Henderiksen et al., 2000). None of the studies reported so far were directed toward the cryotolerance potential of oocytes from different follicle sizes. Our results showed that 35.4, 9.6, and 3.2% vitrified oocytes from small sized follicles were able to cleave and yield morula and blastocysts, respectively, in contrast to 42.8, 16.6 and 7% of vitrified oocytes from medium sized follicles. There was no interaction noticed between vitrification and follicle size of oocytes for cleavage, but there was interaction for morula and blastocyst development, showing a greater rate of decline in development for oocytes from small follicles compared to oocytes from medium sized follicles.

3.5.2 Paternal Influence on In Vitro Embryonic Development

The influence of sire on the in vitro embryonic developmental competence of small and medium sized vitrified and control oocyte was revealed (Table 3-2). There was no interaction between sire and follicle size of oocytes in the cleavage but there was significant interaction noticed in the blastocyst developmental rate. Similarly a sire treatment interaction was noticed for cleavage and blastocyst development. A marked difference among bulls at the blastocyst stage was recorded in the earlier in vitro studies (Shamsuddin and Larson, 1993; Ward et al., 2001). Variation in IVF characteristics among bulls was reported (Eyestone and First, 1989; Ward et al., 2001), but has not been reported with vitrification of oocytes. Individual batches of bovine oocytes fertilized with the semen of different bulls vary between 0 and 36% in their capacity to produce embryos in vitro (Parrish et al., 1986; Lacalandra et al., 1993). Fertility differences among bulls in vivo and in vitro are well established (Saacke et al., 1988; DeJarnette et al., 1992; Eid et al., 1994). Bulls of high fertility sired embryos that were more likely to
develop to the blastocyst stage than bulls of lower fertility. Bulls can differ in embryo generating capacity, even if they display the same fertilization in vitro (Van Soom et al., 1997). Our study indicates that there is variation in developmental rates of embryos among bulls both in the vitrified and control oocytes, due to significant bull effects (Parrish et al., 1986).

The ERCR is a phenotypic predictor of bull fertility, expressed as relative conception rate. It is estimated that a Bull with +2 or higher ERCR value with repeatability >90% would have an impact on fertility (Pursley, 2004). The Bull I with +1 ERCR and 75% repeatability and Bull II with -3 ERCR and 97% repeatability showed higher cleavage and blastocyst rates than Bull III and IV. Our study showed that Bull IV, with -3 ERCR and 89% repeatability had a lower cleavage and blastocyst rate development compared to other bulls. Bull III with +2 ERCR and 99% repeatability, showed a higher cleavage rate than Bull IV, but a similar blastocyst rate. This shows that Bull III may not be a high fertility sire based on the selection criteria of this study. Additionally, Cornwell et al. (2006) showed a high deviation in the ERCR values and the repeatability from time to time. This might be another reason giving contradictory results between in vivo and in vitro studies.

Although a correlation between IVF outcome and bull fertility was found in the previous studies (Hillery et al., 1990; Marquant Le Guienne et al., 1990; Shamsuddin and Larson 1993), our study contrasts the above results. We did not observe correlations between ERCR estimates of the 4 tested bulls and the developmental rates of embryos, or for a significant interaction with sire in vitrified and control oocytes. Contradictory estimates of the relationship between non return rate and cleavage or blastocyst rates were reported earlier (Marquant Le Guienne et al., 1990; Ward et al., 2001). Our study and most of the above studies compared the in vitro results obtained using one or two ejaculates with the cumulative field fertility of the bulls over a long
period of time. The discrepancy in the results may be due to differences among bulls in fertilization rates (Marquant Le Guienne et al., 1990) and in embryo cleavage and development to the morula and blastocyst stages (Eyestone and First, 1989; Hillery et al., 1990; Shi et al., 1990; Zhang et al., 1997). Astiz Blanco et al. (1999) reasoned that differences in embryo development can be related to hereditary factors within individual bulls. In a comparative study using frozen semen from high and low fertility bulls, a significant difference between the two groups was found in the cleavage and blastocyst rates (Zhang et al., 1995). We evaluated 4 bulls that differed in in vivo fertility. There was little evidence from this study that post IVF outcome provided a useful predictor of field fertility of bulls based on ECR. Final conclusions on the usefulness of in vitro results will require work on a large scale.

In conclusion, nylon mesh vitrification in a closed container is a cheap and useful method for oocyte cryopreservation, resulting in high survival of vitrified oocytes, and allowing a reliable isolation of oocytes from liquid nitrogen. Follicle size for oocytes has direct impact on the success of vitrification of oocytes. Additionally, no relation exists between post-IVF outcome and field fertility based on ECR.
3.6 LITERATURE CITED


CHAPTER FOUR

APOPTOSIS IN VITRIFIED OOCYTES; UPSTREAM AND DOWNSTREAM SIGNALING

4.1 ABSTRACT

The present study describes a method for quantification of transcripts from low numbers of bovine oocytes using real time RT-PCR. The objective of this study was to unravel the expression pattern of apoptotic genes (Fas, FasL, Bax, and Bcl-2) in vitrified-thawed oocytes. Oocytes were evaluated at germinal vesicle (GV) stage, at 15 h of maturation, after vitrification and thawing at 15 h of maturation, and at 9 h of additional maturation post vitrification and thawing. Oocytes were collected from follicles of two size categories (small vs. medium). We analyzed the mRNA expression profile of Fas- FasL, Bax- and Bcl-2. There was no significant effect of follicle size for oocytes. All transcripts showed an increase of at least 1.2-fold change post vitrification thawing, but the levels tended to decrease at 9 h of maturation post vitrification-thawing. Transcript abundance for Fas mRNA was 1.4-fold for oocytes after vitrification and thawing. The level of Fas mRNA upon maturation was 0.8-fold. The increase in the abundance of FasL mRNA was 2.1, while it was 0.5-fold relative to control oocytes at 15 h of maturation. Vitrification resulted in 1.5-fold change in Bax mRNA expression in oocytes. After 9 h of maturation post vitrification-thawing, the level for Bax mRNA was 0.6-fold in oocytes. The mRNA for Bcl-2 was nearly the same after vitrification and thawing relative to control oocytes. The relative abundance of mRNA for Bcl-2 was 1.2-fold in vitrified oocytes relative to control oocytes. The level of Bcl-2 was reduced ($P = 0.05$) to 0.5 at 9 h of maturation post vitrification and thawing. The oocytes post vitrification-thawing showed a Bax:Bcl-2 ratio of 1.2, and the trend was almost the same (1.3) following 9 h of maturation. The up-regulation of apoptotic
genes in vitrified oocytes may be an early indicator of reduced developmental competence following vitrification. However, the higher Bax:Bcl-2 ratio favors death inducer levels. Yet, results from terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and caspase assays did not support the evidence of the downstream apoptotic signaling pathway in embryos derived from vitrified oocytes.

