ADVANCES IN THE FUNDAMENTAL CRYOBIOLOGY OF MAMMALIAN OOCYTES

A Dissertation presented to the Faculty of the Graduate School at the University of Missouri-Columbia In Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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OF MAMMALIAN OOCYTES

presented by Steven Francis Mullen,
a candidate for the degree of doctor of philosophy, and hereby certify that, in their opinion, it is worthy of acceptance.

______________________________
Professor John K. Critser

______________________________
Professor Yuksel Agca

______________________________
Professor Heide Schatten

______________________________
Professor Randall Prather

______________________________
Professor Edmund Rucker III
My parents

more than anyone else

are responsible for who I have become.

I would like to dedicate this work to my Mother

Kathleen Marie (Bird) Mullen

and my Father

Timothy Jerome Mullen.
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ADVANCES IN THE FUNDAMENTAL CRYOBIOLOGY OF MAMMALIAN OOCYTES

Steven Francis Mullen

Dr. John K. Critser, Dissertation Supervisor

ABSTRACT

Having effective means to cryopreserve mammalian oocytes could increase the efficiency of managing populations of laboratory animals, increase the effectiveness of breeding programs for livestock, and improve the means by which assisted reproductive therapy is applied to human patients. Unfortunately, for most mammals oocyte cryopreservation suffers from inefficiencies. The work completed in this dissertation was directed at advancing our knowledge of the fundamental cryobiological properties of oocytes from cows, pigs, and humans. The first series of experiments was designed to determine the likelihood of damage to the metaphase II spindle from osmotic stress. Increasing levels of hypotonic and hypertonic stress resulted in an increased proportion of oocytes displaying a damaged spindle as assessed by immunocytochemical staining. Human oocytes appeared more sensitive to hypertonic stress compared to oocytes from cows and pigs. Hypotonic stress caused more damage to cow oocytes compared to human and pig oocytes. Pig oocytes were also shown to lose in vitro developmental potential, and the proportion damaged was greater compared to the proportion showing damage to the spindle. The permeability of mature human oocytes to ethylene glycol and water was also determined. It was shown that the permeability was temperature dependent. The results from the osmotic tolerance and membrane permeability studies for human oocytes was used to develop a theoretically-optimized procedure for vitrifying human oocytes in standard 0.25 cc straws.
CHAPTER 1. AN INTRODUCTION TO MAMMALIAN OOCYTE CRYOPRESERVATION

Outline of the Study

The research described in this thesis was conducted to improve our understanding of the fundamental cryobiology of metaphase II (MII) oocytes from humans, cows, and pigs. With the exception of the first and last chapters, which present an overview of the current state of oocyte cryopreservation, and a discussion on future prospects, respectively, the remaining chapters are designed to be stand-alone manuscripts published in peer-reviewed journals and technical books. The second chapter describes the immunochemical staining technique which was developed as a means of assessing the morphology of the MII spindle in mammalian oocytes. The third and fourth chapters describe the effects of anisosmotic exposure on the morphology of the MII spindle from human and bovine oocytes. The fifth chapter describes the effects of anisosmotic exposure on the morphology of the MII spindle and in vitro developmental potential in porcine oocytes. The sixth chapter describes the permeability of human MII oocytes to ethylene glycol (EG) and water in the presence of EG, the associated activation energies, and an analysis of the data by which a method for vitrification of human oocytes in a closed system was developed.
The Science of Cryobiology*

Introduction

The demand for effective bio-preservation methods in the medical community continues to increase with advances in transplantation and transfusion medicine (Komender, 2004). In reproductive medicine, pre-implantation embryo cryopreservation has become an integral component of overall patient care, increasing the success rate per oocyte retrieval cycle (Toner et al., 1991; Schnorr et al., 2000). Oocyte cryopreservation is becoming increasingly important due to legal restrictions on the creation and transplantation of supernumerary pre-implantation embryos as well as ethical considerations surrounding the cryopreservation of pre-implantation embryos (Benagiano and Gianaroli, 2004; Bankowski et al., 2005).

Early investigations into the effects of sub-physiologic temperatures on living cells have been reviewed in great detail (Luyet and Gehenio, 1940). The current chapter will attempt to provide a broad overview of cryobiology, and refer to the reproductive biology literature when appropriate. Readers interested in learning more details are directed at several excellent texts and reviews on the various

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*This section will be published as a full chapter in the text, titled Oncofertility, edited by Woodruff, T and Snyder, K. to be published in 2007 by Springer.
Anatomy of Cryopreservation

Cryopreservation is the successful preservation of the normal function of cells or tissues by a reduction in temperature below which normal chemical reactions take place. It is not the long-term storage of cells at these temperatures that is damaging, but the progression to these temperatures and back to normothermia that results in cryoinjury. Cryopreservation nearly always entails the use of one or more compounds that confer protection to cells during freezing. These so-called cryoprotectants (CPA) are typically very simple, low molecular weight molecules with high water solubility and low toxicity. One feature that is common among these compounds is their ability to interact with water via hydrogen bonding (Ashwood-Smith, 1987). Application of CPA is done (in most cases) simply by incubating the cells in solutions in which these compounds are dissolved. After this exposure, the cells are cooled to a low sub-zero temperature (specimens are typically held at the temperature of liquid nitrogen; -196 °C). At the appropriate time, the specimen is warmed, washed free of the CPA, and used in whatever manner is deemed appropriate. While this seems like a
relatively straightforward procedure, many types of injuries can result from any one of the steps; thus numerous lethal effects need to be avoided.

**The Effects of Water Precipitation (as ice) During Cooling**

**Ice Nucleation, Crystallization, Vitrification, and Devitrification**

Figure 1.1 shows a phase diagram for a generic aqueous solution. The major physical transitions which occur as a result of cooling are described with such a diagram. The temperature at which these transitions occur depends upon the concentration of solutes in the solution. The curve labeled $T_m$ describes the melting temperature of the solution (which is also the equilibrium freezing temperature). The dashed extensions of the curves represent extrapolations across hypothetical boundaries. As the solute concentration of the solution increases (moving along the X-axis from left to right), the melting temperature of the solution decreases. This is a well known result of the colligative effects of the solutes (Williams *et al.*, 1978). The curve labeled $T_h$ characterizes the homogenous nucleation temperature. Homogenous nucleation is defined as the nucleation of ice crystals in the absence of nucleating agents. Aqueous solutions will usually crystallize at temperatures much higher than this due to impurities which act as effective nucleators. A solution containing pure water (*i.e.* free of heterogeneous nucleators) will remain liquid down to $-39 \, ^\circ C$, at which point the
Figure 1.1. A phase diagram for a hypothetical solution is shown. The concentration and temperature dependent physical transitions, including melting ($T_m$), homogenous nucleation ($T_n$), glass formation ($T_g$), and devitrification ($T_d$), are described by the respective curves. The use of such diagrams allows the calculation of variables which are important considerations for freezing injury (Cocks and Brower, 1974). Figure reproduced from (Fahy et al., 1984) with permission from Elsevier. See text for further details.
entire solution will crystallize (the point where $T_h$ intersects the Y-axis) (Fahy et al., 1984). This temperature decreases with increasing concentration of solutes.

The reason why water can remain liquid below its melting point is because the creation of a crystal entails the creation of a liquid-crystal interface with an associated interfacial free energy. The size of a thermodynamically-stable crystal (i.e. one that will continue to grow by the addition of water molecules) is dependent upon temperature (smaller crystals are more stable at lower temperatures). So as the temperature is lowered, the probability of formation of a stable crystal increases until $T_h$, where the probability is 1 (Fahy et al., 1984). The curve labeled $T_g$ represents the glass transition temperature. At this temperature, liquid solutions will transition to a stable glass (vitrify) and remain vitreous upon further cooling.

In region I of this chart (solutions with concentrations $\leq \sim 45$ wt% (weight/weight) in this example), achieving true vitrification is practically impossible. Nucleation (both homogenous and heterogeneous) is essentially unavoidable (at least with practical cooling rates). In region II, the difference between the $T_h$ and $T_g$ curves is small enough that vitrification can be achieved with practical cooling rates. However, a solution which does attain a vitreous state in this region is thermodynamically unstable. In the regions marked III and IV, vitrification is easily achievable. Notice that the $T_h$ curve actually intersects $T_g$ at the transition
between regions II and III. Heterogeneous ice formation will not occur at
temperatures below the glass-transition temperature.

If a sample with a composition described by regions II and III is cooled fast
enough to vitrify, ice may still form during warming due to nucleation and ice
growth. The temperature at which this happens is described by $T_d$, the
devitrification temperature. Essentially, between $T_g$ and $T_m$ (melting
temperature), a solution free of ice is in a metastable state. Because the solution
is no longer a glass (the glass “melts” to form an unfrozen liquid with the
molecules having translational mobility), nucleation and ice growth can occur
during warming.

Why is this so? Consider the following two facts: 1) The probability of ice
nucleation increases as temperature decreases (notice that as temperature
decreases, the sample will get closer to $T_h$; 2) crystal growth, however, being a
kinetic phenomenon, will be faster at higher temperatures. Now, imagine the
following scenario. A sample with a solute at a concentration of 40 wt% is cooled
very quickly so that nucleation occurs only at the temperature around $T_h$ (~ -70
°C). However, at -70 °C, ice crystal growth is very slow (at least relative to higher
temperatures). Thus, crystal growth is minimal, especially considering that
cooling is still taking place. However, during warming, once the sample gets
above $T_g$ (~ -120 °C), ice crystals can grow from the nucleation sites. However,
it will still be slow, but will increase as the sample warms. Because there were no
ice nuclei above -70 °C during cooling, ice growth at higher temperatures could not happen. However, ice nuclei are present during warming and crystal growth can occur until $T_m$. Therefore, if nucleation does occur during cooling, warming must be very fast to avoid crystal growth at temperatures below $T_m$. The striking differences between the critical cooling rate (cooling rate necessary to achieve vitrification) and the critical warming rate (defined as the warming rate necessary to avoid more than 0.2% crystallization during warming) are illustrated by the analysis of Baudot and Odagescu (Baudot and Odagescu, 2004). According to their calculations, for a 40 % (w/w) solution of ethylene glycol in water, the critical cooling rate is 569 °C/min, but the critical warming rate is $1.08 \times 10^{10}$ °C/min.

**Solute Concentration as a Result of Ice Crystallization, the Associated Osmotic Effects, and Cell Death at Supra-optimal Cooling Rates**

As water precipitates from a solution as ice during cooling, only water molecules comprise the ice crystals. As a result, all other components (salts, etc.) become concentrated in the remaining solution. As the solution concentration increases, the chemical potential of the water in the solution decreases. Water will continue to crystallize until the chemical potential of the water in the liquid phase equals the chemical potential of the water in the solid phase. In other words, the remaining solution will reach its equilibrium freezing point (the curve defined by $T_m$). Therefore, the concentration of the remaining liquid phase can also be determined from a phase diagram.
For example, assume that Figure 1.1 represents the phase diagram of a sodium chloride-water binary solution. If you start with an isotonic saline solution (0.9 wt%) and cool it to -20 °C, ice will form until the remaining solution is at its equilibrium melting point. In this example, the remaining solution will attain a concentration of ~ 45 wt% (note the point where the T_m curve reaches -20 °C). In this hypothetical example, the unfrozen solution would be roughly 14 M sodium chloride (compared to 0.15 M initially). In reality, sodium chloride will only concentrate ~ 4 M at -20 °C, which is still an impressive concentration effect.

When cells are frozen in suspension, the cells are sequestered in the channels of concentrated unfrozen medium. The high concentration of the remaining unfrozen solution establishes an osmotic gradient across the cell membrane, and as a result, water will flow out of the cell via exosmosis (Soodak and Iberall, 1978). Below a cell’s equilibrium freezing point, the cytoplasm is in a supercooled state. If the sample is cooled slowly enough, exosmosis occurs to a sufficient degree to keep the cytoplasm in a near-equilibrium state with the extracellular solution. Such a situation will preclude intracellular ice formation (IIF). On the other hand, if the cooling rate is relatively rapid, water cannot leave the cell fast enough to maintain a near-equilibrium state with the extracellular solution, and at some point equilibrium will be re-established by ice formation (see Figure 1.2). This situation is described schematically in Figure 1.3. The formation of intracellular ice is usually (but not necessarily) fatal to cells (see
below for more details). Direct cryomicroscopic observation of intracellular ice formation in mouse oocytes (similar to that which is seen in Figure 1.2) and the correlation to cell survival were some of the most convincing data to support the assertion that ice formation was the lethal.

The rate at which water flows out of a cell is dictated by the cell membrane water permeability. The permeability of cells to water is dependent upon several factors including temperature and the presence of CPA. For example, in the absence of cryoprotectant, human sperm water permeability is 1.84 μm/min/atm at 22 °C, but is reduced to 1.23, 0.84, 0.77 and 0.74 μm/min/atm in the presence of propylene glycol (PG), dimethylsulfoxide (DMSO), glycerol, and ethylene glycol (EG), respectively (Gao et al., 1997). Furthermore, water permeability can vary greatly across cell types. For example, water permeability for human erythrocytes (Terwilliger and Solomon, 1981) is an order of magnitude higher than the value for human oocytes (Hunter et al., 1992). Because intracellular ice formation is dependent upon the degree of supercooling (hence the water content), the rate of cooling which results in intracellular ice formation differs widely across cell types. The theory of cell death due to intracellular ice formation resulting from the interaction of cooling velocity and water loss outlined above was developed quantitatively by Peter Mazur (Mazur, 1963); (cf. Mazur 2004 for a recent review). The practical benefit of this theory comes from its potential to predict optimal cryopreservation procedures. For example, if one
Figure 1.2. Photomicrographs of intracellular ice formation in mouse oocytes cooled at 100 °C/min in an isotonic solution is shown. In Panel A, intact oocytes are shown prior to ice crystal formation; note the well-defined oolemma within the zona pellucida. As cooling proceeds, ice forms in the extracellular solution and eventually, the intracellular solution. The darker background in Panel B is due to ice, and the “blackening” of the oocytes indicates that intracellular ice formation has occurred. When the solution is warmed and the ice melts (Panel C), the cell membrane within the zona pellucida is no longer visible, indicating cell lysis. The speckled appearance of the background in Panels A and C is due to atmospheric water precipitation on the cold glass surface of the cell chamber, and not due to ice crystals.
Figure 1.3. The cooling-rate-dependent fate of intracellular water resulting from extracellular ice formation is shown. As the temperature of the solution is cooled below the equilibrium freezing point, ice will form in the extracellular solution. As a result, water is driven out of the cell by an osmotic gradient across the cell membrane. If cooling is slow (upper cell, right side), sufficient water leaves the cell and intracellular ice formation does not occur. If the cooling rate is faster, ice will form inside the cell, and the amount of ice that forms and the size of the crystals will depend upon the cooling rate. Intermediate cooling rates (middle cell, right side) result in partial cell dehydration, larger crystals, and less intracellular ice. Very rapid cooling rates result in virtually no cell dehydration, a greater amount of ice formation, but smaller crystals. If cells cooled very quickly (lower cell, right side) and warmed slowly, the small crystals will aggregate to form larger crystals (a process known as recrystallization). This will be more damaging to the cell compared to very rapid warming. Figure adapted from (Mazur, 1977) with permission from Elsevier.
knew the degree of cell dehydration necessary to prevent damaging ice formation and the membrane permeability, it would be possible to predict the cooling rate which would prevent intracellular ice formation and the temperature at which cooling could stop and the sample could safely be transferred to liquid nitrogen (Karlsson et al., 1993). See the original description of mammalian embryo cryopreservation for a relevant example (Whittingham et al., 1972).

Ice formation in the cytoplasm of cells is not necessarily damaging. Studies over the years have investigated the correlation between the morphology of cytoplasmic ice, cooling and warming rates, and survival (Bank, 1973; Shimada and Asahina, 1975). These studies showed that larger ice crystal size correlated positively with cell death. As discussed above for bulk solutions, devitrification and recrystallization can occur to cell water/ice during warming (Karlsson, 2001) resulting in the growth of large ice crystals (Bank, 1973). Recrystallization is the phenomenon whereby small ice crystals fuse to become larger crystals which are more stable at a given temperature. According to a more recent study, the formation of small ice crystals may actually be beneficial to cell survival (Acker and McGann, 2003). Such a result is likely due to the reduced level of osmotic stress.

Attempts have been made to explain the mechanism(s) which cause intracellular ice formation, and to date several theories have been put forth. As explained in more detail by Mazur (Mazur, 2004), theories of intracellular ice formation must
account for several experimental facts: (1) in order for ice formation to occur in cells above −30 °C, extracellular ice must be present, and the proximity of the cells and ice is important; (2) extracellular ice is not a necessary precondition for intracellular ice below −30 °C; (3) intracellular ice formation usually happens immediately if the cells and the surroundings are supercooled -15 to -20 °C and extracellular ice is rapidly initiated; (4) if extracellular ice forms near the cell’s equilibrium freezing point, intracellular ice usually does not form; and (5) the nucleation temperature decreases substantially if the extracellular solute concentration increases.

Several lines of evidence suggest that intracellular ice formation can be triggered from more than one mechanism. It is generally agreed that ice formation below -30 °C in the absence of extracellular ice is due to the presence of intracellular nucleators (or as a result of homogenous nucleation at lower temperatures). The fact that intracellular ice formation above this temperature requires the presence of extracellular ice strongly suggests that the extracellular ice is acting to nucleate the intracellular ice. As extracellular ice does not nucleate intracellular ice at low degrees of supercooling, an intact plasma membrane effectively blocks the passage of ice into the cell. However, the plasma membrane is implicated mechanistically in the major theories put forth to explain the initiation of intracellular ice formation.
Mazur (Mazur, 1965) hypothesized that ice crystals can grow through membranes via protein pores like aquaporins. While evidence exists that ice can grow through channels which connect cells (i.e. gap junctions (Acker et al., 1999; 2001; Irimia and Karlsson, 2005)), the pore size in these channels is much larger than those in aquaporins, making ice growth more likely. Mazur and colleagues are currently using genetic engineering techniques in oocytes as a means to test this hypothesis directly (cf. (Mazur et al., 2005a; 2005b; Guenther et al., 2006) for results from initial experiments). Toner and colleagues (Toner et al., 1990; 1993) have suggested that ice interaction with the plasma membrane causes a structural change to the inner membrane surface, resulting in an increase in the efficiency of ice nucleation. Muldrew and McGann (Muldrew and McGann, 1990; 1994) have put fourth a different mechanism altogether which suggests that ice grows through the membrane after the formation of a lesion as a result of the osmotic pressure gradient and resultant water efflux. A similar argument regarding the formation of a membrane lesion as a prelude to intracellular ice formation had been proposed by Steponkus and colleagues several years earlier (Dowgert and Steponkus, 1983; Steponkus et al., 1983).