4.2 INTRODUCTION

Several studies showed that oocyte survival was controlled by a number of cell death regulatory molecules responsible for either activation or suppression of cell death (Tilly, 2001). One reason for the prenatal loss of oocytes during ovarian development is apoptosis and programmed cell death (PCD) is the cause for the loss of oocytes during follicular atresia (Tilly, 1996). It is generally thought that apoptosis is the reason for the degeneration of the ovulated oocytes which fail to fertilize (Fujino et al., 1996; Perez, et al., 1999). Yang and Rajamahendran (2002) reported that in vitro matured oocytes degenerate via apoptosis. Structural injuries consequent to vitrification have been studied. A majority of oocytes undergo compromised development after vitrification. Men et al. (2003) showed that cryopreserved oocytes degenerate via apoptosis during subsequent culture. Apoptosis is one of the factors that contribute to cellular injury and death in vitrified oocytes (Shaw et al., 2000). Hence, it is imperative to investigate the molecular alterations caused by vitrification procedures.

The study of apoptosis and its influence on oocyte and embryo quality has great relevance for the in vitro production of embryos where a very heterogeneous oocyte population, at different stages of growth and atresia, is used to produce embryos (Anguita, 2007). Research has been directed toward characterizing the expression and, in some cases, the possible function of gene products that regulate PCD in oocytes, granulosa cells, and corpora lutea. But, little is
known about cell death triggered by Fas binding to FasL at the oocyte surface and expression of death regulating genes in vitrified oocytes. Hence, the objectives of this study were to examine whether: 1) the mechanism of cell death in vitrified oocytes can be induced via Fas signaling pathway, through measurement of the abundance of Fas and FasL mRNA expressions in matured cryopreserved oocytes isolated from small (≤4 mm) and medium (4 to 10 mm) follicles; 2) the effect of vitrification yielded differences in relative mRNA abundance between the 2 groups of vitrified oocytes for genes related to apoptosis, Bax and Bcl-2; and 3) vitrification induced changes in caspase activity and DNA fragmentation parallel to patterns of apoptosis.

4.2.1 Upstream Signaling of Apoptosis

Real Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Classical techniques of RNA analysis such as RNAse protection assays, northern blotting, and in situ hybridization are not suitable for detecting mRNA in single cells, mammalian oocytes or preimplantation embryos because of inherent technical limitations and the lack of sensitivity of these techniques. Yet, gene expression analysis was successfully introduced to study quantitative aspects of gene transcription which occurs during oocyte growth, maturation, and embryo development. Presently, the method of choice to quantify mRNA levels is real time RT-PCR, especially when small amounts of mRNA are present from as little as one cell (Steuerwald et al.1999, 2000). Real time RT-PCR was applied for the detection of quantitative abundance of mRNA in oocytes and embryos (Robert et al., 2002; Lonergan et al., 2003; Fair et al., 2004; Bettegowda et al., 2006).

The variables that need to be controlled in gene expression studies are the amount of starting material, enzymatic efficiencies, and the differences between tissues or cells in overall transcriptional activity (Vandesompele et al., 2002). Generally gene expression is normalized by
the amount of starting material, but a problem for oocytes is low RNA yield for reliable quantification and hence, there is high probability of variation in its reverse transcription. Additionally, it involves normalization of the amount of RNA by adding a known amount of external control RNA or utilizing endogenous transcript for housekeeping genes as internal controls. Donnison and Pfeffer (2004) reported that for the proper use of external control, quantification of starting material is a must, and not a preferred method when using small amounts of starting material. Under controlled conditions, the gene transcript number can be standardized to the number of cells (Vandesompele et al., 2002), but the enumeration of cells is often difficult when the starting material is a solid tissue.

The reliability of real time PCR quantitative results lies in the accurate application of the procedures including quality RNA isolation, cDNA synthesis, dilutions made, pipetting, appropriate controls, and final analysis in addition to good experimental and primer designs (Gal et al., 2006). The quantitative gene expression studies have been carried out under various experimental conditions like stress, growth, or cell differentiation. But, the problems with those treatments are a wide variation in transcript levels in the cells or tissues in response to experimental treatment. Consequently, the best way to control for all variables, including the amount of input material, is normalization against internal control genes. Therefore, an mRNA that is not affected by the treatment or one that is stably expressed should be included in the analysis to normalize the mRNA fraction. To date most of the validations are done with reference genes such as β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18S rRNA (Suzuki et al., 2000). Still, various reports (Thellin et al., 1999; Bustin, 2000; Suzuki et al., 2000) provided evidence that the housekeeping gene can vary considerably. Normalization of the data using these controls can lead to false conclusions regarding transcription levels.
Therefore, validation of internal control genes is critical for the accurate analysis of the data and must be carried out before any experiment. Distinct temporal regulation of mRNA abundance for β-actin, GAPDH, and histone (H2A) were reported in germinal vesicle (GV) and MII oocytes (Bettegowda et al., 2006). Additionally, an increase in H2A transcript level by follicle size was reported (Mourot et al., 2006). The 18S rRNA was suggested as a valid internal standard in situations where other commonly used standards were found not suitable (Thellin et al., 1999). In support, human T-lymphocytes gave the most reliable results using 18S rRNA followed by β-actin and GAPDH (Bas et al., 2004). Nonetheless, before using any standard internal control quantification of oocyte transcripts require validation (DeSousa et al., 1998).

Considering the factors mentioned above, we decided to carry out quantitative PCR using 18S rRNA, which was an accurate standard. Hence, the objective of this study was two fold. The first was to validate the stable expression of internal reference gene, 18S rRNA. The mRNA expression of 18S rRNA was determined in GV oocytes, at 15 h (MI) of maturation, post vitrification thawing at 15 h of maturation, and in vitrified thawed oocytes cultured for the remaining 9 h (MII) of maturation in small and medium oocytes. The experimental protocol allowed quantification of four apoptotic genes under study. The second goal was to measure the abundance of Fas-FasL, Bax and Bcl-2 mRNA expressions in cryopreserved oocytes isolated from small and medium oocytes.

4.2.2 Downstream Signaling of Apoptosis

Caspase Activity and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Apoptosis plays an important role in mammalian development as a quality control mechanism to eliminate cells that are damaged, nonfunctional, abnormal, or even infected (Byrne et al., 1999; Neuber et al., 2002). In bovine oocytes (Roth and Hansen, 2004) and
embryos (Paula-Lopes and Hansen, 2002; Walters et al., 2005) the occurrence of apoptosis was reported when it was induced with stress stimuli. Normally, a majority of in vitro produced bovine blastocysts contain one or more apoptotic cells. Brison et al. (1997) reported that a decrease in cell number coupled with increased number of apoptotic cells relates to the reduction in embryo quality. The first appearance of apoptosis is species dependent and in bovine embryos using TUNEL assay the appearance was reported around the 8-cell stage, i.e., at the time of embryonic genome activation (Byrene et al., 1999; Matwee et al., 2000). The disadvantage of TUNEL is that it detects apoptosis only at the later stage. Collins et al. (1997) reported that earlier stages of apoptosis occur from 2- to 8-cell embryos. In support, Jurisicova et al. (2003) reported mRNA transcripts of caspase-3 transcripts in all stages of human preimplantation embryos, indicating the presence of full apoptotic machinery in early cleavage embryos.

Another group reported that reliable information on the level of apoptosis could be gained by detection of specific proteins from the apoptotic cascade such as caspases, than by TUNEL or annexin staining (Levy et al. 1998; Martinez et al. 2002) to avoid false positive results in some cases of necrosis. Of the 6 caspases present in cattle, caspase-3 is involved in both the receptor mediated and intrinsic pathway of apoptosis (Earnshaw et al., 1999). Hence, we decided to detect the activity of caspase-3, an apoptotic marker, in early cleaving embryos and apoptotic index in the blastocysts using TUNEL assay.

4.3 METHODS

4.3.1 Collection of Oocytes for RT-PCR

The oocytes with compact multilayered cumulus investment and homogenous ooplasm from medium and small sized follicles were separately aspirated for mRNA extraction. Briefly,
oocytes, n = 25, were denuded of adherent cumulus cells by treatment with 0.3% hyaluronidase (Sigma-Aldrich, St. Louis, MO), followed by 5 min of constant gentle vortexing at room temperature. Denuded oocytes were rinsed in 0.1% polyvinyl pyrolindone (PVP; Sigma-Aldrich) in phosphate buffered saline (PBS) and were lysed in 22.5 µL of lysis buffer containing DNAse (1:100; Ambion, Austin, TX) and stored at -20°C. Similarly, oocytes, n = 25, at 15 h maturation (control oocytes), post vitrification-thawing at 15 h of maturation, and 9 h maturation post vitrification-thawing were collected.

4.3.2 Real time RT-PCR

**Preparation of Cell Lysate.** Cell lysis was performed using the TaqMan Gene Expression Cells to C, kit (Ambion, Austin, TX) as per the manufacturer’s instructions. Each treatment consisting of 25 granulosa-freed oocytes was counted under a microscope and transferred to a nuclease free microcentrifuge tube with minimum amount of PBS. The pelleting of the oocytes was conducted by centrifugation for 1 min. The supernatant, PBS, was removed by aspiration to ensure the maximum removal of PBS without disturbing the pellet. The discarded PBS was checked under microscope for the presence of oocytes. The oocytes were lysed in 22.5 µL lysis solution with DNAse (1:100 µL). The lysis reaction was mixed by pipetting up and down 5 times followed by mixing with gentle vortexing to ensure complete lysis of the cells. This lyses cells, releasing RNA into the lysis solution, which inactivates endogenous RNAses. The reaction was incubated at room temperature (24°C) for 5 min. Stop solution, 2.5 µL, was pipetted into the lysis reaction and was mixed thoroughly by pipetting up and down 5 times followed by gentle vortexing. The reaction was incubated for 2 min at room temperature. Lysate was then stored at -20°C for 1 wk.
**Reverse Transcription.** Reverse transcription reactions were performed in a final volume of 50 µL consisting of 2 x RT buffer 25 µL, 20 x RT enzymes 2.5 µL, and 22.5 µL cell lysate. MultiScribe Reverse Transcriptase and random primers were used for the RT. The thermal cycler conditions set up for the lysate for reverse transcription were as follows: 37°C for 60 min, then at 95°C for 5 min to inactivate the reverse transcriptase enzyme in the RT mix, followed by cooling to 4°C.

**Real-time PCR.** The quantification of 18S rRNA, Fas-FasL, and Bax- Bcl-2 transcripts in oocytes subjected and not subjected to vitrification procedures was carried out by real time PCR in an ABI Prism 7300 sequence detector (Applied Biosystems, Foster City, CA, USA). The RT product was immediately used for PCR. The internal control 18S rRNA was pre-designed and pre-made. All custom TaqMan Gene Expression Assays (Table 4-1) were designed and synthesized by an assay design service within Applied Biosystems. The probes used were MGB (Minor Groove Binder) probes that are slightly shorter than conventional probes, but, because of the MGB feature, they are actually more specific. Primers were constructed over exon/exon junctions to make them specific to mRNA transcripts. The PCR were performed in 20 µL reaction volume containing 10 µL TaqMan Gene Expression Master Mix (2x) containing AmpliTaq Gold DNA polymerase, which amplifies cDNA, 1 µL TaqMan Gene Expression Assay (20x) which contains two primers (900 nM) and TaqMan MGB probe (6-FAM dye labeled) (250 nM) specific for each target sequence of interest, 5 µL nuclease free water, and 4 µL cDNA equivalent to 2.25 oocyte. The PCR protocol involved an incubation step of 50°C for 2 min which allows an enzyme, uracil DNA glycosylase (UDG), to digest any PCR amplicons that may contaminate the reaction mix. The next incubation of 95°C for 10 min activates the Taq. Finally, there were 40 cycles of PCR for 15 s at 95°C followed by final extension at 60°C for 1
min. The expression value of each gene was normalized to the amount of an internal control gene (18S rRNA) in cDNA to calculate a relative amount of RNA in each sample. The expression value of each gene in non-treated controls was arbitrarily defined as 1 unit. For each treatment, real-time PCR assays were carried out in duplicate for each replicate. For each treatment, a relative quantitative fold change was determined using the \(2^{-\Delta \Delta Ct}\) method (Livak and Schmittgen, 2001).

**Table 4-1. Primers used for real time PCR**

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer sequences</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>Pre-made (no sequence information available)</td>
<td>Hs99999901_s1</td>
</tr>
<tr>
<td>Fas-F*</td>
<td>5’GGGCTAGAAGTGGAAACAAACTGTA3’</td>
<td></td>
</tr>
<tr>
<td>Fas-R*</td>
<td>5’AAGTTCGATTTCATGACTGACTTG3’</td>
<td></td>
</tr>
<tr>
<td>FasL-F</td>
<td>5’ACAGCATCATCTTTGGAGAAGCAA3’</td>
<td></td>
</tr>
<tr>
<td>FasL-R</td>
<td>5’CTTGGCCTGTTAAATGAGCTGCTTGT3’</td>
<td></td>
</tr>
<tr>
<td>BAX-F</td>
<td>5’GCTCTGAGCAGATCATGAAGACA3’</td>
<td></td>
</tr>
<tr>
<td>BAX-R</td>
<td>5’CCATTGCCTGCTCGAT3’</td>
<td></td>
</tr>
<tr>
<td>BCL-2-F</td>
<td>5’GCACCTGCACACCTGGAT3’</td>
<td></td>
</tr>
<tr>
<td>BCL-2-R</td>
<td>5’CGCATGCTAGGGCCATACAG3’</td>
<td></td>
</tr>
</tbody>
</table>