Despite the debate as to the nature of intracellular ice formation initiation, the evidence that IIF is a very damaging (and usually lethal) event is overwhelming. Hence, preventing ice formation in the cytoplasm (at least to a large degree) during cryopreservation is critical.
Cell Death at Sub-optimal Cooling Rates and the Role of Cryoprotectants in Mitigating Cell Damage

Given that the probability of IIF decreases proportionally with cooling rate, an obvious question is: why not just cool cells at very slow rates to prevent damage from intracellular ice? The simple answer is because IIF is not the only cause of cell damage during freezing, and some of the other causes are more detrimental at lower cooling rates. When cell survival is plotted as a function of cooling rate, an inverted “U-shaped” curve is generated (Figure 1.4). The peak of this curve represents the optimal cooling velocity, cell viability being lost at both higher and lower cooling rates. The shape of this curve has been interpreted to suggest at least 2 mechanisms of cell damage, each of which is oppositely dependent upon cooling rate. As described above, damage due to IIF can explain the loss of cell viability at cooling velocities higher than optimal (the right side of the curve). The mechanisms responsible for the loss of viability at lower than optimal cooling rates are varied, and we still do not have a comprehensive understanding of the nature of these injuries. Nevertheless, a general idea has emerged as to the nature of some injuries which occur at slow cooling rates. One of the principal mechanisms of cell injury at suboptimal rates of cooling is directly tied to the nature of CPA.
Figure 1.4. The relationship between cooling rates and cell survival, for different cell-types, is shown. Higher water permeability allows faster cooling rates to be applied without a high probability of cell death due to intracellular ice formation. The specific optimal rate is also dependent upon other factors such as the presence of CPA and the warming rate. Figure redrawn from Mazur et al., 1970. See text for more details.
Cryoprotectants: Beneficial Effects

Cryoprotectants are defined in a functional manner as compounds that allow a higher degree of cell survival during freezing in their presence than in their absence (cf. Karow, 1969; Fahy, 1986; Ashwood-Smith, 1987; Fuller, 2004). Although our knowledge of the mode of action of CPA is still incomplete, it is likely that the effects of CPA are multi-factorial, and CPA of different classes (e.g. alcohols, sugars, diols, amides, large polymers) may act by different mechanisms (Fuller, 2004; Acker, 2007). One of the earliest theories of the mechanism of action of CPA was developed from a series of experiments investigating the protective action of glycerol on erythrocytes. James Lovelock, a physical chemist by training, studied the effects of salt concentration on hemolysis. In his initial experiment (Lovelock, 1953a) he investigated the relationship between the salt concentrations in partially frozen saline solutions which caused cell damage with the degree of cell damage when cells were exposed to the same salt concentrations without freezing. He determined that the degree of damage could be explained by the increase in salt concentration due to ice precipitation (Figure 1.5).

Since that time, others have repeated these experiments and confirmed Lovelock's original findings (Pegg, 1987; Pegg and Diaper, 1988). Lovelock proceeded to show that when cells are frozen in solutions containing glycerol, the temperature at which hemolysis began was progressively lower as the
amount of glycerol was increased (Lovelock, 1953b). As discussed above, solutes depress the equilibrium freezing point of a solution. By adding glycerol to a cryopreservation solution, the amount of water that freezes at any given temperature will be reduced. As a consequence, the final concentration of the salts in the remaining solution will also be reduced. Lovelock’s experimental results supported the conclusion that the colligative depression of the freezing point and concomitant reduction in salt concentration explained the protective mechanism by which glycerol exerted its effect (Figure 1.6). Hemolysis always began at the same concentration of sodium chloride (~0.8 M).

Several other modes of action have been proposed for CPA. One effect includes interacting with water molecules and altering the water structure in a solution, and reducing the ability of water to join the ice phase (Nash, 1966; Korber et al., 1982). Polymers can also facilitate vitrification upon cooling and reduce the concentration of permeating CPA necessary to attain a glassy state (Fahy et al., 1984). CPAs have also been shown to either directly interact with or be preferentially excluded from biosurfaces (e.g. the surface of lipid bilayers or proteins) (Carpenter and Crowe, 1988; Anchordoguy et al., 1990; Anchordoguy et al., 1991; Xie and Timasheff, 1997). The apparent opposite nature of these modes of interaction seems to suggest opposite effects.
Figure 1.5. The correlation between hemolysis and salt concentration for cells frozen or only exposed to salt is shown. The striking correlation lead Lovelock (Lovelock, 1953a) to conclude that the concentration of salt resulting from ice formation was a primary mechanism of cryodamage. Others have argued that the effect of the salt concentration on cell volume was the true cause of cell damage (Meryman, 1971). Redrawn from Pegg 1987. See text for more details.
However, each mode of interaction can be beneficial to the stability of these structures. In addition, Rudolph and Crowe (Rudolph and Crowe, 1985) have shown that trehalose and proline can prevent freezing-induced fusion of lipid vesicles. For more details on these mechanisms, interested readers are directed to recent reviews (Crowe et al., 1988; Crowe et al., 1998; Crowe et al., 2004; Acker, 2007) and references therein.

Perhaps it is not surprising that many organisms living in climates where freezing temperatures are encountered have evolved to include the metabolic production of CPA as a survival strategy. As discussed by Erica Benson (Benson, 2004) and reviewed by Ken Diller (Diller, 1997), the cryoprotective properties of sugars and glycerol in plants were described by Nikolay Maximov in the early 20th century (following on the work of others). The farsighted nature of his conclusions is remarkable considering what has been learned about CPA and their mechanisms since that time.

A great deal of research has been conducted to understand the response of various members of the animal kingdom to freezing temperatures. The metabolic production of CPA is also a common strategy in these organisms. Inhibition of freezing at high sub-zero temperatures is one strategy among arthropods and fish, and is accomplished by regulative supercooling. In many instances the freezing point of physiological solutions is regulated by thermal hysteresis proteins (Barrett, 2001). The tertiary structure of these proteins allows them
Figure 1.6. The presence of CPA reduces the salt concentration at a given sub-zero temperature due to a colligative reduction in the freezing point of the solution (shown here for a glycerol and sodium chloride solution mixture). Notice that, as the concentration of glycerol increases, the salt concentration is significantly reduced. Figure reproduced from (Mazur et al., 1981) with permission from The Biophysical Journal.
to directly interact with ice crystals due to polar residues along the protein backbone (Knight et al., 1984). These proteins lower the freezing point of water without significantly altering the melting point (this phenomenon, the separation of the temperature of ice growth and the melting point of a solution has been termed thermal hysteresis (Kristiansen and Zachariassen, 2005)). Thus, their mode of action is not colligative. According to a general model for their activity, these proteins bind to ice crystals and alter the radius of curvature of the growing crystal, which reduces the temperature at which it is thermodynamically favorable for additional water molecules to join the crystalline phase (Hew and Yang, 1992; Wilson, 1994) (cf. (Raymond et al., 1989) for more detail on an early model of the mechanism of fish hysteresis proteins, and (Kristiansen and Zachariassen, 2005) for a recent review on the mechanism of action). Overall, these proteins restrict ice growth when the environmental temperature is slightly below the equilibrium freezing temperature of the body fluids.

As an alternate strategy, organisms across many phylogenetic groups have developed mechanisms of regulating ice formation in situ. In naturally freeze-tolerant organisms, avoiding the formation of intracellular ice is managed by actively promoting and regulating the formation of extracellular ice. This allows freeze-induced dehydration of the cells and prevents ice from forming in the cytoplasm. As a coupled strategy, mechanisms to avoid the damaging consequences of cellular dehydration and ischemia that accompany freezing have also evolved.
Ken and Janet Storey, in a review on the subject (Storey and Storey, 1988), described four requirements for the successful freeze tolerance in animals: (1) ice must be confined to extracellular spaces and damage from ice crystals must be minimized; (2) the rate of freezing must be slow and controlled; (3) cell volume reduction beyond a minimum tolerable volume must be avoided; and (4) mechanisms must be present to prevent damage from resulting ischemia. These requirements are often met through behavioral and physiological adaptations. For example, slow, controlled temperature change is often facilitated by the chosen hybernaculum of the organisms. Controlled ice nucleation can be performed by specific ice nucleating proteins in the blood (Wolanczyk et al., 1990; Storey et al., 1992). Cryoprotectant synthesis (e.g. glucose production) in some organisms is initiated by freezing (Vazquez Illanes and Storey, 1993) (see the review by Storey and Storey (Storey and Storey, 1992) for more details). Membrane adaptations to cold have also been described (Hazel, 1984). As improved cryopreservation methods are sought, it is likely that attention paid to nature’s laboratory will provide insights into appropriate means to avoid cryodamage.

**Cryoprotectants: Detrimental Effects**

As their name implies, CPA are beneficial during freezing. Their use, however, is not necessarily benign. Since the time of Lovelock’s original work, it has been
pointed out (Fahy and Karow, 1977) and experimentally confirmed (Pegg, 1987) that the correlation between the freezing damage in the presence of glycerol and the associated increase in salt concentration is strongest at low levels of hemolysis. In addition, as the concentration of glycerol is increased, the concentration of salt that causes a given degree of hemolysis decreases, suggesting that high concentrations of glycerol contribute to cell damage during freezing (Figure 1.7). This suggests that high glycerol concentrations (particularly as a result of ice precipitation) contribute to the damage of cells frozen slowly. Similar results have also been shown for dimethylsulfoxide (Kahn, 1978; Fahy, 1980).

Injury from CPA is not limited to those which occur during freezing. Exposing cells to solutions containing CPA prior to cooling can be damaging due to an osmotic effect. Almost all of the commonly used permeating CPA have lower plasma membrane permeability coefficients compared to that of water. This relationship results in cells experiencing osmotically-driven volume excursions during cryoprotectant addition to and removal from the cell during the course of a cryopreservation procedure. It has been shown in numerous cell types that damage to cells can occur as a result from volume excursions alone (Williams and Shaw, 1980; Armitage and Mazur, 1984; Armitage et al., 1985; Mazur and Schneider, 1986; De Loecker and Penninckx, 1987; Gao et al., 1998; Zieger et al., 1999; Agca et al., 2000; Blanco et al., 2000; Koshimoto et al., 2000; Pukazhenthi et al., 2000; Songsasen et al., 2002; Adams et al., 2003; Agca et
al., 2004; Men et al., 2004; Mullen et al., 2004; Walters et al., 2004; Agca et al., 2005; Men et al., 2005; Walters et al., 2005). Furthermore, many studies have demonstrated a beneficial effect of prolonging cryoprotectant addition and/or removal a process which reduces the associated volume excursions (Shaw et al., 1991; Fiéni et al., 1995; Isachenko et al., 2004).

The rate of water movement across the plasma membrane is determined by several factors and can be described by equation 1:

\[
\frac{dV_w}{dt} = -L_p A R T \left( M_s e + M_n e - \frac{n_s}{V_w} - \frac{n_n}{V_w} \right)
\]  

[1]

where \( dV_w/dt \) represents the change in the cell water volume over time, \( L_p \) and \( A \) represent the cell membrane hydraulic conductivity and surface area, respectively, \( R \) and \( T \) represent the gas constant and temperature, \( M \) represents molal concentration, \( n \) represents the number of moles and \( v \) represents the cell volume (collectively, the terms in parentheses represent the concentration gradient across the cell membrane). The letters e, i, s, and n in the super- and subscripts represent the extra- and intracellular compartments, and permeating (s) and non-permeating (n) solutes respectively.

A concentration gradient of permeating CPA will also result in movement of these compounds across the cell membrane. The rate of change in intracellular
Figure 1.7. The correlation between hemolysis and salt concentration is greatest at low levels of cell damage. As the ratio of glycerol to sodium chloride (R) increases, the correlation becomes weaker, particularly at high levels of hemolysis. Figure redrawn from (Pegg, 1987).
cryoprotectant resulting from such a gradient can also be described by an
ordinary differential equation (equation 2):

\[
\frac{dn_s^i}{dt} = P_s A \left( M^e_s - \frac{n_s^i}{v_w} \right) ;
\]  

[2]

where \( \frac{dn}{dt} \) represents the change in the number of moles of intracellular
cryoprotectant over time, \( P_s \) represents the membrane permeability, and the
remaining variables are equivalent to those in equation 1. Here we have shown
the so-called 2-parameter membrane transport model (\( L_p \) and \( P_s \) are the
phenomenological parameters defining the permeability of the cell membrane to
water and cryoprotectant). A 3-parameter model incorporating an interaction
coefficient (\( \sigma \)) was proposed by Kedem and Katchalsky on the basis of
irreversible thermodynamics for membranes where water and solute move
through a common pathway (Kedem and Katchalsky, 1958). It has been argued
that the interaction coefficient is not applicable to biological membranes as water
and CPA usually travel through independent pathways. Furthermore, being
phenomenological in nature, a 3-parameter model is less parsimonious than a 2-
parameter model. Interested readers can find more details on this debate in a
recent review (Kleinhans, 1998).

Osmotic damage is often ascribed to the associated volume reductions
(Meryman, 1971; Williams and Shaw, 1980). Cell volume response can be
controlled during cryoprotectant addition and removal by modifying the
procedures for adding and removing these compounds (Gao et al., 1995). As a result, cryoprotectant addition and removal can be accomplished in a manner that prevents injury due to excessive volume excursions. Because the volume response of cells can be modeled on a computer when the parameters in equations 1 and 2 for the cells are known, one can proactively predict optimal methods for this process (cf. Gao et al. 1997 for a more thorough discussion).

True chemical toxicity is also a concern associated with the use of CPA (Baxter and Lathe, 1971; Fahy, 1986). This is particularly true for vitrification methods (see below) as very high concentrations of these compounds are necessary to achieve and maintain a vitreous state (a solid state free of crystalline structures). The precise nature of the toxic effects of CPA remains, to a large degree, uncertain. Fahy and colleagues have concluded that protein denaturation is not a general effect of CPA (Fahy et al., 1990). They offered an argument that effects on membranes could provide an alternate explanation to a direct effect on proteins that would be consistent with some data and proposed models. CPA have been shown to alter cytoskeletal components in mammalian oocytes, particularly the filamentous actin network and meiotic spindle (Johnson and Pickering, 1987; Vincent et al., 1990; Vincent and Johnson, 1992). Repolymerization after treatments is common, but the particular organization of the polymers often does not represent those of untreated oocytes. Frequently, toxicity is argued to be a significant cause of cell death in oocyte cryopreservation studies. However, rarely is the chemical effect isolated from the
osmotic effect in such experiments. In a previously unpublished experiment in our laboratory, the osmotic effects associated with exposure to 2.5 M PG and EG were controlled when assessing the effects of exposing mouse oocytes to CPA by including a treatment simulating the volume excursions associated with cryoprotectant addition and removal. The results suggested that the damage associated from exposure to PG was not a result of the osmotic effects, but a true chemical effect. Exposure to the same concentration of EG was not detrimental to mouse oocyte survival (Figure 1.8).

It was mentioned earlier that most CPA have hydrogen bonding capability, and altering water structure is one of the mechanisms by which CPA are hypothesized to function. It is also recognized that the toxic concentration differs for different CPA. In a more recent report, Fahy and colleagues have determined that a compositional variable they call $q v^*$ is directly associated with the toxic properties of a cryoprotectant when toxicity is non-specific. The proposal these authors make is that $q v^*$ is related to the degree of hydration of a cryoprotectant. In their report, Fahy et al. show that the total concentration of CPA was not as strong of a predictor of toxicity as was the ratio of the molarity of water ($M_w$) in the solution to the molarity of polar groups in the solution ($M_{PG}$; i.e. $q v^* = M_w / M_{PG}$). Polar groups were defined as hydroxyl groups (-OH), sulfoxide groups (-S=O), carbonyl groups (-C=O), and amino groups (-NH$_2$) on the cryoprotectant. In their experiment, they initially tested the toxicity of various CPA with differing $q v^*$ indices using rabbit renal cortical slices and examined the $K^+ / Na^+$ ratio after
exposure. As $q_v^*$ increased in the range from 2 to 6, the $K^+/Na^+$ ratio decreased from ~ 80% to ~ 10% relative to the controls.

The significance of the polar groups is such that they account for the interaction with water molecules, and compounds with a lower $q_v^*$ can interact with fewer water molecules. Such compounds do not form a vitreous state (i.e., form a glass) as easily as those with a higher $q_v^*$. Hence, weak glass forming CPA are less toxic. Using this new information, the investigators were able to predict and confirm that substitution of PG (a very good glass former) with EG (a very poor glass former) in a previously developed vitrification solution (VS41A) would be a superior vitrification solution using rabbit renal cortical slices and mouse oocytes. In previous reports, others have shown that the protective potential of CPA was correlated to the molarity of potential hydrogen bonding groups (Doebbler and Rinfret, 1962; cf. Mazur, 1970). As discussed below, reducing cryoprotectant toxicity is one approach to improving vitrification methods.

**Cell Death at Suboptimal Cooling Rates (continued)**

Many of the factors which might contribute to cell damage as a result of freezing are interdependent. For example, ice crystal formation may have deleterious mechanical effects on cells in suspension or in tissues (Sjostrom, 1975; Mazur, Rall and Rigopoulos, 1981; Pegg, 1987; Muldrew and McGann, 1990) and the amount of ice formed and the crystal structure is dependent upon cooling rate,
warming rate, and the presence of CPA. Not surprisingly, the existence of an optimal cooling rate for a cell is an oversimplification. The cooling rate at which cell survival is highest is dependent upon other factors such as cryoprotectant concentration and warming rate (Mazur and Schmidt, 1968; Mazur et al., 1970). Intracellular CPA have noticeable effects on IIF (Mazur et al., 1970; Rall et al., 1983; Myers et al., 1989; Harris et al., 1991; Mazur et al., 2005a; Mazur et al., 2005b). In general, in the presence of permeable cryoprotectant, cell water crystallizes at slower cooling rates than in its absence (Diller, 1979). This effect is likely a result of several factors. One includes the reduction in the water permeability of cell membranes in the presence of cryoprotectant as mentioned above. A second is likely a result of lowering the freezing point of the cytoplasm which causes a general reduction in the temperature at which a given driving force for water efflux is present. Because of the temperature dependence of water permeability, less water can move out of the cell in a given amount of time under such circumstances (Myers et al., 1989; Karlsson et al., 1994); as discussed by Mazur (Mazur, 2004).

The generic term “solution effects” has been coined to collectively describe the various forms of injury to cells cooled slowly enough to preclude damaging IIF (Meryman et al., 1977). This term reflects a notion that the damage results from the solution conditions created by ice formation as described above. Meryman and colleagues suggested that damage was a physical and not a biological
Figure 1.8. The effect on cell viability of exposing mouse oocytes to 1,2-propanediol or ethylene glycol (2.5M final with 0.3M sucrose) is shown. A solution containing 0.5M sucrose which simulates the osmotically-driven volume excursions of the other treatments was included as a control. Neither osmotic stress nor ethylene glycol exposure had an effect on oocyte viability after a 6-hour incubation. Exposure to 1,2-propanediol resulted in a dramatic loss in cell viability (cell lysis). These data are unpublished from the author’s experiments.
event (Meryman, Williams and Douglas, 1977), resulting either from the osmotic dehydration of the cells and the resulting stress placed upon the cell membrane due to cell volume reduction, or a direct osmotic effect on the membrane itself. In earlier work, Meryman described a hypothesis of cryoinjury based upon the cell reaching a minimum critical volume (Meryman, 1970). This later work supports this theory.