*F- forward; *R-reverse

### 4.3.3 Caspase Activity

Caspase activity was detected using 5 \(\mu M\) PhippiLux-G1D2 (OncoImmunin, Inc; Gaithersburg, MD) a cell-permeable fluorogenic substrate. To measure caspase activity, embryos were washed three times in 50 \(\mu L\) drops of SOF-IVF (pre-warmed at 38.5°C) and were incubated in 25 \(\mu L\) microdrops of SOF-IVF containing 5 \(\mu M\) PhippiLux-G1D2 at 38.5°C for 40 min in the dark. The negative controls were incubated in SOF-IVF only. Following incubation, embryos were washed twice in 50-\(\mu L\) drops of SOF-IVF and were placed in 2-4 \(\mu L\) drops of SOFH-IVF in a glass bottom microwell 35 mm petridish (MatTek Co Ltd, Ashland, MA). Caspase activity
was determined using laser scanning confocal microscope (LSCM; Zeiss; LSM 510; software version 3.2). An argon laser at 488 nm wavelength and single channel scanning was used for detection of fluorescein isothiocyanate (FITC). Both excitation and emission light were focused through a C-Apochromat 40 x/1.2W Corr objective. The image stacks were reconstructed with the Zeiss LSM 510 software (version 3.2).

### 4.3.4 TUNEL Labeling

The method used for the TUNEL labeling was adopted earlier in our laboratory (Walters et al., 2005), but with slight modification, using the In Situ Cell Death Detection Kit (Fluorescein; Roche Diagnostics Corp.; Indianapolis, IN). Briefly, blastocysts at d 8 of culture were washed 3 times in 100 µL drops of PBS with 1 mg/mL PVP (PBS-PVP), and fixed in 4% (w/v) paraformaldehyde solution [4% (w/v) in PBS, pH 7.4] for 1 h at room temperature, and were stored in PBS-PVP at 4°C for up to 2 to 3 wk before assay. On the day of assay, the fixed embryos were removed from cold storage, allowed to return to room temperature and then incubated in permeabilization solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 30 min at room temperature. After washing 3 times in PBS-PVP, both the positive and negative controls were incubated in a 50 µL drop DNase (50 U/mL) at 37°C for ~20 min.

Both treatment and positive control embryos were placed in 25 µL drops of TUNEL reaction mixture covered with paraffin oil in a 35 mm Petri dish and incubated in the dark for 1 h at 37°C in a humidified chamber. The negative controls were incubated in the absence of the enzyme terminal transferase. Following the TUNEL reaction the embryos were washed and incubated in 500 µL of RNase A (50 µg/mL) for 1 h at room temperature in the dark.

Propidium iodide was used for counterstaining by incubation of the treatment and positive controls in 500 µL of propidium iodide (Roche Diagnostics Corp.; 0.5 µg/mL) for 30
min at room temperature in the dark. The embryos were washed in PBS-PVP to remove excess propidium iodide, placed on poly-L-lysine coated slides, and mounted with 16 µL mounting medium containing Antifade (Molecular Probes, Eugene, OR) as recommended by the manufacturer. The embryos were examined by using LSCM. Positive reactivity was indicated by a bright green/yellow fluorescence indicating apoptotic cells and the total cell number was determined by red fluorescence. An apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of cells per embryo. Both excitation and emission light were focused through a C-Apochromat 40 x/1.2W Corr objective. An argon laser at 488 and 568 nm wavelengths and two channels scanning was used to detect FITC and propidium iodide, respectively. Complete Z-series of 40-80 optical sections at 3-4 µm, each one with an image resolution of 512 x 512 pixels, were acquired from each embryo. The image stacks were reconstructed with the Zeiss LSM 510 software (version 3.2).

4.3.5 Statistical Analysis

For the gene expression study, the relative fold change of mRNA normalized to internal control 18S rRNA was determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) in 3 replicate experiments using oocytes on three different days. The data were analyzed using PROC GLM procedures in SAS (9.1). The model included the main effects of treatment, follicle size and the interaction between follicle size and treatment. PDIFF with Tukey’s procedure of SAS were performed to determine differences among Ct values of 18S rRNA at 4 levels of oocyte maturation including vitrified thawed oocytes at 15 h of maturation. Data are presented as least squares means ± SEM, and differences between treatments were considered significant at $P < 0.05$. 
The total number of cells and the total number of TUNEL positive cells at 8 d embryos derived from medium sized follicles vitrified and control oocytes for Bull I were determined. The apoptotic index was analyzed for vitrified and control blastocysts using PROC GLM procedures in SAS (9.1). Differences between treatments were considered significant at $P < 0.05$.

### 4.4 RESULTS

#### 4.4.1 Expression of 18S rRNA Levels in Oocytes in Response to Vitrification Following In vitro Maturation

The amplification profile of real time RT-PCR with specific detection involving TaqMan fluorescent probes which has the ability to specifically bind the amplified product is shown in Figure 4-1. For all genes studied, the standard curve was composed of 4 samples at four time points, utilizing a 2.25 oocyte equivalent cDNA for each PCR reaction. The Figure clearly shows the amplification profile of all genes studied in the four samples tested. Oocytes from small and medium follicles were collected separately in groups of 25 in triplicate samples immediately after aspiration form ovaries. Oocytes after 15 h of maturation (control oocytes) and those subjected to vitrification and thawing after 15 h of maturation, and vitrified thawed oocytes in culture for the remaining 9 h of maturation were also harvested in groups of 25. At each time point, careful cell counting was conducted by microscopic inspection followed by immediate lysis of the cells. Reaction samples consisted of 2.25 equivalent bovine oocytes at the GV stage and at different time points of in vitro maturation including vitrified thawed oocytes. Each RNA sample was subsequently subjected to RT-PCR analyses for determination of 18S rRNA in parallel with determination of mRNA expression levels for apoptotic genes. Individual experiments were repeated with 3 biological samples to evaluate the degree of variation. Amplification was evident from all samples.
**Figure 4-1.** A representative amplification curve profile (log phase) in real time RT-PCR. Graphic illustration for intensity of fluorescent signals after subtraction of background. The amplification of 18S rRNA (curves in black), and Fas-FasL, Bax- Bcl-2, (multi colored curves) in medium oocytes employing 2.25 oocyte equivalents of cDNA are depicted.

The Figure 4-2 presents the amplification cycle of mRNA for 18S rRNA by C<sub>t</sub> values in the real time RT-PCR at 4 time points. Though statistically significant ($P < 0.05$), the differences in C<sub>t</sub> values for 18S rRNA among 4 time points in the small or medium follicle oocytes were within 0.8 and 0.5 cycles, respectively. The 4 candidate genes showed differential mRNA levels at 4 time points. The results showed that the mRNA level of 18S rRNA was stable across the different time points and demonstrated a valid approach for quantitative analysis of mRNA abundance. This study supports the use of 18S rRNA as a valid internal control in studies.
investigating the gene expression pattern in bovine oocytes. Based on this, the quantitative real-time PCR confirmed the relative abundance of 4 transcripts.

![Graph showing Ct values for 18S rRNA in different conditions](image)

**Figure 4-2.** Quantitative analysis of 18S rRNA by real time RT-PCR using TaqMan sequence detection system. Data of Ct values are collected from 3 replicate experiments represented least squares means ± SE. There was significant difference ($P < 0.05$) in the Ct values among the 4 time points in 18S rRNA. (GV- germinal vesicle oocyte).

### 4.4.2 Changes in the Fas and FasL Abundance in Vitrified Oocytes

This study was conducted to identity the mRNAs encoding two apoptotic genes, Fas and FasL. The expression of Fas and FasL in the vitrified oocytes is shown in Figure 4-3. The control oocytes used for the study were medium and small follicle oocytes at 15 h of maturation without vitrification. There was no interaction between treatment and follicle size of oocytes for the expression of Fas and FasL mRNA. Interestingly, both Fas and FasL mRNA showed almost similar patterns of expression consequent to vitrification and thawing, and following 9 h of maturation post vitrification. Transcript abundance for Fas mRNA was 1.4 for oocytes after vitrification and thawing. The follicle size of oocytes had no impact on the relative abundance of Fas mRNA. The decrease in the level of Fas mRNA upon 9 h maturation was 0.8-fold, relative to
control oocytes at 15 h of maturation; the effect was not significant from vitrified-thawed oocytes (P > 0.05).

The increase in the abundance of FasL mRNA was 2.1-fold relative to control oocytes at 15 h of maturation; the effect was not significant between follicle sizes of oocytes (P > 0.05). There was a discernible effect (P = 0.06) in the abundance of FasL mRNA between the vitrified thawed oocytes and those at 9 h of maturation following vitrification and thawing. The level of FasL mRNA decreased to 0.45-fold relative to control oocytes at 15 h of maturation.

![Relative mRNA expression of Fas-FasL in the vitrified-thawed and post vitrified oocytes at 9 h of maturation vs. oocytes at 15 h maturation (control) as determined by real time RT-PCR, normalized against 18S rRNA. Data of quantification are calculated by the 2^{-ddCt} method. Values are shown as the mean value ± SEM for each group.](image)

**Figure 4-3.** Relative mRNA expression of Fas-FasL in the vitrified-thawed and post vitrified oocytes at 9 h of maturation vs. oocytes at 15 h maturation (control) as determined by real time RT-PCR, normalized against 18S rRNA. Data of quantification are calculated by the $2^{-\text{ddCt}}$ method. Values are shown as the mean value ± SEM for each group.

### 4.4.3 Comparative Bax-Bcl-2 Abundance in Vitrified Oocytes

The expression of the differential expression of Bax-Bcl-2 genes was examined between vitrified oocytes recovered from small and medium follicle oocytes. The control oocytes were small and medium follicle oocytes at 15 h of maturation without vitrification. There was no interaction between treatment and follicle size of oocytes for the expression of mRNA for Bax
and Bcl-2. There was a higher mRNA abundance for Bax in oocytes subjected to vitrification and thawing relative to control oocytes. Vitrification resulted in 1.5-fold increase in Bax mRNA expression in oocytes. After 9 h of maturation post vitrification-thawing, the level for Bax mRNA fell to 0.6-fold in oocytes. There was no significant effect of follicle size of oocytes on Bax mRNA expression, but the gene expression values showed a difference ($P = 0.08$) between the vitrified-thawed oocytes and those undergoing maturation 9 h after vitrification (Figure 4-4). Yet, the mRNA for Bcl-2 level was nearly the same after vitrification and thawing relative to control oocytes. The relative abundance of mRNA for Bcl-2 was 1.2-fold relative to control oocytes. The pattern of Bcl-2 mRNA expression at 9 h of oocyte maturation resembled that of Bax, but the decrease was almost significant ($P = 0.05$). The level of Bcl-2 was reduced to 0.48 at 9 h of maturation post vitrification and thawing (Figure 4-4).

**Figure 4-4.** Relative mRNA expression of Bax-Bcl-2 in the vitrified-thawed and post vitrified oocytes at 9 h of maturation vs. oocytes at 15 h maturation (control) as determined by real time RT-PCR, normalized against 18S rRNA. Data of quantification are calculated by the $2^{-\Delta\Delta Ct}$ method. Values are shown as the mean ± SEM for each group. a,b Means without common superscripts differ at $P<0.05$. 
4.4.4 Bax-Bcl-2 Ratio in Vitrified Oocytes

From the above data a Bax-Bcl-2 ratio was generated at 3 time points. Figure 4-5 presents the ratio of Bax:Bcl-2 in the control, vitrified-thawed oocytes, and oocytes at 9 h maturation post vitrification and thawing. The oocytes post vitrification-thawing showed a Bax:Bcl-2 ratio of 1.2, and the trend was almost the same (1.3) following 9 h of maturation relative to control oocytes (15 h maturation), with a Bax:Bcl-2 ratio of 1.

![Figure 4-5. Bax-Bcl-2 ratio in the vitrified-thawed and post vitrified oocytes at 9 h of maturation and control oocytes, as determined by real time RT-PCR normalized against 18S rRNA.](image)

4.4.5 Caspase Activity

Representative digital images illustrating caspase activity in embryos derived from vitrified oocytes from 1-cell to embryos at >6 cell stage are shown in Figure 4-6. The 1-celled embryo did not show caspase activity in the cytoplasm (Figure 4-6a). Remarkably, no induction of caspase activity could be detected in all stages of embryo development. A barely detectable level of caspase was seen near the area of polar body degeneration and the borders of few blastomeres (Figure 4-6a to 4-6e).
Figure 4-6. Representative confocal projections showing fluorescent labeling of active caspase through d 4 bovine embryos derived from vitrified medium sized follicle oocytes. a) 1-cell embryo, no caspase activity detected in the cytoplasm; b) 2-cell embryo showing a slight dispersed pattern of caspase in the border of blastomere; note (arrow) the presence of caspase in the area of polar body extrusion in a) and c). d) embryo with caspase localized within a blastomere (arrow); e) the pattern of caspase distribution is detectable, dispersed and not localized within blastomere.
4.4.6 TUNEL

The representative confocal images of bovine blastocysts derived from oocytes of medium sized follicles subjected to TUNEL analysis (Figure 4-7). A blastocyst derived from a control oocyte (medium) is shown in Figure 4-7c. Arrows point to TUNEL positive nuclei. Figure 4-7d shows blastocyst derived from an oocyte (medium sized follicle) subjected to vitrification. Note that this blastocyst has no incidence of TUNEL positive blastomeres. Indeed, the percentages of blastomeres undergoing apoptosis were not significantly different ($P > 0.05$) in blastocysts originating from control and vitrified oocytes. Propidium iodide staining demonstrated that vitrification reduced ($P < 0.05$) total number of cells per embryo derived from vitrified oocytes than control oocytes.