Another interesting hypothesis has been put forth in relation to the formation of ice. In a series of studies, Mazur and colleagues investigated the effects of the fraction of the solution which remained unfrozen on cell damage (Mazur, Rall and Rigopoulos, 1981; Mazur and Rigopoulos, 1983; Mazur and Cole, 1985; Mazur and Cole, 1989). Their data showed a strong correlation between survival and the unfrozen fraction when the unfrozen fraction was low (5-15%). They proposed that as the unfrozen fraction is reduced, the cells are damaged by mechanical effects of the ice and/or close apposition with other cells. When the unfrozen fraction is increased, damage was less strongly dependent on that variable and more on the salt concentration until the effects of the unfrozen fraction were lost. When the unfrozen fraction is not a damaging mechanism, the loss was attributed to the osmotic effect of the solute (both during exposure and dilution) (Zade-Oppen, 1968).

The interpretation of these data came under scrutiny as Pegg and Diaper (Pegg, 1989) pointed out that the unfrozen fraction variable was confounded by the
treatments used to change the unfrozen fraction (changing the initial osmolality of the solution). Such treatments would systematically alter the volume excursions which the cells would undergo during the experiment, and this difference could also result in the outcome seen. Mazur (Mazur, 2004) goes into greater detail about this debate and additional evidence for the unfrozen fraction hypothesis.

Perhaps the most important message to get from this particular debate is the difficulty in designing experiments to isolate the effect of a single variable on cell damage during freezing when numerous potential variables are interdependent (see (Pegg, 1987; Pegg and Diaper, 1991) for an elaboration). Another good example of this is the challenge to the explanation for slow-cooling injury resulting from increased salt concentration. As discussed above, concentrated solutes cause exosmosis and result in a reduction in cell volume. Thus, either high salt concentration or volume reduction could explain the damage (it could also be an interaction of the 2 factors). The minimum volume hypothesis was strongly supported by the results of Williams and Shaw with erythrocytes (Williams and Shaw, 1980) following up on earlier work by Meryman (Meryman, 1971).

In more recent years, the molecular mechanisms of cryodamage, particularly the induction of apoptosis, have been investigated (Baust et al., 2000). These authors have suggested that the trigger for apoptosis is not necessarily an
immediate effect of the cryopreservation stresses, but can be delayed for several hours (Baust et al., 2001). Clearly, we are far from understanding all of the mechanisms which result in cryodamage.

**Chilling Injury / Cold Shock**

Even in the absence of ice, sub-physiological temperatures can have profound effects upon cells. Injury from cooling cells is often differentiated by the degree to which the rate of cooling causes the specific event. Injuries from rapid cooling are usually categorized as cold shock injuries. These types of injuries occur quickly after cooling, and are generally independent of the warming rate. In the context of cryopreservation, a significant body of literature has been produced which describes the effects of cold shock on cell membranes, particularly for spermatozoa (Morris and Watson, 1984; Watson and Morris, 1987).

A description of the liquid crystalline model of cell membranes can be found in a standard cell biology text (Alberts et al., 2002). In general, amphipathic lipid molecules form a bilayer structure with various proteins being integrated throughout. At physiologic temperatures, there is a general fluidity such that molecular mobility is high and many of the proteins and lipids are free to diffuse laterally within the bilayer (opposite faces of the bilayer are not identical, and moving from one face of the bilayer to the other is energetically unfavorable). The structure that lipids can take in solution is more diverse than this, however.
A lamellar (*i.e.* bilayer) structure is common, but micelles, inverted micelles (micelles within the bilayer), hexagonal-II, and cubic phase structures can occur (see Figure 1 in the review by Quinn (Quinn, 1985)). The particular arrangements lipids take is dependent upon factors such as water activity, temperature, pH, salt concentration, and interaction with other molecules (*e.g.* proteins).

When membranes are cooled, they exhibit thermotropic behavior; that is to say they tend to undergo phase transitions. As membranes are cooled, the lipids tend to transition from a liquid-like state to a gel-like state, with the molecules being arranged in an orderly, crystalline fashion with a characteristic hexagonal arrangement (Chapman, 1975). Due to the complexity of biological membranes, a transition is not like a crystallization event in a simple solution (*i.e.* a rapid precipitation), but more like a (relatively) slow lateral separation of membrane lipids into distinct domains (see Figure 1.9). Nevertheless, this transition is a distinct change from the usual lipid arrangement, and can have significant effects on membrane function.

The temperature at which this transition occurs is dependent upon several factors, including the length of the hydrocarbon chain in the lipid group, the presence and location of cis-unsaturated bonds (transition temperatures decrease as the position of the bond moves away from the polar group and toward the middle of the chain), and the concentration and valence of cations in
the solution. An increase in the concentration of polyvalent cations increases the phase transition temperature, whereas monovalent cations increase lipid fluidity and decrease the phase transition temperature. The presence of cholesterol in a membrane can also affect phase transition behavior by (1) altering the ability of lipid species to transition to a gel-like configuration; and (2) increasing the disorder of the gel phase.

The propensity to develop a lamellar or hexagonal-II structure varies across lipid species. Different species tend to aggregate into domains during the phase change, and the creation of inverted micelles (hexagonal-II structures) within a bilayer can occur as a result (Figure 1.9) (Sen et al., 1982). Rearrangements such as these can result in alteration to the selective permeability of membranes and loss of cell homeostasis.

Changes in the biochemistry of spermatozoa as a result of cold shock have been examined. A reduction in anaerobic glycolysis and respiration, ATP levels, Cytochrome C loss from the mitochondria, and release of numerous intracellular enzymes have all been described (reviewed in (Watson, 1981; Watson and Morris, 1987)). Furthermore, changes in the distribution of intracellular ions have also been noted.

Numerous compounds have been shown to confer protection to spermatozoa from cold shock. Protective agents include glycerol, phosphatidylserine, egg
yolk, lecithin, milk, and albumin. The low density lipoprotein fraction of egg yolk is particularly effective at preventing cold shock injury (Pace and Graham, 1974), with phosphatidylcholine being a particularly active component (Quinn et al., 1980).

The results from Quinn and colleagues (Quinn et al., 1980) suggest that the effect is a result of interactions with the surface of the membrane, and not as a result of components intercalating within the lipid bilayer. The mechanisms of these compounds are not fully understood, but one model for the effect of adhering CPA on phase separations of membranes has been put forth (Quinn, 1985) and is shown schematically in Figure 1.10.

Studies during recent years have focused on the genetic regulation of the response to cold shock (Fujita, 1999; Phadtare et al., 1999; Rieder and Cole, 2002). While it seems a bit ironic, members of the heat-shock protein family are often upregulated as a response to cold stress. Other proteins that have been referred to as cold-shock proteins are also upregulated as a response to cold temperatures. Compared to that which is known in prokaryotes, our knowledge of the genetic response to cold temperatures in eukaryotic cells is just developing. For further review of these topics, see (Inouye and Phadtare, 2004; Phadtare and Inouye, 2004; Al-Fageeh et al., 2006; Al-Fageeh and Smales, 2006) and references therein.
Vitrification as an Alternate to Equilibrium Freezing

As has been alluded to several times in this chapter, vitrification can be employed as an alternative to equilibrium freezing. The obvious benefit of this approach is that the damage due to IIF can be completely avoided. Unfortunately, other kinds of damage are much more likely to occur when using this method. Recalling the initial discussion of phase transitions in solutions during cooling, it was mentioned that with a sufficiently high solute concentration, ice formation could be avoided altogether. The easiest way to achieve vitrification would be to use a solution that has a concentration as indicated in section IV of Figure 1.1. Water in solutions with such a composition will not crystallize nor de-vitrify even at cooling rates applicable to equilibrium freezing methods. Unfortunately, the toxicity of such solutions is too high to make them practical. Similarly, solutions with concentrations around 60 weight % (Region III) are often too toxic to be useful, although they can also be cooled slowly without crystallization. Various strategies have been described to counter the potential toxicity of solutions, and include: (1) the use of a combination of solutes, each of which is below a concentration that is very toxic, yet in combination will facilitate vitrification; (2) the substitution of polymers in the extracellular medium for the smaller permeating agents; (3) the application of hydrostatic pressure; (4) the use of compounds which counteract the toxicity of other agents (e.g. acetamide with dimethylsulfoxide (Fahy et al., 1990)); and (5) reducing the time for which and/or the temperature at which the biomaterial is exposed to high
Figure 1.9. A model of temperature-induced phase changes to membranes is described in this figure. In the upper portion of the figure, a typical biomembrane is shown, with various integral membrane proteins and lipid species. As the temperature is reduced from physiologic to hypothermic (10 °C in this instance), lateral redistribution of the various molecules occurs, with lamellar-forming lipid species (represented with white polar groups) and hexagonal-II-forming lipid species (represented with black polar groups) separating into distinct domains. Upon warming, an inverted micelle structure is created by the hexagonal-II-forming lipids. Such a configuration could result in a significant disruption of the membrane selective permeability, and the possibility of membrane failure and cell death. Figure adapted from (Parks, 1997), which was originally adapted from (Quinn, 1985).
Figure 1.10. A model of the effect of CPA on preserving biomembrane stability as described in Quinn 1985 (Quinn, 1985) is shown. In the presence of CPA (not shown schematically), the hexagonal-II –forming lipid species preferentially associate with membrane proteins during cooling (compare middle panel to middle panel in Figure 10) and only lamellar-forming lipid species segregate into distinct domains. When the cell is warmed, the hexagonal-II-lipid – protein interactions prevent a non-lamellar transition, promoting the return to a normal bilayer configuration.
concentrations of CPA. Hydrostatic pressure has an effect by shifting the $T_h$ (lower) and $T_g$ curves (higher) such that their intersection point occurs at lower concentrations of solutes.

As discussed above, when solution concentrations are reduced, the likelihood of devitrification during warming increases. Hence, the warming rate is an especially important consideration when designing vitrification strategies. In the first report of successful vitrification of mammalian embryos, Rall and Fahy (Rall and Fahy, 1985) used a combination of these strategies (1, 4, and 5) to overcome the toxicity associated with the vitrification solution (VS1). Mouse embryos (8-cell) could survive exposure to the solution for up to 15 min at 4 °C; survival dropped precipitously with increasing time. Furthermore, loss of viability occurred when samples were cooled slowly compared to rapidly when the concentration of VS1 used was reduced by 25%, and when the warming rate was reduced to 10 °C/min from 300 or 2500 °C/min.

These results support the idea that vitrification at moderate solute concentrations (region II in Figure 1.1) can be precarious. This region is labeled “doubly unstable” on the chart, as the use of solutions in this range are likely to be highly nucleated upon cooling, and prone to devitrification and recrystallization upon warming. Reducing the solute concentrations in combination with higher cooling rates is a strategy being currently employed for vitrification of mammalian oocytes (Martino et al., 1996; Lane and Gardner, 2001; Kuwayama et al., 2005).
However, modification of the cooling and warming rate is not the only strategy that might lead to improvements (Otoi et al., 1998). As has been discussed throughout this chapter, several types of cryoinjury exist, and the development of optimal cryopreservation strategies will require that all of these factors are taken into account, along with the specific cryobiological properties of the cells under study (Woods et al., 2004).

**Mammalian Oocyte Development: General and Comparative Aspects**

In mammals, ovarian follicles begin to form during the early stages of fetal development (Baker, 1963), for humans, it has been estimated that, at birth, the ovary contains between 250,000 and 500,000 follicles (Forabosco et al., 1991). Most of these follicles are in the primordial stage of development, characterized by low metabolism (Fajer, 1983) and having small oocyte surrounded by a single layer of squamous, pregranulosa cells (Hirshfield, 1991). The entire structure is encapsulated by a basement membrane. Entry of these quiescent follicles into the growth phase occurs at slow rates over time, with the majority remaining in the primordial state for months to years. The regulation of this transition remains ill defined, although factors such as the Kit receptor and its ligand (Kitl) and anti-Müllerian hormone (AMH) have been implicated as possible contributors (Kuroda et al., 1988; Huang et al., 1993; Durlinger et al., 1999). When these follicles begin to develop, the first sign of a change occurs in the somatic cells (see
Figure 1.11). They transition from having a squamous to a cuboidal morphology and are positively labeled with $[^3]$H thymidine (Hirshfield, 1991); at this stage the structure is termed a primary follicle (~80 – 100 μm in diameter in humans). The somatic (granulosa) cells then undergo clonal expansion, resulting in significant proliferation and an increase in the number of cells which surround the oocyte. Over the course of several weeks, additional changes occur to the developing follicle. Proliferation of the granulosa cells continues, the zona pellucida begins to form, and a second layer of somatic cells (theca) develops outside of the granulosa cells. At this stage, the diameter of the follicle in humans attains a size ranging from approximately 100 – 150 μm.

The early stages of follicular development appear to be directed by factors contained within the ovary (Rajkovic et al., 2006); however, later stages of follicular development (early-antral stages and beyond) require pituitary hormones (i.e. follicle-stimulating hormone; (Cattanach et al., 1977; Kumar et al., 1997)). As granulosa cell proliferation continues, the follicle increases in size and fluid-filled cavities begin to appear among the cells. Over time, these cavities coalesce to form a single antrum, and in humans, the follicle attains a size of about 2 mm. This process of cell division and antral expansion continue over the course of another few weeks, and, if selected to continue growth, the pre-ovulatory follicle in humans eventually approaches 2 cm in size. Final oocyte maturation is triggered by the mid-cycle gonadotropin surge, culminating in ovulation.
The mature oocyte is one of the largest cells in mammals, with a diameter of approximately 70 μm in mice and 120 μm in humans, cows, and pigs. In most vertebrates, oocytes progress to the second meiotic division stage near the time of ovulation, and maintain a meiotic arrest until fertilization (Figure 1.12). As such, the chromosomes are maintained in a condensed state and positioned on the metaphase plate of the meiotic spindle.

While in general oocytes from mammals are similar (Pincus, 1936), some differences do exist that are thought to be important determinants to cryosurvival (Parks and Ruffing, 1992). As mentioned above, mouse oocytes are smaller in size than oocytes from humans, cows, and pigs (although they all are approximately spherical in shape). The larger oocytes have a smaller surface-to-volume ratio, which makes the rate of water flux under osmotic influences slower, all else being equal. This difference could influence the probability of IIF (cf. Mazur, 2004), as for a given cooling rate, more water will remain trapped inside of the cell over time (again, assuming the membrane permeability properties are equal). Another notable difference in oocytes across mammalian taxa is the amount of cytoplasmic lipid inside of the cell (Szöllösi, 1972; McEvoy et al., 2000) (see Figure 1.13). Removal of cytoplasmic lipids from porcine embryos rendered them less sensitive to cooling (Nagashima et al., 1994), and presumably the presence of lipid in the cytoplasm of oocytes also makes them chilling sensitive (Isachenko et al., 2001; Park et al., 2005).
Figure 1.11. A diagram showing the development of a hypothetical ovarian follicle is shown. Primordial germ cells initially enter the gonadal ridge and eventually develop into primordial follicles. After re-initiation of development, the follicle progresses through several developmental stages (primary, secondary, pre-antral, and antral) with proliferation of the inner granulosa cells and outer theca cells. If the follicle is selected to continue development, it progresses to the preovulatory follicle stage, containing a large fluid-filled cavity (antrum) with the oocyte surrounded by cumulus granulosa cells.
Figure 1.12. Immunocytochemical staining of the MII spindle in mouse oocytes (red) and counterstaining of the chromosomes (green). The smaller green spots on the edge of some of the oocytes represent nuclear staining of granulosa cells that remained attached to the oocytes after collection.
Figure 1.13. Photomicrographs of oocytes from mice (smallest; n=5), cows (n=2, upper right), and pigs (n=2; lower right) are shown. Notice the difference in darkness of the cytoplasm in these examples, particularly in the cow and pig oocyte compared to the oocytes from the mouse. A human oocyte has a lipid concentration similar to a mouse. The high concentration of lipids is one proposed means that renders oocytes (and embryos) from pigs and cows more sensitive to cooling compared to oocytes from mice. See text for more details.
The shape and orientation of the meiotic spindle also differs between human, cow, pig, and mouse oocytes. The spindle in mouse oocytes is barrel-shaped, with a pole-to-pole distance longer than the distance across the metaphase plate (see figure 1.13). However, spindles from cow and pig oocytes are nearly spherical. Human oocyte spindles are intermediate to these two shapes. The MII spindle of mouse oocytes is positioned tangentially to the oolemma at metaphase arrest, with the metaphase plate being orthogonal to the oolemma. The spindle from bovine, porcine, and human oocytes is positioned such that the metaphase plate is parallel to the oolemma (or orthogonal relative to the spindle in mouse oocytes (see figure 1.14). Another interesting biological difference between oocytes from mice (and other rodents) and most other mammals is the inheritance pattern of the centrosome. In most mammals, centrosomal proteins are biparentally inherited and the centrioles are contributed to the oocyte by the fertilizing spermatozoon. In mice, however, centrosomes are contributed wholly by the oocyte with centrioles being generated de novo around the blastocyst stage (Schatten, 1994; Sun and Schatten, 2007). The metaphase spindle in mammals has been shown to be easily disrupted by reduced temperatures and cryoprotectants (Vincent and Johnson, 1992). One significant difference in the spindle properties that is relevant to cryobiology is that the spindle in mouse oocytes is easily repaired after such damage (Pickering and Johnson, 1987); this is not the case in oocytes from other mammals, however (Pickering et al., 1990; Aman and Parks, 1994; Wang et al., 2001; Songsasen et al., 2002). In general, a higher proportion of mouse oocytes can be cryopreserved more easily compared
Figure 1.14. Immunostained MII spindles in a human oocyte (left) and a mouse oocyte (right) are shown. Notice the difference in size and orientation of the spindles in the human oocyte compared to the mouse oocyte.
to oocytes from other mammalian taxa (See tables 2-5). This difference has lent credibility to the hypothesis that disruption of the MII spindle is a primary mechanism for cryoinjury in oocytes from most mammals.
The Significance of Mammalian Oocyte Cryopreservation

Although adequate means for the cryopreservation of preimplantation embryos are available across many taxa (Whittingham, 1971; Leibo et al., 1974; Gordon, 2003; Paynter et al., 1997), methods for cryopreserving oocytes for these species are less successful (Van Blerkom and Davis, 1994; Bouquet et al., 1995; Critser et al., 1997; Eroglu et al., 1998; Saunders and Parks, 1999). Mature oocytes have several unique characteristics, such as the large intracellular water volume, cortical granules, and meiotic spindle, which make them vulnerable to damage during cryopreservation (Toner et al., 1991; Agca, 2000; Shaw et al., 2000). To date, many studies have been published describing attempts to cryopreserve oocytes from mammals using an empirical approach without taking into consideration the fundamental cryobiological properties of the cells. It is my thesis that developing a comprehensive understanding of the fundamental cryobiology of oocytes will yield insight into developing efficacious means to cryopreserve these cells.