No differences ($P > 0.05$) in the apoptotic index were found in embryos generated from the control vs. vitrified oocytes. The apoptotic index for the blastocysts derived from control oocytes was 10.9 vs. 11.8 for the blastocysts derived from vitrified oocytes.
Figure 4-7. Confocal images of apoptotic nuclei in bovine blastocysts by TUNEL assay. a) control blastocyst, propidium iodide (red) b) control, fluorescein isothiocyanate (green), c) control, superimposed image, d) vitrified blastocyst, superimposed. Arrows point to TUNEL positive nuclei

4.5 DISCUSSION

The TaqMan Gene Expression Cells to C\textsubscript{T} technology enabled reverse transcription and RT-PCR analysis of lysates employing 25 oocytes, constituting 2.25 equivalent oocytes per PCR reaction without isolating or purifying RNA. A recent study (Hartshorn et al., 2005) employed a single tube approach utilizing a single embryo or blastomeres processed from lysates to RT-PCR eliminated loss of material and allowed precise quantification of genes. In our study bypassing the RNA isolation step helped to eliminate possible loss of template molecules, thereby avoiding procedures such as phase separation and recovery, repeated washing and resuspension of nucleic acid pellets, and elution from binding matrices substantially expediting and simplifying gene expression analysis of oocytes. Although quantitative analysis of transcription levels in single
cells or small numbers of cells remains technically difficult, several methods of RNA isolation from small samples have been available from manual and commercially available kits. Most of the methods worked well when the starting material was comprised of hundreds of cells or more, but many methods are not suitable for nucleic acid extraction from very few cells. The reason is that most procedures require a number of steps that can lead to incomplete RNA recovery or low RNA yield when elution is attempted in very small volume from small numbers of cells (Hartshorn et al., 2005). The method employed in our study is easy to use, eliminates loss of material, and RT and PCR could be carried out immediately after cell lysis, avoiding cumbersome and lengthy nucleic acid preparation procedures. This method is sensitive and accurately quantifies mRNA in small amounts of samples such as 2.25 equivalents of oocytes. Increased sensitivity to detect transcripts was illustrated by the fluorescence signals from TaqMan probes, which was useful in preventing primer dimers or nonspecific products.

To our knowledge the abundance of apoptotic gene transcripts in vitrified oocytes derived from follicles of varying sizes, small (≤4mm) vs. medium (4 to 10 mm), has not yet been reported. For the evaluation of candidate genes in real time RT-PCR an appropriate housekeeping gene is critical in gene expression studies. The ideal internal control should be expressed at a constant level among cells at all stages of development and should be unaffected by various experimental conditions (Bustin, 2000). Using this method, 18S rRNA transcripts over the period of in vitro maturation including post thaw vitrified oocytes varied within a narrow range of 0.8 and 0.5 cycles respectively, for oocytes derived from small and medium follicles, which establishes the accuracy of the method and reflects the stability of its expression during IVM. Our results support Aswal et al. (2007) with stable expression of 18S rRNA over the period of in vitro maturation (IVM) of buffalo oocytes at 5 time points.
4.5.1 *Upstream Signaling of Apoptosis in Vitrified Oocytes*

The results show that Fas, FasL, Bax, and Bcl-2 mRNA are present in small and medium oocytes from GV to MII stage. The expression of these genes in vitrified oocytes from medium sized follicles was not different from that of oocytes from small sized follicles. These findings indicate that oocytes contain the biochemical machinery responsible for programmed cell death to ensure rapid removal of dead cells at appropriate times.

The Fas-FasL signaling interaction is one of the fundamental measures for the induction of apoptosis. The Fas antigen belongs to a conserved family of membrane receptors known as the tumor necrosis factor receptor or TNFR family. Fas is a cell surface receptor that exists in two forms: transmembrane and soluble. The former triggers apoptosis when bound by FasL, activating cascade caspases that results in death (Nagata, 1997). Fas ligand exists as a soluble and cell associated molecule that engages the Fas antigen and initiates programmed cell death (Suda et al., 1993). Fas ligand mRNA was found both in cumulus cells and oocytes, but its protein was found only in cumulus cells (Rubio-Pomar et al., 2004).

In this study we found 1.4 and 2.13-fold increase of Fas and FasL mRNA in vitrified oocytes compared to the 15 h control maturation group, indicating a potential role for these molecules in the initiation of apoptosis. These results showed that oocytes irrespective of their size have an endogenous Fas pathway that was activated under conditions of stress. Surprisingly, the levels for both Fas and FasL mRNA showed a similar pattern of reduction at 9 h of maturation post vitrification. Altered expression for apoptosis genes, Fas, survivin and caspase-3, and reduced developmental competence subsequent to vitrification was reported in bovine blastocysts (Park et al., 2006).
The fate of mRNA transcribed during the oocyte growth can differ considerably. Some RNA becomes translated immediately; others are temporarily stored and recruited for translation at defined periods of oocyte growth, maturation or embryogenesis. Still, others are either deadenylated or degraded at specific stages of maturation (Eichenlaub- Ritter and Peschke, 2002). There are reports in mouse (Bachvarova, 1985) and bovine (Dalbies- Tran and Mermillod, 2003) oocytes that a large proportion of the transcripts stored within the oocyte cytoplasm were believed to disappear during maturation. This degradation may be coupled with active translation that occurs during oocyte maturation and results in protein synthesis pattern (Kastrop et al., 1991). Therefore, in our study it is not known whether an increase in Fas antigen protein occurs concomitantly with increased Fas antigen mRNA. Additionally, one class of proteins known as inhibitors of apoptosis (IAP) prevents apoptosis in response to a variety of stimuli by inhibiting caspase activity (Liston et al., 1996; Deveraux et al., 1997) in healthy granulosa and theca cells. As long as survival pathways like IAP are functional within the healthy cell, it is likely that FasL fails to induce apoptosis. Additionally, Vickers et al. (2000) reported that ovarian cells were resistant to FasL mediated killing in vitro until activated by cytokines. An elevated expression of the apoptotic genes Fas-FasL in our study could clearly establish that Fas-FasL pathway is operational in vitrified oocytes, but based on the findings described above, Fas-FasL as the ultimate pathway leading to apoptosis in vitrified oocytes needs further downstream studies. Though an increase in expression of mRNA for Fas and FasL detected in vitrified oocytes, the significant reduction in the abundance upon maturation necessitates analyzing the expression of proteins for these mRNA post vitrification maturation period.