The Role of Oocyte Cryopreservation in Human Reproductive Medicine

For many individuals, having a family defines their sense of self and place in their community, and the desire to have children is one of the strongest
emotional drives in humans. Unfortunately, many couples (estimated to be as high as 40%) of reproductive age would be infertile without medical intervention (Crosignani and Rubin, 1996). For many individuals, the experience of infertility is a serious life crisis (Menning, 1980; Alesi, 2005). Women often describe infertility as being the worst crisis they have ever faced; having symptoms of depression and anxiety that are similar in women facing heart disease, cancer, and incurable infections (Domar et al., 1992; Domar, 1997). Emotions such as anger, grief, frustration, and guilt are often felt by persons experiencing infertility (Dunkel-Schetter and Lobel, 1991).

The Society for Assisted Reproductive Technology and The American Society for Reproductive Medicine in conjunction with the Centers for Disease Control and Prevention compile annual statistics on human assisted reproductive technology (ART) activities performed in the United States. In 2002, nearly 86,000 cycles of ART treatments were initiated. Just over 14,000 transfers using cryopreserved embryos were performed during that year with approximately 3500 deliveries reported from those cryopreserved embryos and a live birth rate per transfer ranging from 28 % for women under the age of 35 to 17 % for women between the ages of 41-42 (National center for chronic disease prevention and health promotion, 2004). Live birth rates per transfer of non-frozen embryos ranged from 43 % to 15 % for these respective age groups. A similar report of the work completed in 2002 from 25 European countries has recently been produced (Andersen et al., 2006). This report documented nearly
325,000 cycles initiated from 631 clinics. In 13 countries for which all clinics reported their data, more than 175,000 cycles were reported, and the clinical pregnancy rate for in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) was approximately 30%. Deliveries per transfer ranged from ~10% to >30% across clinics.

When IVF is utilized as a treatment option, a series of interventions are utilized. Initially, the woman's menstrual cycle is artificially controlled through the administration of hormones to increase the number of oocytes available for fertilization. In the majority of cases, more oocytes are collected than initially used; yet due to the lack of adequate methods for preserving oocytes, it is common for the entire cohort to be subjected to IVF. This usually results in the creation of supernumerary embryos. Commonly, the remaining embryos that look healthy are cryopreserved so future transfers can be made in the event that the first transfer fails to produce a child (Toner et al., 1991; Schnorr et al., 2000; El-Toukhy et al., 2006). As a result of this practice, the number of human preimplantation embryos currently in cryostorage is estimated to be on the order of several hundred thousand (Hoffman et al., 2003).

Unfortunately, there are significant social concerns when it comes to human embryo cryopreservation (Beyler et al., 2000; Bankowski et al., 2005). These issues have lead to increases in societal regulation and concomitant increases in costs. Having successful methods to cryopreserve oocytes would help to
alleviate these concerns by allowing the creation of only the numbers of embryos deemed appropriate for transfer, yet would maintain the potential for future attempts at pregnancy should the initial one fail. Such an approach has been mandated by law in Italy, leading to a significant increase in the numbers of oocytes being subjected to cryopreservation cycles (Benagiano and Gianaroli, 2004).

Additionally, having successful methods available to cryopreserve human oocytes would allow the establishment of oocyte banks to assist those female patients who cannot produce viable oocytes (Karow, 1997). Oocyte cryopreservation would not only obviate cycle synchronization between donor and recipient, a method which is not always successful, but it would allow sufficient time for seroconversion of the donor in the event of a recent infection with a transmittable disease (Critser et al., 1997). Such practices are routine in semen banks and are facilitated by the ability to cryopreserve human semen (Hornicek et al., 2002).

Another area in human medicine where oocyte cryopreservation could prove beneficial is cancer therapy (Abir et al., 1998; Gosden, 2001; Posada et al., 2001; Gosden and Nagano, 2002; Sonmezer and Oktay, 2004; Wallace et al., 2005). With the use of aggressive chemo- and radiotherapy, as well as bone marrow transplantation, greater than 90 percent of many cancers of children and young women can be cured (Greenlee et al., 2001). One unfortunate iatrogenic
side effect of these treatments is the loss of gonadal function and subsequent infertility. Although promising, ovarian tissue freezing is still experimental in nature (Newton, 1998; Oktay, 2001). Additionally, *in vitro* maturation techniques for ovarian follicles are presently experimental (Chian *et al*., 2004). Having a means to preserve mature oocytes could preserve the fertility of young women undergoing such therapy. Oocyte cryopreservation is also important given that the average age of childbearing continues to increase (Centers for Disease Control and Prevention, 1989) and embryo cryopreservation is not desirable for many women without partners.

**The Role of Oocyte Cryopreservation in the Use of Animal Models for Human Health and Disease**

Cryopreservation of reproductive cells dates as early as 1776, with the report by Spallazani of sperm freezing (*cf*. Luyet and Gehenoio, 1940; Leibo, 2007). The use of slow cooling rates as well as CPA has led to improvements in overall post-thaw viability in cellular cryopreservation. Successful freezing of mouse embryos was first demonstrated in 1972 (Whittingham *et al*., 1972; Wilmut, 1972). Continued research has improved post-thaw viability significantly, with embryo cryopreservation often yielding post-thaw survival rates greater than 90 percent. Oocyte cryopreservation, on the other hand, is much less successful (Critser *et al*., 1997).
Two technological advances occurred in the 1980’s that had enormous implication in the ability to study physiology and disease in mammals on a genetic level. The successful isolation of mouse embryonic stem cells reported in 1981 (Evans and Kaufman, 1981) and the development of targeted gene disruption strategies, pioneered by two groups (Thomas and Capecchi, 1987; Koller et al., 1989) allow investigators to alter the genomes of mice with the precision of a single base-pair. Since these techniques have become routine, hundreds of genetically-modified mouse strains have been created (Critser and Russell, 2000).

Given their value as models for biological systems, it is desirable to preserve these strains indefinitely for future studies. Historically, this has been performed by the maintenance of breeding colonies. Expenses for this maintenance not only include food and bedding, but also building maintenance and disease monitoring and control. Therefore a substantial cost is being borne by the research community, thus society, for the care of these animals (Sharp and Mobraaten, 1997). Cryopreservation of germplasm offers a way to reduce these costs by allowing the maintenance of "frozen" colonies as a backup in the event of a disease outbreak or other emergency, or as a full replacement of breeding colonies that are not currently in use but whose maintenance is desired for future research (Critser and Russell, 2000). Additionally, a frozen colony derived from founder animals would help alleviate inbreeding depression and genetic drift, thus maintaining overall genetic vigor while minimizing the number of animals
necessary for maintenance of a live colony (Whittingham, 1974). While oocyte cryopreservation may not offer the most efficient means to preserve such rare genotypes, it may play a role in special cases such as when sperm cryopreservation in inefficient.

Genetic modification of animals such as sheep, cattle and swine also offers benefits to progress in biomedicine (Wheeler and Walters, 2001; Prather et al., 2003), including the creation of pharmaceuticals (Schnieke et al., 1997; Edmunds et al., 1998; Lindsay et al., 2004), and may even allow the production of tissues and organs for xenotransplantation (Cozzi and White, 1995; French et al., 1998; Cooper et al., 2002; Lai et al., 2002). Furthermore, in some instances, these organisms are preferred models for human physiology and pathology (Turk and Laughlin, 2004). Genetic engineering technology can be applied to livestock species for the creation of transgenic and knockout organisms for physiological studies, similar to that described for mouse models above (Hao et al., 2006).

Due to the lack of embryonic stem cell technology in livestock species, the use of nuclear transfer (NT) technology is relied upon for the creation of targeted genetic modifications in animals such as sheep, cattle and swine (Ward and Brown, 1998). At the present time, NT technology is relatively inefficient, and relies on the ready access to mature oocytes. Having an efficient means to cryopreserve oocytes would ensure a steady supply of these cells for NT
procedures, and could alleviate inefficiencies associated with seasonal variability of oocyte quality (Rutledge et al., 1999). The value of a ready supply of oocytes for research is evident by the development of commercial operations that sell oocytes (www.BOMED.com). Under the current system, oocytes have to be shipped and used shortly after acquisition from abattoir-derived ovaries. Having effective means to cryopreserve oocytes might improve operational efficiencies by loosening this restriction, and also allow stockpiling of oocytes during the best season as just described.

The Role of Oocyte Cryopreservation in the Management of Domestic Livestock

Initially with the use of artificial insemination (AI) with frozen-thawed semen, and continuing with other methods of assisted reproductive technologies (ART), increases in the average value of domestic animals progressed much more rapidly than would have been the case using natural mating (Gearheart et al., 1989). This is due to the much broader dissemination of reproduction events from elite animals than would be feasible without ART.

Harvesting oocytes can offer significant additional increases in offspring potential of females. One estimate suggests that up to 300 offspring can be produced from a single animal (Kruip et al., 1991). By using in vitro produced (IVP) embryos, significant improvements in reducing the generational interval can be
attained (Presicce et al., 1997; Fry et al., 1998). This technology would also allow the rescue of gametes from otherwise infertile animals, as well as the use of abattoir-derived oocytes that are increasingly being used in research (Day, 2000). Although maximizing reproductive potential of individual animals can have detrimental inbreeding effects, it is expected that with careful management this can be held to acceptable levels (Nicholas, 1996). Genetic engineering technology also has the potential to benefit food and other farm-related products (Damak et al., 1996; Zuelke, 1998; Nagashima et al., 1999; Lai et al., 2006; Prather, 2006),

The maximum potential of such improvements are unrealizable without efficient methods to preserve oocytes. The viability of mature oocytes is measured on the scale of hours. Even though methods exist to mature bovine oocytes in vitro, this only prolongs their potential by an additional 24 hours. Additionally, the creation of highly valuable transgenic animals as discussed above will make successful cryopreservation critical. For one thing it will allow international distribution of these genotypes. Secondly, it will facilitate the creation of repositories in the event of a catastrophe such as disease outbreak or natural disaster. Any event that has the potential to destroy the living animals must be considered, and preparations for such events must be made. While application of embryo cryopreservation has the potential to address these issues to a large degree, having cryopreserved gametes will allow more flexibility in future decision-
making. For example, it would allow planned genetic recombination through fertilization, allowing the creation of cross-bred animals.
The Sensitivity of MII Mammalian Oocytes to Various Steps in the Cryopreservation Process - Focus on Oocytes from Mice, Cattle, Pigs, and Humans

Sensitivity of MII Oocytes to Cooling Below Physiologic Temperatures without Intracellular Ice Formation

Effects on Development

Many cell types are adversely affected by cooling below the temperatures at which they normally function (cf. Watson and Morris, 1987). The rate of cooling can play a significant role in determining the viability of cells after cooling. As described earlier, cold shock is a term defined as an adverse effect of abrupt cooling to a lower temperature. It has been shown that cooling cells slowly to temperatures at which they are susceptible to cold shock injury can be innocuous. The term chilling injury is used when an adverse effect occurs irrespective of the cooling rate. Mammalian oocytes are susceptible to chilling injury or cold shock. However, oocytes from some mammals are less sensitive than others, and this difference lends some insight into possible mechanisms which result in the loss of viability after cooling. Investigations into injury from cooling oocytes (and early embryos) were pioneered by M.C. Chang in the middle of the 20th century using a rabbit model (Chang, 1952; Chang, 1953). In
his first report, he showed that oocytes could undergo cleavage divisions after having been stored at 10 or 0 °C for 24 hours (~80% and 40% respectively). In the second report, however, investigation into further development after storage at either temperature for 6-7 hours or 30-31 hours showed that only 3-11% developed normally for 20 – 26 days (average gestational length for rabbits is approximately 31 days (McNitt et al., 1996)).

Injury to oocytes, as a result of cooling below physiologic temperatures, from cattle, mice, humans and pigs has also been investigated, to various degrees. The earliest investigation on the effects of cooling oocytes from these groups appeared in 1959, with the report on mouse oocytes by Sherman and Lin (Sherman and Lin, 1959). They showed that mouse oocytes can tolerate cooling to 0 °C (at 0.2 °C/min or 1.0 °C/min) with immediate re-warming. Viability was unaffected as measured by morphological assessment and supravital staining. Furthermore, the proportion that survived IVF and embryo transfer was not different in comparison to untreated oocytes (gestation was allowed to proceed for 19 days; gestation for mice is 19-20 days (Whittingham and Wood, 1983)). A lower temperature (-10 °C in the presence of 5% glycerol) or longer storage times (6 or 24 hours) proved deleterious, with very low survival after -10 °C exposure, or no survival after 24 hour exposure at any sub-physiological temperature. About 50 % of the oocytes were able to survive storage at 5 °C for 6 hours relative to those abruptly cooled to and then immediately warmed from this temperature (differentiating the effects of chilling injury versus cold shock). In
1995, two additional reports appeared which described similar experiments. The robust nature of mouse oocytes to cooling to 0 ºC at relatively slow rates (the precise rate was not given in the report, but the cells were cooled from room temperature in a ¼ cc straw to near-zero by placing the straw into an ice bath) was confirmed by Bouquet and colleagues (Bouquet et al., 1995), but somewhat contradicted by Hunter and colleagues (Hunter et al., 1995). In the later study, both rapid cooling (> 1000 ºC/min) and slow cooling (3 ºC/min) to 0 ºC were investigated. The proportion undergoing fertilization and further development to hatching blastocysts was recorded. Both rates of cooling affected the proportion of viable cells, with fertilization being reduced from 95 % to 75 % and 83 % for the rapid and slow cooling groups, respectively. Further development was also reduced to 67 and 69 % for the respective groups compared to 92 % for the controls.

It has been shown that oocytes from cattle are extremely sensitive to cold temperatures, with very short exposures at some temperatures having dramatic effects on developmental potential. Arav and colleagues, in a study focusing on the effects of cooling bovine oocytes to 13 or 4 ºC, showed that polyspermy was more prevalent in oocytes rapidly cooled to these temperatures (20 – 25 % polyspermy in cooled oocytes vs. 12% in controls) (Arav et al., 1996). Further development was not reported. Martino et al. reported on the effects of cooling in vitro matured bovine oocytes to 20, 10, or 0 ºC for 30 min, and the effects of holding oocytes at 0 ºC for various times up to 30 min, on in vitro development.
All cooling was conducted at rates of about 120 °C/min. While cooling to 20 °C showed no effect on fertilization and development, cooling to 10 or 0 °C was damaging, with a reduction in blastocyst development to 8 and 3 %, respectively (25 % of untreated oocytes developed to blastocysts). They also showed that polyspermy increased as a result of cooling. They proceeded to analyze the kinetics of injury at 0 °C, and demonstrated that cooling for short periods of time (as short as 30 seconds) had a noticeable effect on development; the proportion of oocytes developing to blastocysts was reduced by 50% after 30 seconds, and nearly all oocytes failed to develop to blastocysts after holding for 30 min. These results were confirmed a short time later by Azambuja et al., where they also showed that cooling in vitro matured bovine oocytes to 10 or 0 °C for 5, 10, or 20 min resulted in the loss of the developmental potential of nearly all of the oocytes (Azambuja et al., 1998). They went on to show that changes to the zona pellucida as a result of cooling could account for some of the developmental failure, with approximately 30 % and 38 % fewer of the oocytes being cooled to 10 and 0 °C, respectively, undergoing fertilization due to effects on the zona (zona free oocytes had a higher probability of sperm penetration).

Only limited basic research into the effects on development of cooling human and porcine oocytes has been performed. In 1992, Bernard and colleagues published a report on the effects of cooling mature human oocytes to 0 °C at one of 2 rates – 3 or 1000 °C/min and holding for 30 min prior to warming (Bernard et al., 1992). They showed that the proportion of oocytes that became fertilized
after cooling at either rate was high, and similar to the untreated control oocytes (≥ 70 % for all groups). Development for an additional 24 hours of 17 of the embryos was also similar between groups. Only one of 16 oocytes used as parthenogenetic controls underwent cleavage. Restrictions on research with human embryos precluded the assessment of further development in this study. Cooling in vitro matured porcine oocytes to high sub-physiological temperatures for 1 hour was shown to be slightly detrimental to fertilization and embryo development (Yuge et al., 2003). The proportion of oocytes undergoing fertilization was reduced to 57, 46, and 43 % after having been cooled to 30, 25, and 20 ºC (compared to 62 % fertilization when the oocytes were maintained at 38.5 ºC) at slow rates of cooling (cooling within ¼ cc straws and transfer to a water bath). The proportion of oocytes undergoing cleavage was 45, 49, and 36 % at those temperatures compared to 46 % for non-cooled oocytes.

Effects on the Oolemma

Cooling can directly affect the plasma membrane of cells, resulting in a transition from a liquid-crystalline phase to a gel phase caused by lowering the thermal energy of the phospholipid molecules. The result is an altering the interactions between the lipids, and hence the physical properties of the membrane. The temperature of this transition is affected by several factors, including the length of the hydrocarbon chain on the particular species, the presence of unsaturated hydrocarbon chains and the degree of unsaturation, cooling rate, type and
concentration of integral membrane proteins, and the composition of the solution surrounding the cell, including the presence of CPA (cf. Quinn, 1985).

The phase transition temperature of MII oocytes has been investigated from some mammalian species. In 1996, Arav and colleagues documented this phenomenon in bovine oocytes, showing that a very broad transition occurs, centered around 10 °C (the temperature of transition ranged between ~20 and 0 °C) (Arav et al., 1996). The presence of cholesterol in a lipid bilayer is known to broaden a phase transition such as that described for bovine oocytes (Chapman, 1975) and may account for this property. These results are in contrast to a more narrow transition for human oocytes centered at ~17 °C (range from ~ 12 to 18 °C; Ghetler et al., 2005).

**Effects on the Metaphase II Spindle**

Many research studies investigating the effects of cooling mature mammalian oocytes have been directed at understanding the effects on the metaphase II (MII) spindle. The first report appeared in 1980 using mouse oocytes (Magistrini and Szöllösi, 1980). It was shown that cooling on ice for 15, 30, 45, or 60 min resulted in progressive depolymerization of the microtubules of the spindle. The majority of the spindle fibers were depolymerized after the shortest treatment, with the continuous, or pole-to-pole fibers being completely depolymerized, and the kinetochore fibers being mostly depolymerized. Nearly all of the kinetochore microtubules had depolymerized by 30 min, and longer treatments completed
the depolymerization process. Rapid warming to different temperatures (30 min at 18, 25, or 37 °C) resulted in different degrees of repolymerization and normal spindle structure, depending upon the temperature. No repolymerization occurred at 18 °C, and little occurred at 37 °C, but complete repolymerization occurred at 25 °C. They went on to show that progressive warming (warming in an 18 °C water bath, and transferring to a 25 °C bath, and finally to a 37 °C bath) resulted in rebuilding of a normal spindle as well.