We determined the expression of Bax and Bcl-2 in the vitrified oocytes from small and medium sized follicles. Analysis of Bax -Bcl-2 mRNA expressions indicated that, vitrification
resulted in an elevation in the levels of Bax, but the effect was not significant between the sizes of oocytes. Although the level of Bcl-2 was nearly the same after vitrification, an increased fold change in Bax expression clearly makes the ratio of Bax:Bcl-2 favor death inducer levels. Following maturation after vitrification, the levels of both Bax and Bcl-2 still remained almost the same; surprisingly the ratio still favored a higher Bax level showing a tendency towards apoptosis. Though Bax:Bcl-2 ratio is high in oocytes subjected to vitrification and thawing and ensuing maturation, the fate (survival or death) of oocytes will finally be determined depending on the nature of other factors such as external stimuli, internal defects, and activation of other apoptotic related genes (Liu et al., 2000). The balance in favor of survival may be restored by embryonic transcription or translational recruitment of maternal mRNAs encoding cell survival factors.

The B cell lymphoma-2 family of protein includes both inhibitors (Bcl-2) and promoters (Bax, Bcl-2 associated X protein) of apoptosis. Bcl-2 is a membrane associated protein that resides in the nuclear envelope and mitochondria. Its pro-survival function is by modulating the mitochondrial release of cytochrome c and the interaction of Apaf-1 with caspase-9, or by binding to Bax, (Luo et al., 1997). Bax is a Bcl-2 homologue that resides either in the cytoplasm or in the cell membrane and thus, can antagonize the protective role of Bcl-2. Inherent differences in the level of gene expression for Bcl-2 family members (Jurisicova and Acton, 2004), which may determine an oocytes developmental potential in the direction of either death or survival were reported in human oocytes. In support, Van Soom (2007) reported that the different balance in the expression of pro- and anti-apoptotic genes may be a crucial factor in determining the quality of the oocyte.
Eichenlaub- Ritter and Peschke (2002) provided evidence that a disturbance in gene expression at an early stage of oogenesis may interfere severely with maturation of oocytes at a much later time. Similarly, alterations in the expression of apoptotic genes in the oocytes consequent to vitrification may interfere with the development of embryos later, because after vitrification a majority of apparently normal oocytes fail to develop during the first few days of culture and undergo degeneration (Men et al., 2003). Since no information is available regarding the regulation of genes under study, this study recommends performing the quantitative analysis of the protein profiles of the tested genes in the vitrified oocytes and surviving embryos with respect to assessment of quality and environmental disturbances since mRNA will be eventually degraded in the developing embryos.

4.5.2 Downstream Signaling of Apoptosis in Embryos Derived from Vitrified Oocytes

In the present study, barely detectable levels of caspase activity could be observed from d 1 to d 4 embryos derived from vitrified oocytes. This clearly shows that there is little change in the degree of apoptosis as embryos undergo further development through d 4 after insemination. Similarly, our study clearly showed that blastocysts derived from vitrified oocytes were similar to those from control non vitrified oocytes with respect to percentage of TUNEL positive cells, though the total cell number of embryos was reduced. Similarly, Roth and Hansen (2004) reported that stress in response to heat shock during oocyte maturation can promote an apoptotic response mediated by capsases, disrupting its ability to support early embryonic development. Notwithstanding, the blastocysts derived from heat shocked oocytes similar to physiologically normal oocytes did not have changes in the total cell number, percentage of TUNEL positive cells and activity of groups II caspases. Our results could support the report by Schroeder et al.
(1990), in which the damaging effects of freezing-thawing were apparent only up to the 2-cell stage and beyond this stage no further effects were observed on the development to the morula or blastocyst stages. In this respect, as mentioned earlier, since inhibitors of apoptotic proteins (IAP) have a role in binding and inhibiting active caspase, it could be better to focus on some detection methods of IAP in embryos derived from vitrified oocytes.

In conclusion, we described a novel, easy and sensitive method for the quantification of gene transcripts using real time RT-PCR. The shift in the abundance of the apoptotic genes determined in the vitrified oocytes may impair the oocyte’s ability to respond to signals for continued development. No evidence of the downstream apoptotic signaling pathway in embryos derived from vitrified oocytes could be detected.
4.6 LITERATURE CITED


CHAPTER FIVE

5.1 SUMMARY

The cryopreservation of gametes is important in animal species considering widespread application of assisted reproductive technologies such as preserving endangered species and providing oocytes for in vitro fertilization (IVF) and somatic cell nuclear transfer (SCNT). Oocyte vitrification resulting in the production of blastocysts was reported in bovine species (Martino et al., 1996) a decade after the first report of successful vitrification of mouse embryos (Rall and Fahey, 1985). Since then, many attempts have been made to improve the multi-step process of vitrification of bovine oocytes by refining the procedures of vitrification. Oocyte vitrification, however, is less successful, because of impaired developmental potential and the absence of comprehensive information on the status of essential biological attributes of oocytes after warming (Ledda et al., 2007). The two most important variables which determine the success of vitrification involves speed of cooling and the concentration of cryoprotectants used. The toxicity of cryoprotectants can be minimized by increasing the cooling rate. Several studies emphasized the hostile effects of cryopreservation at the organelle/sub cellular level. According to Fahy (1986), no cryopreservation technique allows 100% cellular survival after thawing.

Special open and closed carriers were used in the process of vitrification during the last two decades. Yet, the most widely emphasized concerns of the vitrification procedures are toxicity effects due to high concentration of cryoprotectants and the danger of contamination due to direct contact with liquid nitrogen (Kuwayama, 2007). Factors most likely to affect the success of bovine oocyte cryopreservation are the particular structural and functional characteristics of the oocyte, such as its size (Liebermann et al., 2002), cumulus oocyte complex (COC), maturation status (Hochi et al., 1998) and dynamic of subcellular organelles during
meiosis (Shamsuddin et al., 1996). A clear correlation emerged between follicular size and development potential of oocytes in cattle (Pavlok et al., 1992). None of the studies reported so far were directed towards the cryotolerance potential of oocytes from different follicle sizes. The study of apoptosis and its influence on oocyte and embryo quality has great relevance for the in vitro production of embryos where a very heterogeneous oocyte population, at different stages of growth and atresia, is used to produce embryos (Anguita, 2007). Every cell has an apoptotic cell death pathway. Several studies showed that oocyte survival was controlled by a number of cell death regulatory molecules responsible for either activation or suppression of cell death (Tilly, 2001). Yang and Rajamahendran (2002) explained that in bovine oocytes the ratio of Bax to Bcl-2 may be used to gauge the tendency of oocytes for either survival or apoptosis. Later, Men et al. (2003) reported that cryopreserved bovine oocytes degenerate via the process of apoptosis during subsequent culture.