These effects were largely confirmed by later studies (Pickering and Johnson, 1987; Sathananthan et al., 1992). One of the differences in the later studies is that direct warming to 37 °C was able to restore a normal spindle structure in the ooplasm.

Cooling also has deleterious effects on the MII spindle of oocytes from other mammals. However, one of the major differences is that the ability of the spindle to undergo normal repair (by which is meant the morphology of the spindle appears the same after warming as before cooling) is reduced. Less than half of human oocytes had normal spindles after cooling to room temperature for either 10 or 30 min and warming to 37 °C for either 1 or 4 hours in one study (Pickering et al., 1990). Several other studies have investigated the effects of cooling human oocytes to various temperatures for various times and observed similar effects (Sathananthan et al., 1988; Wang et al., 2001; Zenzes et al., 2001). In the study by Zenzes and colleagues, they showed that evidence for microtubule
depolymerization could be observed in as little as 1 minute at 0 °C, although only slight effects were discernable. However, after 2 min it was more noticeable, 4-5 min caused substantial disruption, and 10 min resulted in complete microtubule depolymerization. Most of these studies have shown that the chromosomes do not disperse far from the spindle area, but clumping and some displacement off of the metaphase plate is frequent. Similar effects have been shown with bovine and porcine oocytes as well (Aman and Parks, 1994; Liu et al., 2003), suggesting one possible mechanism for the significant difference of the cooling sensitivity of oocytes from mice and other mammalian species as discussed above.

**Effects on Cortical Granules Exocytosis**

Cooling oocytes has also been shown to affect the zona pellucida, presumably as a result of cortical granule exocytosis. Johnson and colleagues showed that rapid cooling to 4 °C resulted in a reduced proportion of oocytes undergoing fertilization after sperm incubation, and this result could be eliminated by removal of the zona (Johnson et al., 1988). As mentioned above, Azambuja and colleagues also showed cooling affects the bovine zona pellucida (Azambuja, Kraemer and Westhusin, 1998).
Sensitivity of MII Oocytes to Intracellular Ice Formation

Freezing of intracellular water has a strong correlation with cell survival during cooling, as described above. Due to their large size and spherical shape (hence low surface-to-volume ratio), mammalian oocytes are particularly susceptible to IIF due to the likelihood of a large volume of water being trapped inside the cells during cooling (please see Figure 1.2). Oocytes were used as a model cell in some of the earliest experiments which documented the relationship between IIF and cell death (Leibo et al., 1978). In this report by Leibo and colleagues, oocytes were cooled at various rates (ranging from ~1 to 32 °C/min) in the presence of 1.0 M DMSO to temperatures as low as -80 °C under a cryomicroscope to observe the incidence if IIF (evidenced by a sudden darkening, or “flashing” of the cell) and the temperatures at which it occurred. The IIF temperature was shown to be slightly related to the rate of cooling, and occurred at a surprisingly low temperature, around -40 °C, on average. The cumulative survival (as determined by the inverse of the incidence of ice formation) was shown to strongly correlate with cooling rate. Rates at or above ~5 °C resulted in IIF in all cells examined, while cooling at 1.3 °C resulted in only 13 % of oocytes exhibiting evidence of IIF. Some of the cells could be cooled to temperatures near -60 °C before exhibiting evidence of IIF.

Toner and colleagues extended this line of investigation to look at IIF parameters for mouse oocytes in the absence of permeating CPA (Toner et al., 1991). They
found that the temperature at which IIF occurred was much higher (median temperature of IIF was ~ -12 °C). This temperature was also shown to be dependent upon the tonicity of the extracellular solution (hence the concentration of water in the cytoplasm). The median temperature of IIF was approximately -8, -12, -17, -27, -29, and -32 at extracellular osmolalities of 200, 285, 510, 735, 820, and 1035 mOsm, respectively. Again, there was not a strong cooling-rate dependence of the IIF temperature, the cooling rate for which 100 % of oocytes exhibited IIF was about 5 °C/min in an isotonic solution. One important conclusion the authors reached is that at least 2 mechanisms of ice formation appeared to be operating. As the temperature of the suspending medium was lowered, the cumulative fraction of oocytes containing ice increased rapidly after reaching ~ -31 °C. The authors speculated that heterogeneous ice nucleation was responsible for the differences in ice formation at temperatures above and below -31 °C, the former being catalyzed by extracellular ice and the later by intracellular nucleating agents. Other important findings in this investigation included a difference in the cumulative fraction of oocytes exhibiting IIF at constant temperatures (lower temperatures tended to result in a higher proportion of oocytes freezing internally), and that the presence of extracellular ice was absolutely necessary for the formation of intracellular ice at temperatures above -20 °C. When cells were supercooled to temperatures around this point, and then ice spontaneously formed outside of the cells, ice would immediately form inside the cells as well.
More recently, Mazur and colleagues investigated IIF temperatures in mouse oocytes in the presence of EG and glycerol (Mazur et al., 2005b). Similar to DMSO, EG and glycerol depressed the nucleation temperature of the oocytes well below that of oocytes in an isotonic solution without permeating CPA. Freezing oocytes in solutions containing one molar CPA resulted in an average flash temperature of -41 and -37 for glycerol and EG, respectively (the temperature was -14 °C for cells in solutions of phosphate-buffered saline (PBS) alone). Interestingly, the presence of the CPA seemed to mitigate the effects of extracellular ice on membrane integrity, such that in a solution of PBS, about half of the oocytes appeared to have lost membrane integrity as a result of the external ice (the lack of “flashing” of the oocytes when plasma membrane integrity was lost was interpreted to be a direct effect of the ice on plasma membrane integrity, such as a mechanical effect and not a nucleating effect). The results from this study also did not support the conclusion of an effect of cooling rate on IIF temperature when cooling rates ranged from 5 to 50 °C/min. The analysis of the solution compositions was extended to temperatures below freezing to determine the fraction of unfrozen solution at the flashing temperatures. The results showed that in the presence of only PBS or a solution with glycerol, flashing took place when approximately 94 % of the water in the solution was converted to ice. This percent was independent of the initial concentration of glycerol (the values were virtually identical at 0, 0.5, and 1.0 M glycerol). In comparison, in the presence of EG, flashing occurred when between 84 to 91 % of the water was converted to ice.
IIF was also investigated in bovine oocytes by Chandrasekaran and colleagues (Chandrasekaran et al., 1990) and Ruffing and colleagues (Ruffing et al., 1993). In the absence of CPA, Chandrasekaran et al. showed that the average temperature for bovine oocyte IIF occurred at -11.2 °C when cooled at 16 °C/min. The report by Ruffing et al. used in vivo matured oocytes for one experiment where the cells were only exposed to an isotonic solution. In vitro matured oocytes were also utilized to investigate IIF after equilibration in 1.5 M EG, PG, or glycerol. The cells were cooled at one of 3 rates (4, 8, or 16 °C/min) from 5 to -60 °C. For in vivo matured oocytes in isotonic medium, a total of 45, 75, and 93% of the oocytes exhibited IIF at the 3 cooling rates. The median temperature for ice formation was -13.3 and -14.2 °C for the cooling rates of 8 and 16 °C/min, respectively. The median temperature of IIF for in vitro matured oocytes in the presence of EG, glycerol, or PG was significantly reduced. Specifically, IIF occurred in half of the cells by -45.6, -46.5, and -34.4 °C when cooled at 4 °C/min; -39.2, -33.5, and -28.1 °C when cooled at 8 °C/min; and -27.8, -20.7, and -19.4 °C when cooled at 16 °C/min in the presence of the respective CPA. The data for mammalian oocyte IIF are summarized in Table 1.

**Sensitivity of MII Oocytes to Anisosmotic Conditions**

As discussed above, cryopreservation usually requires the exposure of cells to one or more cryoprotective compounds. Base solutions used for freezing cells
are approximately isosmotic with serum. Therefore, the addition of CPA to such solutions makes the overall solutions hyperosmotic. Exposing cells to these solutions results in osmotically-driven volume excursions which can be detrimental to cell viability. However, the degree of volume change associated with exposure to solutions containing permeating CPA can be controlled in a manner which reduces the absolute volume excursions and can prevent the cells from exceeding their osmotic tolerance limits. This means of cell damage is particularly acute for oocytes, as these cells are spherical, having a minimum surface-to-volume ratio. In order to minimize the deleterious effects of osmotic volume excursions, the tolerance of cells to changes in volume must be defined. The results of studies investigating the tolerance of mammalian oocytes from some species to anisomotic solutions have been reported in the literature.

In 1997, MII mouse oocytes from an outbred stock (ICR) were exposed to isotonic or hypotonic concentrations of PB1 medium, the later made by dilution of an isotonic PB1 solution with double-distilled water (Pedro et al., 1997). The concentrations of the hypotonic solutions were 0.5, 0.3, 0.25, and 0.2 X isotonic (150, 92, 77, and 62 mOsm, respectively). After exposure and return to an isotonic culture medium, the oocytes were allowed to recover for 30 to 60 min before morphological assessment. Only the oocytes having been exposed to the 150 mOsm solution were not affected, on average. Oocytes exposed to the other hypotonic solutions showed a loss of viability directly related to the solution concentration (~ 90, 60, and 20 % viable cells, respectively). Oocytes having
previously been vitrified showed a similar pattern when immediately exposed to the hypotonic solutions, with a slightly higher proportion of oocytes undergoing lysis after the treatments. A recovery period in culture of 1.5 – 2 hours before exposure to the hypotonic solutions eliminated this effect.

The effect of anisosmotic exposure on MII bovine oocytes has also been investigated (Agca et al., 2000). Hypo- and hypertonic solutions of sodium chloride (final osmolalities of 75, 150, 600, 1200, 2400, and 4800 mOsm) were used as the treatments. In this experiment, the oocytes were not denuded of cumulus cells prior to the treatment. All of the treatments affected the viability of the oocytes as measured by post-IVF development. The proportion of oocytes able to develop to the blastocyst stage decreased as the solution concentrations diverged from isosmotic. The proportion of oocytes developing to blastocysts (normalized to the controls) was approximately 37, 68, 60, 50, 48, and 19 % for the treatments, respectively. These results show that even brief exposure (10 min) to anisosmotic solutions can lead to a significant loss of developmental competence of bovine oocytes.

Only limited information on the effect of anisosmotic exposure to human oocytes has been reported to date. Exposure to anisosmotic solutions ranging in concentration from 0.25 to 1.5 M had no effect on the immediate survival of MII human oocytes as measured by a vital dye assay (McWilliams et al., 1995). Unfortunately, due to restrictions on human oocyte research, no attempt to
determine viability after fertilization and development was conducted, limiting the conclusions that can be drawn from this study.

**Sensitivity of MII Oocytes to Cryoprotectant Exposure**

**Effects on Cellular Components**

The effect on mammalian oocytes of exposure to some permeating CPA has also been investigated. Johnson and Pickering showed that exposure to DMSO changed the polymerization pattern of nearly all of the microtubules in the MII spindle of mouse oocytes (Johnson and Pickering, 1987). Exposure to a 1.5 M concentration at 37 °C for 10 to 20 min resulted in the poles of the spindle showing an astral pattern of microtubules, compared to the normal anastral pattern seen in untreated oocytes. Furthermore, other cytoplasmic asters of microtubules formed, focused around pericentriolar material not associated with the spindle. This pattern was also seen after transfer to 4 °C. In both cases, the overall shape of the spindle was preserved. Exposure to 4 °C without DMSO was associated with rapid depolymerization of the spindle, as expected. Prolonged exposure (60 min, with or without transfer to 4 °C) resulted in disappearance of the spindle microtubules, with the tubulin monomers becoming incorporated into the cortical asters. This effect was also associated with chromosomal dispersal from the metaphase plate. Restoration of the spindle after DMSO removal occurred in some oocytes (between 56 to 60 %, depending
upon the treatment) after incubation at 37 °C for 30 min, but none of the spindles were restored in oocytes that remained at 4 °C. More oocytes (89 %) were able to restore a normal spindle apparatus if the DMSO was added in increments instead of in one step, with no cooling below 37 °C, suggesting that the osmotic effects of the cryoprotectant addition and removal play a factor in spindle stability. Changes to the actin cytoskeleton in mouse oocytes were also investigated after a direct, 30 minute exposure to DMSO at concentrations of 0.25, 0.5, 1.0 and 1.5 M (Vincent, Pickering, Johnson and Quick, 1990). Only 1.0 and 1.5 M concentrations affected the cortical actin meshwork, with irregular patterns shown after the treatment. The effects on the actin meshwork appeared to be fully reversible after a 30 minute wash at 37 °C. In this study, chromosome scattering was also evident due to the treatment, and not always reversible.

In both of these studies, exposing mouse oocytes to DMSO at 4 °C resulted in far less disruption to the spindle or actin meshwork in mouse oocytes. These authors also showed that exposure to DMSO resulted in hardening of the zona pellucida surrounding mouse oocytes and that exposure to this compound at 4 °C reduced the proportion of oocytes affected (Vincent et al., 1990). They also showed that a stepwise procedure for addition and removal was more detrimental to the zona hardening effect, and increasing osmotic stress (due to exposure to increasing concentrations of a solution containing sucrose up to 1.0 M) had little effect on the zona (Johnson, 1989). Cortical granule exocytosis resulting from exposure to DMSO in mouse oocytes was confirmed and
extended to human oocytes, and was also shown to be caused by PG (Schalkoff et al., 1989). As a logical conclusion to these experiments, it was shown that exposure to DMSO at 4 °C improved fertilization compared to exposure at 37 °C. This result was subsequently extended to human oocytes (Pickering et al., 1991).

Depolymerization of the spindle microtubules in mouse oocytes also results from exposure to PG, but more strongly at lower concentrations (0.5 or 1.0 M) (Joly et al., 1992). Higher concentrations (1.5 and 2.0 M) resulted in less disruption. All of these concentrations resulted in minor disruption of the cortical actin band, with the exception of the actin-rich region just above the metaphase spindle.

Similar effects on the spindle and actin cytoskeleton were shown in bovine oocytes after exposure to 1.5 M EG, but to a lesser degree (Saunders and Parks, 1999). The proportion of oocytes displaying a normal spindle was reduced from 75 – 80 % in control oocytes to ~ 65, 55, and 50 % after exposure for 20 min, 1 hour, and 3 hours respectively. The proportion of oocytes exhibiting normal actin staining patterns was reduced from ~ 85 % to ~ 65 – 70 % with the same incubation times. The EG addition occurred in a single step, and the removal proceeded in a stepwise fashion at ~ 25 °C in this study. Rho and colleagues demonstrated an effect of exposure to 3.2 M EG and 2.36 M DMSO on microtubules (only 64% of the cells had a normal spindle pattern vs. 92 % for
controls) and mitochondria membrane potential (74 % normal vs. 82 % for controls) in bovine oocytes (Rho et al., 2002).

In a different study, bovine oocytes were exposed in a single step to DAP213 (2 M DMSO, 1 M acetamide, and 3 M PG) for 1.5 or 5 min, then transferred to an isotonic solution supplemented with 0.5 M sucrose for 5 min and then to an isotonic solution (Fuku et al., 1995). In this study, the expanded cumulus cells had been partially removed prior to the treatment. The oocytes were subsequently processed for IVF or transmission electron microscopy (TEM). Ultrastructural changes were affected to a minor degree by the 1.5 minute treatment, but more so by the 5 minute treatment. Specifically, a higher proportion of oocytes exhibited a loss of cytoplasmic cortical granules (90 % vs. 8 % for the controls) and an increase in the number of cytoplasmic vesicles (53 % vs. 16 % for the controls). While the proportion of oocytes with abnormal microvilli and mitochondria was doubled in the 5 minute treatment group, the results did not reach statistical significance.

Several studies have shown a detrimental effect of exposure to vitrification solutions on porcine oocytes. Rojas and colleagues exposed oocytes to increasing concentrations of EG in a stepwise manner (final concentration of 40 %; 7.14 M) after exposure to cytochalasin B (7.5 µg/ml) for 15 min (Rojas et al., 2004). The EG was removed by transferring the cells to an isotonic solution containing 0.75 M sucrose (5 min), then to 0.5 M sucrose (2 min), then to 0.25 M
sucrose (2 min), then to an isotonic medium for a final wash. The cells were returned to culture for 2 hours prior to fixation and cytoskeletal visualization with fluorescence microscopy. Only 41% of the oocytes displayed a normal spindle pattern after this treatment (compared to 84% of the controls), and only 45% displayed a normal actin pattern (vs. 73% of the controls). Shi et al. showed that exposure to 20% EG (3.57 M) and 20% DMSO (2.8 M) plus 18% ficoll and 0.3 M sucrose in a stepwise addition and removal procedure resulted in only 39% of the oocytes maintaining a normal MII spindle (Shi et al., 2006). A similar treatment in a separate study (Shi et al., 2006) showed that 68% of spindles were normal after exposure to 2.4 M EG and 1.77 M DMSO with 0.5 M sucrose. This study also documented a small reduction in mitochondria membrane potential staining resulting from the treatment (72% normal vs. 83% normal for untreated oocytes).

**Effects on Development**

There is conflicting evidence for an effect of exposure to DMSO on mouse oocyte developmental potential. The study by Carroll et al. (Carroll et al., 1990) showed that exposure to a concentration of 1.5 M caused a small but significant loss of developmental potential to the blastocyst stage and Bouquet et al. showed a significant effect on development to 2-cell embryos (Bouquet, Selva and Auroux, 1995); although freezing and thawing had a more pronounced effect. Yet George et al. showed that there was no effect of 1.5 M DMSO (George et al., 1994) and Van der Elst et al. showed no effect of an exposure to
a concentration of 3.5 M (Van der Elst et al., 1993; Van der Elst et al., 1998) on development to blastocysts, although the cell number was reduced in the treated group compared to controls, and the number of polyploid embryos was increased, albeit slightly in each case. In each study, the B6CBF1 strain was used. One difference is that in the study by Van der Elst et al, the oocytes were allowed to recover in culture for 1 hour prior to IVF. A recovery time was also shown to be beneficial to development by Eroglu et al (Eroglu et al., 1998). Bos-Mikich showed that exposure to 6 M DMSO for 110 seconds caused a significant reduction in the oocytes ability to progress to the first meiotic metaphase after Sr++ activation (29% progressed normally) compared to an exposure for 90 seconds (65% normal). However, the number of embryos developing to the 2-cell stage did not differ from controls when the incubation was 110 seconds. O'Neill et al. also showed that exposure to 6 M DMSO decreased the proportion of oocytes that were able to develop to blastocysts (58 vs. 65%; (O'Neill et al., 1991)). However, the addition of polyethylene glycol to the medium (1mg/ml) increased the developmental potential to 58%, which was not statistically different than the controls. Men et al. showed no effect of 1.5 M PG (in the presence of 0.2 M sucrose) but a slight negative effect of 3.5 M DMSO (in the presence of 0.5 M sucrose) on oocyte fertilization using Kunming mice (Men et al., 1997).