Though several workers have extensively investigated structural and morphological damages induced by cryopreservation, very little information is available on biochemical and molecular events related to apoptosis induced by cryopreservation on mouse embryos and bovine oocytes.

The use of nylon mesh provides an efficient and attractive method of vitrification of large numbers of cumulus-intact murine zygotes. The method is inexpensive and easy to perform. This method is promising, since around 40 mouse zygotes at a time could be vitrified successfully with high developmental rates. Although in vitro development of nylon mesh vitrified embryos differed from control non vitrified embryos, the similar result for the hatched blastocyst rates, obtained in the vitrified and control embryos shows the usefulness of the method. Despite the effectiveness of this method, there was a high rate of degeneration noticed in embryos after
thawing, and in the culture, indicating further refinement of the procedures like rate of cooling, nature and concentration of the cryoprotectants used. The differences in gene expression, consequent to vitrification may explain some of the observed differences in further development of the preimplantation embryos. We provide strong evidence that fragmented embryos consequent to vitrification degenerate through an apoptotic cell death mechanism after warming and there is increase in the relative abundance of apoptosis related genes in the vitrified pre-implantation embryos. The presence of Fas-FasL mRNA in the 4-cell and morula stage embryos showed that the apoptotic processes might be present in these developing embryos. There was an earlier report that Fas expression may not always lead to apoptosis (Payne et al., 1999). Though the apoptotic process is triggered in embryos, further development to blastocyst and increased hatched blastocyst rates showed the rescuing of the pre-implantation embryo from undergoing apoptosis so as to facilitate implantation.

The refinement of the vitrification protocol adopted for mouse zygote vitrification for bovine oocyte vitrification resulted in high survival of bovine oocytes, a rate higher than the earlier reports for bovine oocyte vitrification using nylon mesh. The pre-cooled cryovials coupled with 20% fetal bovine serum (FBS) might have had some beneficial effects on the survivability of oocytes following vitrification. In contrast to small diameter devices like open pulled straw (OPS), in this method removal of the cumulus cells partially or fully before vitrification protocol is not required. The striking feature of this method was retention of the cumulus cells post vitrification thawing. The role of cumulus cells at the time of in vitro insemination to maximize the incidence of the acrosome reaction in frozen-thawed bovine spermatozoa and fertilization and subsequent development in vitro should not be disregarded at this point. Furthermore, 6 to 8 h of maturation (inductive phase) is the stage at which the
cumulus cells act in a particularly crucial supportive and regulatory role in RNA synthesis (Gordon, 2003). In our study oocytes were vitrified after 15 h of maturation and even if there were alterations in the morphology of cumulus cells, by the time of vitrification their supportive role might have been completed. The degree of oocyte maturation, from the germinal vesicle stage (GV) to the metaphase-II (MII) stage, was shown to affect survival post-vitrification (Parks and Ruffing, 1992). The nuclear maturation at the time of vitrification of oocytes in our study was the MI stage. In the MI stage oocyte microtubules were well organized near the meiotic spindles. Though the temporary loss or clumping of microtubules might have been induced by vitrification on MI oocytes, they may be able to repair such changes during 9 h of further incubation. This is the second report of MI bovine oocyte vitrification in the literature besides Hochi et al. (1998), but we showed twice as high blastocyst rates. In comparison to recent results obtained for bovine oocyte vitrification from different laboratories, the results obtained in our study are similar to or higher than most of the recent reports. Our results provided compelling evidence that follicle size for oocytes had a high impact on the developmental rates of embryos from cleavage to blastocyst stage. Nylon mesh vitrification in a closed container is a cheap and useful method for oocyte cryopreservation, resulting in high survival of cumulus-intact vitrified oocytes, and allows a reliable method for isolation of oocytes from liquid nitrogen.

In an attempt to find the relationship between post IVF outcome and field fertility based on 70 d non return rate using calculated estimated relative conception rate (ERCR), there was little evidence from our study that post IVF outcomes provided a useful predictor of field fertility of bulls based on ERCR.
Our study describes a novel method for the quantification of gene products from small samples like oocytes using real time RT-PCR without isolating or purifying RNA. The method employed is easy to use, eliminates loss of material, and opens new opportunities for the understanding of molecular events following cryopreservation. This method is sensitive and accurately quantifies mRNA in small amounts of samples such as 2.25 oocyte equivalents.

The Fas-FasL signaling interaction is one of the fundamental measures for the induction of apoptosis. The level of Bax:Bcl-2 determines whether a cell should undergo death or should survive. The follicle size has no impact on the expression of apoptotic genes under study. All transcripts showed an increased change post vitrification thawing. The up-regulation of apoptotic genes might be an early indicator of poor survivability of oocytes post vitrification and thawing. Surprisingly, the levels tended to decrease following 9 h maturation post vitrification. The increased Bax:Bcl-2 ratio after post vitrification thawing remain unaltered even after 9 h of maturation post vitrification thawing, showing a ratio towards apoptosis. Both terminal deoxynucleotidyl transferase dUTP nick end (TUNEL) labeling and caspase assays did not show any evidence of apoptosis in blastocysts derived from vitrified oocytes. This shows the absence of a downstream apoptotic signaling pathway in embryos derived from vitrified oocytes. This suggests that the damaging effects of vitrification and thawing might have not passed to the subsequent embryos.

5.1.1. Future Research

By adopting the method of nylon mesh vitrification, cryopreservation of large number of embryos of any species can be attempted within a short period of time. The factors responsible for the rescuing of embryos from the process of apoptosis triggered in the developing embryos
consequent to vitrification will be helpful to decrease the rate of demise in the in vitro developing embryos, and thus, increase the chances of implantation in any species.

Men et al. (2003) reported that more than 90% of oocytes were viable just after warming. Nevertheless, the detrimental effect of cryopreservation was expressed gradually during in vitro culture. Since no information is available regarding the regulation of apoptotic genes under study, this study recommends performing the quantitative analysis of the protein profiles of the tested genes in the vitrified oocytes and surviving embryos with respect to assessment of quality and environmental disturbances since mRNA will be eventually be degraded in the developing embryos. Since no caspases could be detected even in 1 and 2-celled embryos derived from vitrified oocytes, some methods to detect inhibitors of apoptosis (IAP) proteins, which have a role in binding caspase and inhibit caspase activity, could be detected in embryos derived from vitrified oocytes. Our study provided strong evidence to question the usefulness of IVF to predict the field fertility performance of bulls based on non return rate. Final conclusions on the usefulness of in vitro results will require work on a large species.
5.2 LITERATURE CITED


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PUBLICATIONS


ABSTRACTS