Conflicting results have also been presented regarding the tolerance of mouse oocytes to PG. In the report by Gook et al., (Gook et al., 1993) a very high
A concentration of 8 M EG was tolerated only if the exposure time was less than 1 minute. In a different report, fertilization rates were high after exposure to 7 M EG in a 1-step or 2-step procedure (67 and 66 % cleavage vs. 75 % for untreated controls), yet the development rate to blastocysts was significantly reduced (31 and 45 % vs. 64 % for controls (Bautista et al., 1998)).

Numerous studies have investigated the effects of cryoprotectant exposure on bovine oocytes. This has particularly been of concern for bovine oocytes since methods to cryopreserve these cells using low cryoprotectant concentrations and slow cooling have been abandoned in favor of vitrification methods, due to the extreme sensitivity of these cells to chilling. Papis and colleagues exposed bovine oocytes to 2, 4, or 6 % EG (0.36, 0.71, and 1.07 M) and showed that only the concentration of 6% had a noticeable effect on the cleavage rate (77 % vs. 94 % for controls; (Papis et al., 2000)). Martino et al. exposed bovine oocytes to solutions containing EG at concentrations of 4 or 5.5 M in a single-step, and determined that there was a significant decrease in development as a result (10
to 15 % development to blastocyst compared to 42 % for untreated oocytes; (Martino, Songsasen and Leibo, 1996)). Rho and colleagues demonstrated a significant decrease in developmental potential from exposure to a vitrification solution containing 3.2 M EG and 2.36 M DMSO (12.5 % blastocysts rate vs. 24.7 for untreated oocytes) (Rho, Kim, Yoo, Balasubramanian, Lee and Choe, 2002). Fuku and colleagues showed that exposure to DAP213 for 1.5 min had no significant effect on fertilization, cleavage or development to the blastocysts stage. A 5 minute exposure, however, resulted in a significant reduction compared to control oocytes as measured by fertilization (56 vs. 79 %), and blastocysts development (4 vs. 13 %) (Fuku, Liu and Downey, 1995).

The study by Rojas et al. also showed that a stepwise exposure to EG at a final concentration of 40% (~7.1 M) had a serious effect on the in vitro development of porcine oocytes (Rojas et al., 2004). Only 11.2 % of the oocytes exposed to the cryoprotectant solutions developed to blastocysts compared to 31 % for untreated oocytes. Shi et al. showed that only 3.8 % of porcine oocytes exposed to a solution containing to 20 % EG (~3.6 M) and 20 % DMSO (~2.8 M) with 18 % ficoll and 0.3 M sucrose developed to blastocysts compared to 26 % of untreated oocytes. Only 11 % of oocytes exposed to a solution containing 2.4 M EG and 1.77 M DMSO with 0.5 M sucrose developed to blastocysts compared to 23 % for controls. Compared to these significant reductions in developmental potential, Somfai and colleagues (Somfai et al., 2006) showed no effect of exposure to a solution of 35 % EG (~6.3 M) with 5 % polyvinylpyrrolidone and
0.3 M trehalose after an exposure to 4 % (0.7 M) EG (25 % blastocysts development compared to 27.5 % for controls).

It should be noted that experiments aimed at determining the effect of CPA rarely take into consideration the possibility of an osmotic effect independent of the chemical toxic effects of the exposure making the true cause of the injury from exposure to such solutions uncertain. This is one possible explanation for some of the apparent discrepancies in the literature.
Human Oocytes

The first report of a pregnancy and subsequent delivery of a baby derived from a frozen and thawed oocyte appeared in 1986 and 1988, respectively (Chen, 1986; Chen, 1988). In the first report, Chen described unsuccessful attempts to freeze oocytes using the method developed for human embryos (Trounson and Mohr, 1983). A modified method was developed using a mouse model that provided high survival, and was subsequently applied to oocytes from IVF patients. This specifics of this method included exposure of the oocytes to pre-cooled DMSO (1.5 M concentration) in a PBS solution with 10% fetal bovine serum for 15 min, and gradual cooling to -7 °C (rate not described). After extracellular ice induction (seeding), the temperature was lowered to -36 °C at a rate of -0.5 °C per minute. The sample was subsequently plunged into liquid nitrogen. Warming was rapid (immediate immersion into a 37 °C water bath; ~2500 °C per minute) and the cryoprotectant was diluted by adding a 4x volume of PBS. Of the 40 oocytes frozen with this method, 32 (80%) survived the freezing and thawing, and 25 of 30 were successfully fertilized. Eighteen of those underwent cleavage divisions. One of the 4 patients receiving embryos became pregnant (as described in the initial report), and the later report described the full-term delivery.
Several other reports appeared in the late 1980’s describing additional attempts to cryopreserve human oocytes (Al-Hasani et al., 1987; Diedrich et al., 1987; van Uem et al., 1987; Feichtinger et al., 1988; Mandelbaum et al., 1988; Todorow et al., 1989). One notable feature of these reports (and many other reports on oocyte and embryo cryobiology) is the lack of fundamental experiments designed to characterize the cryobiology of human oocytes. Instead, simple changes to a standard equilibrium protocol were made and outcomes were assessed. Changes included altering the addition and removal of the cryoprotectant (stepwise and at room temperature; Diedrich et al., 1987), and assessing the effects of PG vs. DMSO (Al Hasani et al., 1987). In general, the outcomes of these early reports were poor and highly variable. For example, immediate survival of 136 oocytes recovered after thawing was 32% (Diedrich et al.), 58% of those survived underwent fertilization, 2 pregnancies ensued, but neither went to term. In the report by Al Hasani et al. (1987), 28 % of the oocytes frozen in DMSO survived, and only half of those fertilized. Oocytes frozen in PG tended to survive better (32 %) and 75 % of those fertilized. The second report of a live birth came in 1987 (van Uem, Siebzehnrubl, Schuh, Koch, Trotnow and Lang, 1987) using an equilibrium method with DMSO as the permeating CPA.

While concerns over possible damage to the meiotic spindle during cryopreservation of oocytes slowed progress in this field during the early years, a few reports in the mid-1990’s offered evidence to refute this notion. Deborah
Gook and colleagues published a series of reports describing the outcome of equilibrium cryopreservation of human oocytes using PG. The first report documented a higher survival of oocytes (64 %) compared to earlier studies (Gook, Osborn and Johnston, 1993). Perhaps more importantly, this report suggested that damage to the spindle apparatus may not be as prevalent as originally thought. Of oocytes untreated, exposed only to the CPA, and those frozen, 81 %, 63 %, and 61 % had normal spindles, respectively, as assessed by conventional epifluorescence microscopy. In a second report, a karyotype analysis was performed on blastocysts having been derived from frozen and thawed oocytes. All 4 which had a chromosome spread that could be analyzed showed a normal karyotype. It should be noted that only those oocytes undergoing normal fertilization and proceeding to blastocysts were assessed, and of the oocytes not undergoing normal fertilization, all had abnormal spindles. This suggests the possibility that aneuploid embryos may be selected against during early embryo culture, biasing these results.

This topic continues to generate controversy, as some reports provide evidence that spindle damage occurs in the majority of oocytes frozen (Boiso et al., 2002) while other reports suggest that this is not the case (Cobo et al., 2001; Stachecki et al., 2004). Using live cell imaging technology, Bianchi and colleagues showed that microtubule density does increase during post-thaw culture, but does not return to the same intensity noted prior to cryopreservation (Bianchi et al., 2005). Given the inherent challenges associated with research on human oocytes and
embryos, including small numbers and restrictions on destroying human embryos, it will be difficult to resolve this controversy. Nevertheless, the fact that a significant proportion of transferred embryos perish *in vitro* and *in utero* supports the hypothesis that a high number of aneuploid embryos are generated from frozen and thawed human oocytes.

In two reports appearing in 1995, ICSI was tested against IVF to determine if ICSI would improve development (Gook *et al.*, 1995; Kazem *et al.*, 1995). Indeed, this was the outcome. These reports seemed to have a significant influence on the practice of human oocyte cryopreservation, for the use of DMSO was wholly abandoned for PG after this time. It should also be noted that at this time, the use of low concentrations of sucrose (0.1 M) in the freezing medium became routine, and likely contributed to the increased post-thaw morphological survival of oocytes with this method (although survival was not always consistently high (*e.g.* 55 % in Tucker *et al.*, 1996 vs. 34 % in Kazem *et al.*, 1995). Another important note is that in the reports by Kazem (1995) and Gook (1994), the investigators showed that a proportion of oocytes lysed in culture after thawing and CPA removal prior to sperm injection.

The first live birth using an equilibrium method with PG and sucrose as CPA appeared in 1997 (Porcu *et al.*, 1997). In this report, 33 % of the oocytes survived freezing morphologically (4 of 12), 2 fertilized normally, one underwent
cleavage and was transferred on day 2. These investigators also utilized sucrose at a concentration of 0.2 M in the freezing medium.

Possibly the most important contribution, from a basic research standpoint that has resulted in improved post-thaw viability appeared in 2001, where Fabbri and colleagues systematically compared the effects of different sucrose concentrations on oocyte survival with the equilibrium method using 1.5 M PG. Increasing the concentration of sucrose from 0.1 M to 0.3M caused a marked improvement in survival, from ~33 % to ~82 %. Due to these results, most reports have utilized a 0.3 M sucrose concentration as a cryoprotectant during human oocyte cryopreservation.

Since 2001, at least 10 reports have appeared describing the results of freezing human oocytes using a standard equilibrium method with 1.5 M PG and sucrose (See Table 2). In most cases, sucrose has been used at a concentration of 0.3 M. This and other subtle differences may explain at least some of the variation in success seen in these reports (see notes in Table 2). Some of these reports show very high survival and early development rates, and this has lead some individuals to emphatically defend human oocyte cryopreservation and suggest that the clinical results are nearly equivalent to the use of fresh embryos (Marina and Marina, 2003). Other investigators have been more cautious in their interpretation (Coticchio et al., 2004), and in two recent reports with a very large number of cycles, the results were still rather poor (Borini et al., 2006; Levi Setti
et al., 2006), especially when compared to the use of fresh embryos (Levi Setti et al., 2006).

Vitrification has also been utilized as a means to cryopreserve human oocytes in recent years, albeit less often that the traditional equilibrium method. In 1999, Kuleshova et al. described a birth resulting from an oocyte vitrified with a solution containing ethylene glycol and sucrose (Kuleshova et al., 1999). For this work, the investigators utilized an open-pulled straw (OPS) for the procedure. Since this time, other reports on human oocyte vitrification have been published (Table 2b), with all of these reports utilizing open container systems to achieve so-called “ultra-rapid cooling”. On average, the results from the vitrification trials have been better than the trials using slow-cooling (as measured by the number of oocytes to achieve a pregnancy). However, the results are still limited in number and general conclusions are difficult to reach at this time.

Overall, progress on human oocyte cryopreservation has been significant during the past few years, as evident by the number of reports appearing in the literature. Furthermore, changes in legislative mandates in recent times (e.g. (Benagiano and Gianaroli, 2004)) have pushed some clinics toward the necessity of freezing human oocytes, increasing the demand for a clinically robust procedure. Progress on investigations into new methods will continue to be slow, however, as dictated by the difficulty in conducting controlled studies with human oocytes.
Mouse Oocytes

The mouse is the first mammal for which MII oocytes were successfully frozen and stored in liquid nitrogen, and subsequently developed into live offspring (Whittingham, 1977). This report appeared in 1977 by the same investigator that contributed to the first successful method for mammalian embryo cryopreservation (Whittingham, Leibo and Mazur, 1972). The procedure used was somewhat similar to the one used for mouse embryos. It included exposing the cells (with or without the cumulus mass) to 1.5 M DMSO at 0 ºC, slow cooling (< 1 ºC/min) to -65 ºC, and slow warming (average of 8 ºC/min). The authors fertilized the oocytes either in vitro or in vivo for this study. Two strains of mice were used as oocyte donors, MF1 outbred and B6CBAF1 hybrid mice. For oocytes having been fertilized in vitro, some oocytes from both MF1 and B6CBAF1 oocytes were transferred to pseudopregnant foster dams at the 2-cell stage. However, only oocytes from the B6CBAF1 strain were cultured to later stages in vitro before being transferred (culture methods had yet to be developed to overcome the 2-cell block in embryos from many strains (Biggers, 1998)).

Overall, the proportion of morphologically normal oocytes after freezing and thawing ranged from 65 to 75 %. For the results for development to the 2-cell stage, freezing had a significant effect as did strain (B6CBAF1 oocytes cleaved at a higher rate), yet the presence or absence of the cumulus mass had no effect. A significant loss of embryos occurred in utero from the oocytes having undergone fertilization in vivo. It was estimated that only about 5 % of the
oocytes frozen with cumulus cells and 15 % without developed to day E14. For oocytes fertilized in vitro post-thaw, about 16 % (vs. 34 % for unfrozen oocytes) from the MF1 strain and 38 % (vs. 45% for unfrozen oocytes) from the B6CBAF1 strain developed to day E14 and live young after having been transferred at the 2-cell stage. Approximately the same proportion (36 %) developed to term having been transferred at the blastocyst stage (control = 25 %). B6CBAF1 oocytes tended to have a higher developmental capacity to term compared to MF1 oocytes. Stage of transfer for the B6CBAF1 oocytes did not seem to have an effect on outcome. Fertilization was in the range of 50 – 80 % and possibly could have been higher had the authors used serum in the freezing medium (as discussed above).

Since this time, dozens of papers have appeared in the literature describing results of mouse oocyte cryopreservation (see Table 3 for a summary of the more important developments).

**Different Cryoprotectants or Solution Compositions**

Todorow and colleagues (Todorow et al., 1989) directly compared the effects of DMSO, PG, or a combination of the two (all at a final concentration of 1.5 M with 0.1 M sucrose) in a slow-cooling procedure. These authors tested a stepwise addition (5 equal molar steps) and a 5-step removal as well as cooling to either -35 or -80 °C at a cooling rate of 0.3 °C /min. The combination of the two compounds gave the best results, and cooling to -80 °C was also superior to -35
°C. They did not see a significant difference in stepwise or single-step CPA addition and removal.

Surrey and Quinn also compared DMSO and PG in a slow-cooling method (Surrey and Quinn, 1990). The temperature at which the controlled cooling was terminated differed between the two compounds (-40 °C vs. -30 °C, respectively). In addition, 0.2 M sucrose was present with the PG, but no sucrose was present with DMSO. Even though survival was lower in the PG group (33 vs. 24 %), fertilization (50 vs. 71 %) and blastocysts formation rates (30 vs. 60 %) were higher. Thus, the overall proportion of oocytes developing to blastocysts was higher in the group frozen with PG compared to DMSO (5 vs. 10 %). Unfortunately, the confounded nature of the study makes it difficult to determine the true cause of the differences between the methods.

Stachecki and colleagues published a series of reports, beginning in 1998, investigating a novel approach to slow-cooling mouse oocytes; namely, modifying the freezing solution to replace sodium ions with choline ions. The rationale for this approach was the hypothesis that the high concentrations of sodium ions that result from the precipitation of water as ice during freezing are responsible for the loss of cell viability. In the first report (Stachecki et al., 1998), they demonstrated a significant preservation of developmental potential after replacing nearly all of the sodium ions with choline. When the sodium concentration was reduced from 167 mM to 30 mM (with a concomitant increase
in choline concentration), 99 % of the cells recovered from the freezing procedure with intact membranes, and 35 % of the total number of oocytes frozen were able to develop to blastocysts. In a second report (Stachecki et al., 1998), they tested several variables of the freezing procedure on developmental potential, including thawing rate, plunge temperature after slow-cooling, and the composition of the holding medium used prior to freezing (choline chloride-based medium vs. NaCl-based medium). In their best results using a choline chloride holding and freezing medium along with a moderate rate of warming (warming in air prior to warming in a 30 °C water bath using ¼ cc straws), they demonstrated that 58 % of the oocytes frozen could develop to blastocysts. In a later report they went further to show that a high proportion of oocytes could develop to term after embryo transfer (Stachecki et al., 2002). Of recipients becoming pregnant, 48 % of the transferred embryos developed to fetuses on E10 – 13 and 27 % of the total number of oocytes frozen could develop to term. The logic of replacing sodium chloride with choline chloride stems from the hypothesis that the increased concentration of salt resulting from water precipitation as ice during cooling is a primary mechanism of cryoinjury when equilibrium freezing methods are utilized, as was described earlier. However, the authors also suggest that choline could interact directly with the membrane and assist in its stabilization during freezing.

Studies such as those described above show how the solution composition can have a great influence on the outcome of attempts to cryopreserve cells.
Solution properties can have differing influences, from the ability to depress the rise in extracellular solute concentration concurrent with ice precipitation (Pegg, 1987), to effects on the hydration level of cells, and direct chemical effects. The last series of studies discussed is particularly insightful in this regard, as the replacement of a common inorganic ion with an organic ion was shown to have a beneficial effect, and the authors suggest the resulting benefit was due to a reduction in the Na⁺ concentration. However, one possibility these authors seem to dismiss is an intracellular role for choline. Choline is a metabolic precursor of betaine, a compound commonly used as an organic osmolyte for counteracting the effects of osmotic stress (Landfald and Strom, 1986; reviewed in Somero and Yancey, 1997). It is possible that choline entry into the cell could act as an intracellular osmolyte during freezing, and the fact that these authors saw an increased protective role when the cells were incubated for longer periods in the choline chloride-based medium prior to freezing lends support to this hypothesis. Regardless of the specific mechanism, these results show how significant the composition of the cryopreservation solution can be on the ability to achieve successful cryopreservation.

**Rapid Cooling without Vitrification**

A small number of experimental reports have been published describing attempts to rapidly freeze mouse oocytes in moderate concentrations of either EG or DMSO. Sathananthan and colleagues reported on freezing mouse oocytes with 3.5 M DMSO or PG with 0.5 M sucrose (Sathananthan et al., 1988).
The time for exposure to the CPA was approximately two min and the straws were then plunged into liquid nitrogen. There was no dilution procedure post-thaw that included an osmotic buffer, however. Of the oocytes frozen, only 33-34% survived morphologically. Of these, 15 % frozen in DMSO and 7 % frozen in PG developed to blastocysts. Ultrastructural analysis was performed with the thawed oocytes within 1 hour of thawing in one group, and another group was fixed at 0 °C immediately after thawing. All oocytes fixed immediately at 0 °C showed signs of severe ultrastructural damage including spindle disorganization and membrane disruption. Various levels of disruption were apparent, but to a lesser degree in the oocytes thawed to 37 °C and held at approximately 22 °C prior to fixation.

Van der Elst and colleagues published two similar reports with such a method (Van der Elst et al., 1993; Van der Elst et al., 1998). In both reports, the authors froze the solution containing the oocytes by rapidly quenching the straws in liquid nitrogen after exposure to 3.5 M DMSO and 0.5 M sucrose. Survival rates were very similar (94 % after CPA exposure in both studies and 78 % and 87 % after rapid freezing in the 1993 and 1998 studies, respectively). The fertilization rate of the frozen oocytes was similar (59 % and 69 % respectively). The development to the blastocyst stage of oocytes reaching the 2-cell stage was better in the report in 1998 compared to 1993 (82 % vs. 49 %). In the 1993 report, the authors compared ploidy in the resulting embryos and determined that exposure to the CPA solution resulted in a small reduction in the proportion of embryos
having diploid nuclei (78 % vs. 81 % for CPA exposure and control, and 74 % vs. 91 % for frozen and thawed oocytes vs. control). Also in the 1993 report, the total number of nuclei was reduced in the frozen group compared to the controls (38 vs. 70) and the proportion of blastocysts that developed to d17 viable fetuses was reduced to one-third of the unfrozen controls (11 % vs. 32 %). In the 1998 report, they also showed that the total cell number per blastocyst was reduced in the frozen group (40 vs. 54) and that the average number of cells in the inner cell mass (14) was less in the frozen group compared to the other treatments.

In 1994, Rayos and colleagues reported on rapid freezing of mouse oocytes using 3 M EG and 0.25 M sucrose or trehalose (Rayos et al., 1994). They tested the effects of different incubation times in the CPA prior to putting the straws into liquid nitrogen (5, 10, 20, and 40 min). Overall, the sugar treatment had no effect. The five minute incubation was the only treatment that resulted in a reduced survival compared to the other times (52 % vs. 67, 77, and 77 %, respectively). The blastocyst development rate was also reduced with the five minute group compared to the others (26 % vs. 38, 44, and 43 %; the control rate was 88 %). Fetal development was also reduced due to freezing (20 % vs. 49 % for controls).

This particular method, rapid freezing in moderate concentrations of CPA, at first appears to be a fair compromise between the potential toxic effects of high concentration of solutes used in true vitrification procedures and the potential for
cooling damage of equilibrium methods. However, the process itself is somewhat precarious. Using a moderate concentration of cryoprotectant may allow true vitrification during rapid cooling, but the likelihood of devitrification and recrystallization during warming is very high. Critical warming rates are usually many orders of magnitude higher than critical cooling rates (the rates needed to avoid ice crystal formation during warming and cooling, respectively; cf. Baudot and Odagescu, 2004), and at the cooling and warming rates attainable with ¼ cc straws, solutions with CPA concentrations in the range used in these studies are very likely to undergo devitrification. Even with higher concentrations of CPA, devitrification can be a serious challenge, resulting in high variability between experimental runs (Shaw et al., 1992).

Vitrification

Reports of vitrification of mouse oocytes appeared in the literature shortly after the successful vitrification of mouse embryos by Rall and Fahy (Rall and Fahy, 1985). In 1988, Kola and colleagues vitrified cumulus-intact oocytes using Vitrification Solution 1 (VS1: 2.5 M DMSO, 2.36 M acetamide, 1.19 M PG, 5.4 % polyethylene glycol) and compared the results to a traditional slow-cooling procedure with 1.5 M DMSO to -80 °C before storage in liquid nitrogen (Kola et al., 1988). Exposure to the VS1 solution alone resulted in morphological changes to the oocytes and reduction in their developmental potential. The fertilization potential of the slowly-frozen oocytes was not affected a great deal, but further development was impaired even more than the oocytes having been vitrified.
The incidence of aneuploidy was slightly higher in the vitrification group compared to the untreated oocytes (2-3X higher); an increase was also seen in the group cooled with slow freezing, but less than the vitrified group.

In 1989, Nakagata and colleagues reported very high survival of oocytes vitrified using VS1 medium when the total time for exposure to the solution was 10 seconds or less and the dilution was rapid (immediate transfer to a 10X volume of 0.3M sucrose). Eighty-eight percent of the thawed oocytes were morphologically normal, and the fertilization rate was very high with control oocytes as well as those only exposed to the modified VS1 medium and vitrified (97 %, 86 %, and 82 %, respectively). Cleavage for these groups after IVF was also high (98, 84, and 78 %), as was the proportion of live young (60, 43, and 46 %). These high survival rates were not repeatable by another group using this method, however (Shaw et al. reported that the use of Nakagata’s original procedure resulted in 54 % morphological survival in their hands (Shaw et al., 1991)). In this report, Shaw et al, used a 90 % concentration of the original VS1 recipe, and made modifications to the CPA addition and removal method original used by Rall and Fahy (1985). They found that extending the CPA dilution procedures (without sugars as osmotic buffers) could improve the overall viability. When the oocytes were only exposed to the vitrification solution and the dilution procedure was extended, reducing the osmotic effects, the morphological survival improved (96 % vs. 77 % for the original method). The proportion that developed to the blastocyst stage from the 2-cell stage was also
greater (95 % vs. 51 %). These results were better, despite the oocytes being in
contact with the dilute vitrification solutions for longer periods of time. When
sucrose was added as an osmotic buffer and the exposure to solutions
containing the permeating CPA was eliminated during the dilution procedure, the
results were also improved. The proportion of oocytes developing to the
blastocyst stage having been vitrified by the original method where the CPA was
removed stepwise without the presence of sucrose was more than doubled by
using a 1-step dilution with sucrose as an osmotic buffer (24 % blastocyst
development vs. 55 % with sucrose dilution). The effect of this procedure when
exposure to the cryoprotectant without cooling was tested was also dramatic (31
% vs. 72 % blastocysts development, respectively). The morphological results
support the conclusion of a protective effect of sucrose on the membrane after
vitrification, for when the longer dilution procedure without sucrose was
employed, fewer oocytes survived morphologically (45 %) compared to when
sucrose was employed (79 %). A report that appeared the same year also
showed that changes to the CPA dilution procedure after vitrification of mouse
oocytes in VS1 could also result in high survival and developmental rates (Kono
et al., 1991). In this study, the authors also performed embryo transfer and
showed that the overall term development rate was approximately 25 % after
vitrification. Other investigators have shown the beneficial effects of extended
dilution procedures on post-thaw oocyte viability after exposure to a high
concentration of EG (7 M), with and without vitrification (Bautista et al., 1998).
The following year, Shaw and colleagues also reported on the effects of brief exposures of mouse oocytes to 90 % VS1 solutions and the subsequent viability (Shaw, Bernard, Fuller, Hunter and Shaw, 1992). They investigated exposing oocytes directly to the solution for 10, 15, 30, 60, or 300 seconds. While exposures up to 1 minute were tolerated well (>85 % morphologically normal oocytes), a 5-minute exposure reduced the number of morphologically normal oocytes to 48 %. Fertilization and cleavage rates were also very high for the treatments lasting ≤ 60-seconds, and blastocyst development was good as well for the 10, 15, and 30-second groups (≥77 %; the group exposed for 60 seconds had a blastocyst developmental rate of 45 %). The results for the groups having been vitrified were markedly lower, with rates of blastocyst development of 22, 32, 12, 10, and 0 for the respective treatments; untreated oocytes developed to blastocysts at a rate of 87 %. The development rates were highly variable within the treatments, and the authors attribute this, at least in part, to devitrification of the medium during thawing, even though the thawing took place in a 37 °C water bath. These results suggest that the cells are able to tolerate high osmotic stress for brief periods of time. The authors mention that there was very little evidence of any volume recovery of the cells for the first 60 seconds of exposure, suggesting that only an infinitesimal volume of cryoprotectant penetrated the oocytes during this time.

The time of exposure to a 6.0 M DMSO solution has also been shown to be important to mouse oocytes (110 seconds yielded lower results than 90 seconds;
Bos-Mikich et al., 1995). These authors used a stepwise exposure to DMSO instead of a direct addition to the final solution. With the short exposure time, the percent of viable fetuses was shown to be similar between the untreated control, DMSO exposure, and vitrified groups.

Results such as these have lead to the development of certain practices for vitrifying oocytes and embryos from mammals, incorporating very brief times of exposure to the CPA. Some authors have argued that restricting the times of exposure to only a few seconds is difficult in practice, and not an optimal method for practical application as there is little room for error (Stachecki and Cohen, 2004). Considering that the 1991 reports by Shaw et al. and Kono et al. have shown that oocytes can tolerate exposures to less concentrated solutions of VS1 for longer periods of time, and similar results have been reported with DMSO, there appears no need to resort to the extreme measure of direct exposure to the final concentration of the vitrification solution for brief periods of time as first described by Nakagata (1989).

Since this time, numerous other reports have appeared in the literature describing successful mouse oocyte vitrification with varying modifications, including the use of modified complex vitrification solutions and vitrified sperm (Nakagata, 1993), ethylene glycol (Hotamisligil et al., 1996), the addition of various macromolecules (O'Neil et al., 1997; O'Neil et al., 1998), and devices (Lane and Gardner, 2001). Table 3 summarized the results of embryo
development from several reports where mouse oocyte cryopreservation was investigated.

In general, rapid cooling methods, including vitrification procedures, are becoming more commonly used to cryopreserve mammalian oocytes (Vajta and Nagy, 2006). As discussed above, the idea that short exposure times are more beneficial when using high concentrations of CPA has become established. However, as results such as those of Shaw and colleagues (Shaw et al., 1991) and Kono and colleagues (Kono, Kwon and Nakahara, 1991) have shown, when a reduction in the osmotic consequences of cryoprotectant exposure are incorporated into the method, short exposure times may not be necessary. This is a very important conclusion, as ensuring that cryoprotectant exposure occurs for only a very brief time is difficult in practice. Furthermore, the likelihood of IIF can be higher than expected if devitrification is considered, and solutions are frequently used that are likely to undergo devitrification during warming. With more careful study design, osmotic stress and devitrification can be avoided, which should result in more robust procedures.

**Aneuploidy**

Due to the known disruptive effects of cold exposure on the metaphase II spindle as discussed above, several investigators have examined rates of aneuploidy and polyploidy after freezing MII stage mouse oocytes. In 1992, Sterzik and
colleagues noted a relatively small increase (4 % vs. 0 % in controls) in the incidence of hyperploidy in 2-cell stage embryos developed from frozen and thawed oocytes using 10 % DMSO and 10 % sucrose exposure at room temperature and applying a standard equilibrium freezing procedure to -32 °C prior to liquid nitrogen storage (Sterzik et al., 1992). The total incidence of all types of aneuploidy and polyploidy were not significantly different compared to controls. Using this freezing procedure, there was also a significant reduction in the proportion of frozen and thawed oocytes that underwent cleavage after IVF compared to unfrozen oocytes (34 vs. 75 %). Bouquet and colleagues published two reports describing an analysis the results of freezing mouse oocytes in 1.5M DMSO to -80 °C. In one report (Bouquet et al., 1993), they determined that the incidence of sister chromatid exchange was about 50% higher in all treatments (CPA exposure at either 0 °C or room temperature, cooling to 0 °C, and complete freeze and thaw cycles) compared to controls. There was no significant difference among the treatments themselves. The proportion of polyploid embryos originating from frozen and thawed oocytes was about twice as high (15.1 % vs. 7.1 %) compared to unfrozen oocytes. There was no difference in the proportion of haploid embryos, suggesting that digyny was responsible for the results. The results in their second report were similar (Bouquet et al., 1995), with the frozen and thawed group showing an increase of ~ 5-fold in polyploidy compared to control oocytes. They also looked at the developmental potential of the embryos after freezing, and the proportion of oocytes undergoing cleavage after IVF was reduced to about half the level of the controls (30 vs. 64 %). Their
results showed a reduction in cleavage for oocytes having been exposed to DMSO for 15 min at 0 °C (49 % cleavage) and room temperature (40 % cleavage). Bos-Mikich and Whittingham (Bos-Mikich and Whittingham, 1995) showed that exposure to DMSO at 37 °C for 60 min resulted in a very high incidence of aneuploidy (76.9 %, with an approximately equal mix of hypo- and hyperploidy) after parthenogentic activation. The average incidence of aneuploidy increased by varying amounts due to varying combinations of exposure to 1.5M DMSO and cold temperature (0 °C: cooling oocytes in the presence of DMSO for 30 or 60 min resulted in a 14 and 11 % aneuploidy rate, respectively; in the absence, 19 and 17 %, respectively). The incidence of retention of the second polar body did not differ across treatments, however.

These results suggest that, while aneuploidy may be a factor in loss of developmental potential after cryopreservation attempts in mouse oocytes, the probability is relatively small. This may be a result of the ability of the MII spindle to undergo normal repair after prolonged cooling, as described above.

**Direct Comparison of Equilibrium Freezing, Rapid Cooling, and Vitrification Methods**

Several investigations have tested slow-cooling methods directly against rapid cooling methods in the same study, with only some of the later methods containing vitrifiable concentrations of CPA. Surrey and Quinn compared rapid
cooling using 3.5M EG plus 0.25, 0.5, or 1.0 M sucrose to slow-cooling with either 1.5 M DMSO or PG, the later containing 0.2 M sucrose (Surrey and Quinn, 1990). For the first three methods, the authors transferred the cells initially to solutions with just sucrose, and then to solutions containing both CPA prior to freezing. The average blastocyst development rates for the respective groups were 49, 59, 5, 30, and 60 percent. Untreated oocytes developed to blastocysts at a rate of 62 %. Aigner and colleagues compared slow-cooling with 1.5 M DMSO to rapid cooling with 3.5 M DMSO plus 0.5 M sucrose (Aigner et al., 1992). They noted ice crystal formation during cooling with the rapidly-frozen treatment. Survival was higher with the rapidly-cooled oocytes compared to the slowly-cooled ones (94 vs. 69 %). After a 1-hour recovery in culture, 67 % of the oocytes cooled slowly had normal spindle morphology compared to 84 % of the rapidly-cooled oocytes (91 % normal spindle morphology in control oocytes). After a 3-hour recovery, the proportions were 72, 87, and 95 %, respectively).

Men and colleagues showed a reduction in fertilization of oocytes cooled with an ultra-rapid method using 3.5 M DMSO and 0.5 M sucrose (59 %) compared to 72 % and 74 % fertilization for cells slowly frozen with 1.5 M PG plus 0.2 M sucrose and vitrified with VS1 solution (Men, Chen, Ji, Shang, Yang and Zou, 1997). Lane and Gardner described superior results using cryoloop vitrification compared to slow-cooling (Lane and Gardner, 2001). The former procedure having been conducted with 2.8 M DMSO and 2.8 M EG plus 10mg/ml Ficoll and 0.65 M sucrose, and the later with 1.5 M PG in a choline chloride based solution. Fetal development was better in the vitrified oocytes (57 %) compared to those
cooled slowly (26 %). Two recent reports described very poor development of oocytes having been frozen slowly with PG compared to vitrified oocytes. In the report by Valojerdi and colleagues (Valojerdi and Salehnia, 2005), none of the oocytes frozen in PG developed to the morula stage, whereas nearly 60% of those vitrified in 30% Ficoll, 10.7 % acetamide, 10 % EG, and 0.5 M sucrose developed to blastocysts. Similarly, in the report by Kim et al, only 3 % of the oocytes frozen in PG survived, whereas 85 % survived vitrification on electron microscope grids in the presence of 5.5M EG plus 1.0M sucrose (Kim et al., 2006).

These results are very insightful in regards to the difficulty of making broad generalizations about the success of equilibrium vs. non-equilibrium methods. According to Aigner and colleagues, rapid cooling is superior to equilibrium freezing (Aigner et al., 1992). According to Men and colleagues, rapid cooling is inferior to equilibrium freezing or vitrification (Men et al., 1997). Lane and Gardner conclude that vitrification is superior to equilibrium freezing (Lane and Gardner, 2001), as do Valojerdi and Salehnia (Valojerdi and Salehnia, 2005) and Kim and colleagues (Kim et al., 2006). However, according to the results of Surrey and Quinn, rapid cooling can be equally effective compared to equilibrium freezing, depending upon the specific nature of the method utilized (Surrey and Quinn, 1990). While their conclusions are all correct in general, the more precise conclusions should be that some methods of equilibrium freezing are as good as or superior to non-equilibrium methods, and some methods of non-equilibrium
freezing are superior to equilibrium methods. The most appropriate question to be asked as a result of such an analysis is why some methods are better than others. Obviously, cooling rate is only one of many factors in a cryopreservation procedure, and the particulars of the other factors may favor one method over the other. Designing experiments to understand the fundamentals behind the reasons is the mark of good scientific inquiry.
Bovine Oocytes

Numerous reports have appeared in the literature describing efforts to cryopreserve bovine oocytes using slow cooling methods (Table 4). Overall, this approach has been unsuccessful for MII bovine oocytes, with very low blastocyst yield in vitro. Investigators have tested several variables, including post-thaw culture period (Asada and Fukui, 2000), different CPA (Glycerol, DMSO, or PG; (Lim et al., 1999)) and at different concentrations (1 or 1.5 M), the use of different sugars as post-thaw osmotic buffers (sucrose, lactose, or trehalose; (Schellander et al., 1994)), different cooling rates (0.3, 0.6, or 0.9 °C; (Otoi et al., 1994)), concentrations of sucrose in the freezing solution (0, 0.1, or 0.2; (Otoi et al., 1995)), and polarization of cytoplasmic lipids (Otoi et al., 1997). None of these variables has had a marked effect on the success of the procedure as measured by overall post-thaw developmental potential.

As discussed above, bovine oocytes are highly prone to chilling injury. Long time periods of exposure to cold temperatures seems to be the common factor among the studies cited above, and is likely the major contributor to the failure of slow cooling as a methodology. As a result, researchers have turned their attention to rapid cooling methods to cryopreserve bovine oocytes. The earliest reports attempted vitrification in ¼ cc straws (Table 4b). In 1992, Fuku and colleagues directly compared a vitrification method to a slow cooling method (Fuku et al., 1992). Surprisingly, the slow cooling method proved superior. However, the
oocytes were exposed to the highly concentrated vitrification solution (2 M DMSO, 1 M acetamide, and 3 M PG; DAP213) in a single step. Thus, the osmotic shock of this procedure may have been responsible for much of the loss of developmental potential. As supporting evidence for this hypothesis, Hamano and colleagues (Hamano et al., 1992) showed that a higher developmental potential could be preserved if this CPA was diluted from the cells in a gradual fashion (12 steps; 10 % blastocyst yield) than in a more rapid fashion (2 steps; 0 % blastocyst yield). A 1-step addition procedure was also used in this investigation.

In 1996, Martino and colleagues (Martino et al., 1996) demonstrated a significant improvement in the cryopreservation of bovine oocytes using a so-called “ultra-rapid cooling” method (Table 4c). In this report, they utilized electron microscope grids as a carrier for the oocytes and vitrification solution and plunged them directly into liquid nitrogen. The blastocyst yield ranged from 10 to 15% with all of the treatments, including the CPA exposure controls, and the authors suggested that these results demonstrated that chilling injury could be overcome when such rapid cooling was employed. Since this time, several other devices/methods to achieve rapid cooling have been developed and tested with bovine oocytes, including OPS (Vajta et al., 1998), solid surface vitrification (SSV; (Le Gal and Massip, 1999; Dinnyes et al., 2000)), gel loading pipette tips (Asada et al., 2002), glass capillaries (Rho et al., 2002), and the cryotop (Chian et al., 2004). While many of these reports demonstrate higher survival and blastocysts development
compared to the use of slow-cooling methods, others have shown more limited success, even when using identical methods (Men et al., 2002). The inventors of these ultra-rapid cooling devices/methods and other authors have argued that the success of these devices suggests that ultra-rapid cooling is a necessity to achieve effective cryopreservation of bovine oocytes. However, others have argued that the use of such devices is cumbersome, and may not be necessary if improvements of the overall vitrification approach are made (Otoi et al., 1998).

In a series of experiments, Otoi and colleagues took a more systematic approach to develop improved vitrification solutions and methods for their addition and removal to try to account for potential osmotic damage during the procedure, and demonstrated that the use of a ¼ cc straw could achieve results comparable to the so-called ultra-rapid cooling devices. Testing various stepwise CPA addition methods, EG concentrations, and CPA removal procedures, this group achieved blastocysts development up to 20 % (the average development for the best procedure was 10% compared to 41 % for untreated oocytes). They determined that a 3-step procedure was superior to a one or two-step CPA addition. Most reports on bovine oocyte vitrification use a 2-step CPA addition with no justification for such an approach. Such improvements provide strong support for utilizing a fundamental approach to improving methods to cryopreserve mammalian oocytes.

Despite the success of the trials by Otoi and colleagues (Otoi et al., 1998), utilization of ultra-rapid cooling devices has become the established paradigm for
mammalian oocyte vitrification. This has had unfortunate side effects (in my opinion) in that the avenues of investigation have been apparently narrowed. Across the field of mammalian oocyte cryopreservation, much of the current research continues to proceed in a trial-and-error fashion with the assumption that the use of ultra-rapid devices is a prerequisite. This has the unfortunate effect of eliminating the consideration of investigations into procedures which may be at least as successful, and perhaps even better from the standpoint of cell viability and user-friendliness. This seems particularly true in the human assisted reproduction community, where a robust method for oocyte cryopreservation is increasingly needed.
Porcine Oocytes

To date, very few investigations focusing on cryopreserving MII porcine oocytes have appeared in the literature. Even though bovine oocyte cryopreservation has been very difficult, porcine oocytes have proven even more recalcitrant to cryopreservation (see Table 5 for an overview). In 1999, Nagashima and colleagues reported only a 6.3 % development rate (to the 8-cell or morula stage) for porcine oocytes after having been vitrified in 40 % EG and 1.0 M sucrose in a standard holding medium with ¼ cc straws, and having been fertilized by sub-zonal sperm injection (Nagashima et al., 1999). The oocytes also underwent lipid removal via centrifugation and micromanipulation prior to vitrification. None of these oocytes developed to blastocysts in culture. The method used to vitrify these oocytes was very unorthodox, and may account for much of the loss of developmental potential. The cells were initially incubated in 10 % glycerol for 10 min prior to transferring them to the vitrification solution described above.

Failure of embryo development after cleavage was also reported by Rojas and colleagues (Rojas et al., 2004). This group used a 7-step CPA addition (0.5 M increments up to 30 %, then transfer to 40 % EG) and a 4-step EG removal process using sucrose as an osmotic buffer. The oocytes were also incubated with 7.5 μg/ml Cytochalasin B prior to vitrification. Ten percent of the vitrified and warmed oocytes (OPS method) underwent cleavage after IVF, and none
progressed further. Somfai and colleagues also investigated the effects of Cytochalasin B at the same concentration on porcine oocyte vitrification using the solid surface vitrification method (Somfai et al., 2006). They used a 2-step CPA addition procedure (4 % EG for 10 – 15 min at 37 °C, then 35 % EG with 5 % PVP and 0.3 M trehalose (≤ 30 sec exposure) as the final vitrification solution). They measured post-warming viability using several assays, including vital dye staining (both propidium iodide and fluorescein diacetate) and artificial activation followed by in vitro culture. In their first experiment they demonstrated that exposure to the CPA without cooling had no effect on viability as measured by the vital dye assays. They went on to show that CPA exposure also had no effect on the proportion of oocytes developing to blastocysts after activation. However, vitrification with or without Cytochalasin B caused a significant proportion of the oocytes to lose their developmental potential (2.5 to 5 % blastocyst development compared to 26 to 27 % with untreated oocytes) Gupta and colleagues used a nearly identical vitrification procedure to that used by Somfai and colleagues just described (Gupta et al., 2007). In an experiment using GV-stage oocytes, they showed a slight improvement in the outcome when a combination of EG and DMSO was used, so they applied this combination to MII oocytes as well. They also determined that this method caused a significant decrease in the developmental potential of the oocytes (only 3.4 % progressed to blastocysts compared to 20.1 % for the control treatment after IVF).
Fujihara et al. investigated the effects of Paclitaxel (Taxol®) on vitrification at concentrations up to 5 µM (Fujihira et al., 2005). These authors used the cryotop device for vitrification and fertilized the oocytes using ICSI. Concentrations of paclitaxel actually reduced the developmental potential in a concentration-dependent manner (7.4, 4.5, 1.2, and 1.2 % blastocysts rate for 0, 0.5, 1.0 and 5.0 µM, respectively). Control oocytes developed to blastocysts at a rate of 38%. These results were similar to those described in a more recent report by Shi and colleagues (Shi et al., 2006). In their study, none of the oocytes vitrified using OPS either in the presence of absence of 1 µM paclitaxel with 20 % EG, 20 % DMSO, 18 % ficoll 70, and 0.3 M sucrose developed to blastocysts. They did see an improvement in the proportion of oocytes undergoing cleavage with paclitaxel, however (24.3 % vs. 5.6 %).

Wu and colleagues noted that OPS vitrification with 40 % EG and 0.6 M sucrose caused all of the MII oocytes to fail to undergo cleavage division after IVF. In an ultrastructural analysis, they showed that the cortical granules in the oocytes were not released as a result of the vitrification procedures, suggesting that zona hardening was not the reason for failure of cleavage. They also showed that the mitochondria appeared normal in their electron micrographs, but a noticeable change in the level of homogeny of the lipid vesicles, and vacuoles appeared in the cytoplasm. They also demonstrated a significant disruption in the microtubule staining patterns (13 % vs. 80 % for the controls) and actin staining patterns (37 % vs. 72 % for the controls).
An interesting note was made in the report by Gupta and colleagues (Gupta, Uhm and Lee, 2007). The authors mentioned that some of the droplets of solution became opaque during cooling or warming, and they were discarded from their analysis. This suggests that the solution they used was not a stable vitrification solution. Their solution was nearly identical to the one used by Somfai and colleagues described above, except sucrose was used as the non-permeating sugar by Gupta et al. (Gupta et al., 2007) instead of trehalose, which was used by Somfai and colleagues (Somfai et al., 2006). Again, this observation reflects the failure of studies investigating procine oocyte cryopreservation to account for some of the fundamental principles of cryobiology. Accounting for some of these basic principles is likely to lead to improvements in the cryosurvival of oocytes (Woods et al., 2004).
Overall Summary

As this review has shown, a very large number of studies have been undertaken to advance our understanding of oocyte cryobiology. Some significant insights into the nature of oocyte cryoinjury have resulted from these investigations. For example, our understanding of the differential sensitivity of oocytes across taxa to cooling has provided a means to explain why slow cooling methods are relatively successful with mouse oocytes, but fail to preserve the viability of oocytes from cattle and pigs. Furthermore, cytoskeletal elements in oocytes (e.g. microtubules and microfilaments) have been shown to be easily damaged by various aspects of cryopreservation. Like all cells, oocytes are damaged by the formation of intracellular ice and high concentrations of cryoprotectants, yet damage from cryoprotectants can be mitigated to a large degree by reducing the osmotic stress associated with their addition and removal. Vacuoles are often seen in ultrastructural analyses of oocyte vitrification, yet the nature of these vacuoles is still uncertain.

As was highlighted throughout this chapter, cryopreservation is a multi-step process, with each step having the potential to cause cell damage. Many of the variables in a cryopreservation procedure are interdependent, as discussed in the section on the science of cryobiology. As a result of this equivocal nature of study outcomes, it is often very difficult to ascribe cryoinjury to a specific component of a cryopreservation method which makes overcoming the injury
quite challenging. Furthermore, as just discussed, the nature of cryoinjury differs across taxa, making the development of a universal method for oocyte cryopreservation unlikely. Therefore, continued progress in this field will require patience, diligence, and perhaps even a bit of fortune.
References Cited


Bianchi V, Coticchio G, Distratis V, Di Giusto N, Flamigni C and Borini A (2007) Differential sucrose concentration during dehydration (0.2 mol/l) and rehydration (0.3 mol/l) increases the implantation rate of frozen human oocytes. Reprod Biomed Online 14, 64-71.


Storey KB and Storey JM (1988) Freeze tolerance in animals. Physiol Rev 68, 27-84.


<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Taxon</th>
<th>CPA</th>
<th>Average IIF temperature (°C)</th>
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<tr>
<td>Leibo et al.</td>
<td>1978</td>
<td>Mouse</td>
<td>DMSO (1 M)</td>
<td>-40</td>
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<td>Toner et al.</td>
<td>1991</td>
<td>Mouse</td>
<td>none</td>
<td>-12</td>
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<tr>
<td>Mazur et al.</td>
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<td>Mouse</td>
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<tr>
<td>Mazur et al.</td>
<td>2005</td>
<td>Mouse</td>
<td>Glycerol (1 M)</td>
<td>-41</td>
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<tr>
<td>Mazur et al.</td>
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<td>Mouse</td>
<td>none</td>
<td>-14</td>
</tr>
<tr>
<td>Chandrasekaran et al.</td>
<td>1990</td>
<td>Bovine*</td>
<td>none</td>
<td>-11.2 (cooling rate of 16 °C/min)</td>
</tr>
<tr>
<td>Ruffing et al.</td>
<td>1993</td>
<td>Bovine*</td>
<td>EG (1.5 M)</td>
<td>-45.6, -39.2, -27.8**</td>
</tr>
<tr>
<td>Ruffing et al.</td>
<td>1993</td>
<td>Bovine*</td>
<td>Glycerol (1.5 M)</td>
<td>-46.5, -33.5, -20.7**</td>
</tr>
<tr>
<td>Ruffing et al.</td>
<td>1993</td>
<td>Bovine*</td>
<td>PG (1.5 M)</td>
<td>-34.6, -28.1, -19.4**</td>
</tr>
</tbody>
</table>

*Data reported here are for in vitro matured oocytes
** These data are for cooling rates of 4, 8, and 16 °C/min, respectively.
Table 2. Results from cryopreservation studies with MII human oocytes since 2001

<table>
<thead>
<tr>
<th>First Author</th>
<th>Year</th>
<th># of patients</th>
<th># of cycles</th>
<th># of oocytes thawed</th>
<th># of oocytes surviving (%)</th>
<th># of oocytes injected with sperm</th>
<th># of oocytes fertilized (%)</th>
<th># of oocytes undergoing cleavage of those fertilized (%)</th>
<th># of transfers performed</th>
<th># of pregnancies (% per transfer)</th>
<th>% pregnancy per patient</th>
<th>Average # of oocytes required to achieve one pregnancy</th>
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<td>Fabbri</td>
<td>2001</td>
<td>112</td>
<td>1502</td>
<td>796 (54)</td>
<td>632</td>
<td>365 (58)</td>
<td>58</td>
<td>33 (57)</td>
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<td>5 (42)</td>
<td>42</td>
<td>54.5</td>
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<td>Quintans</td>
<td>2002</td>
<td>12</td>
<td>109</td>
<td>58 (53)</td>
<td>58</td>
<td>33 (57)</td>
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<td>5 (42)</td>
<td>42</td>
<td>57</td>
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<tr>
<td>Notes: Choline chloride used in medium</td>
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</tr>
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<td>Fosas</td>
<td>2003</td>
<td>7</td>
<td>7</td>
<td>88</td>
<td>79 (90)</td>
<td>79</td>
<td>58 (73)</td>
<td>7</td>
<td>4 (57)</td>
<td>57</td>
<td>22</td>
<td>22</td>
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<tr>
<td>Notes: Oocytes donated from young women (18-25 yrs)</td>
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<td>Boldt</td>
<td>2003</td>
<td>16</td>
<td>90</td>
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<td>66</td>
<td>39 (59)</td>
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<td>2003</td>
<td>7</td>
<td>7</td>
<td>49</td>
<td>6 (12%)</td>
<td>6</td>
<td>3 (50)</td>
<td>2</td>
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<td>Notes: Standard method, 0.1M sucrose in medium</td>
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<td>Borini</td>
<td>2004</td>
<td>68</td>
<td>86</td>
<td>737</td>
<td>273 (37)</td>
<td>273</td>
<td>124 (45)</td>
<td>107 (86)</td>
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<td>15 (25)</td>
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<td>0.1M sucrose in freezing medium</td>
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<td>Chen</td>
<td>2005</td>
<td>21</td>
<td>159</td>
<td>119 (75)</td>
<td>119</td>
<td>80 (67)</td>
<td>73 (91)</td>
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<td>7 (33)</td>
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<td>Notes: Results compare favorably with zygote freezing.</td>
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<td>Borini</td>
<td>2006</td>
<td>146</td>
<td>201</td>
<td>927</td>
<td>687 (74)</td>
<td>589</td>
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<td>404 (90)</td>
<td>185</td>
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<td>Notes: Limited number of oocytes thawed to comply with Italian Law</td>
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<td>Levi Seti</td>
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<td>120</td>
<td>159</td>
<td>1087</td>
<td>687</td>
<td>464 (68)</td>
<td>413 (89)</td>
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<td>18 (12)</td>
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Table 2a (continued). Results from equilibrium cryopreservation studies with MII human oocytes since 2001

<table>
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<th>First Author</th>
<th>Year</th>
<th># of patients</th>
<th># of cycles</th>
<th># of oocytes thawed</th>
<th># of oocytes surviving (%)</th>
<th># of oocytes injected with sperm</th>
<th># of oocytes fertilized of those injected (%)</th>
<th># of oocytes undergoing cleavage of those fertilized (%)</th>
<th># of transfers performed</th>
<th># of pregnancies (% per transfer)</th>
<th>% pregnancy per patient</th>
<th>Average # of oocytes required to achieve one pregnancy</th>
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<tr>
<td>Chamayou</td>
<td>2006</td>
<td>39</td>
<td>40</td>
<td>337</td>
<td>263 (78)</td>
<td>221</td>
<td>150 (68)</td>
<td>116 (77.3)</td>
<td>37</td>
<td>2 (5)</td>
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<td>Notes: Standard method with 0.3M sucrose</td>
<td></td>
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<tr>
<td>Boldt</td>
<td>2006</td>
<td>46</td>
<td>53</td>
<td>361</td>
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<td>216</td>
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<td>Notes: Frozen in vials, 0.2 or 0.3M sucrose did not have apparent difference. Sodium depleted medium used (e.g. choline chloride).</td>
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<tr>
<td>De Santis</td>
<td>2007</td>
<td>65</td>
<td>65</td>
<td>506</td>
<td>123 (24)</td>
<td>114</td>
<td>61 (54)</td>
<td>44 (72)</td>
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<tr>
<td>Notes: 0.1 M sucrose group. 2 infants were born and one pregnancy was ongoing</td>
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<td>Notes: 0.3 M sucrose group. 1 infant was born and 3 pregnancies were ongoing</td>
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<td>Bianchi</td>
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<td>403</td>
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<td>180 (94)</td>
<td>80</td>
<td>17 (21)</td>
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<td>Notes: Fertilization includes only normal fertilization. For a comparison, non-frozen cycles resulted in 80 % fertilization, and 21 % pregnancy.</td>
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<td>Year</td>
<td># of patients</td>
<td># of cycles</td>
<td># of oocytes thawed</td>
<td># of oocytes surviving (%)</td>
<td># of oocytes injected with sperm</td>
<td># of oocytes fertilized of those injected (%)</td>
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<td># of transfers</td>
<td># of pregnancies (% per transfer)</td>
<td>% pregnancy per patient</td>
<td>Average # of oocytes required to achieve one pregnancy</td>
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<td>Kuleshova</td>
<td>1999</td>
<td>4</td>
<td>17</td>
<td>11</td>
<td>11</td>
<td>5 (45)</td>
<td>3 (60)</td>
<td>3</td>
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<td>Notes: Open-pulled straw used</td>
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<td>34</td>
<td>34</td>
<td>474</td>
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<td>Notes: EM grids, avg. 4.5 embryos transferred per patient</td>
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<td>Katayama</td>
<td>2003</td>
<td>6</td>
<td>6</td>
<td>46</td>
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<td>91%</td>
<td>90%</td>
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<td>33</td>
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<td>Notes: Cryotop, limited information given in this case report</td>
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<td>Notes: Cryotop, only one patient data reported</td>
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<td>6.8M EG</td>
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<td>5.0M EG</td>
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<td>Notes: Cryotop, cleavage rates are blastocyst rates, not enough info given to complete table. Many embryos developed to blastocyst prior to transfer</td>
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<td>Notes: 15% each of EG and DMSO with the cryopop.</td>
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Table 3. Results from cryopreservation studies with MII mouse oocytes

<table>
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<tr>
<th>First Author</th>
<th>Year</th>
<th>Strain</th>
<th>Method of cryopreservation</th>
<th>Permeating cryoprotectant</th>
<th>Final concentration</th>
<th>CPA addition method</th>
<th>CPA removal</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
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<tbody>
<tr>
<td>Sathananthan</td>
<td>1988</td>
<td>B6CBF1</td>
<td>Rapid Freezing</td>
<td>DMSO or PG</td>
<td>3.5 M</td>
<td>single step</td>
<td>single step</td>
<td>34</td>
<td>0.5 and 0.3 for the respective CPAs</td>
<td>60 and 32</td>
<td>18 and 9</td>
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<tr>
<td>Nakagata</td>
<td>1989</td>
<td>B6C3F1</td>
<td>Vitrification</td>
<td>VS1</td>
<td>**</td>
<td>single step</td>
<td>stepwise</td>
<td>88</td>
<td>64</td>
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<tr>
<td>Schroeder</td>
<td>1990</td>
<td>B6SJLF1</td>
<td>Slow cooling</td>
<td>DMSO</td>
<td>1 M</td>
<td>single step</td>
<td>stepwise</td>
<td>84</td>
<td>74</td>
<td>50</td>
<td>77</td>
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<tr>
<td>Surrey</td>
<td>1990</td>
<td>B6C3F1</td>
<td>Slow cooling</td>
<td>DMSO or PG</td>
<td>1.5 M</td>
<td>single step</td>
<td>33 and 24</td>
<td>10</td>
<td>14</td>
<td>62</td>
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<tr>
<td>Surrey</td>
<td>1990</td>
<td>B6C3F1</td>
<td>Rapid Freezing</td>
<td>DMSO</td>
<td>3.5 M</td>
<td>single step</td>
<td>42, 61, and 13</td>
<td>21, 36, and 0.65</td>
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<tr>
<td>Shaw</td>
<td>1991</td>
<td>B6CBF1</td>
<td>Vitrification</td>
<td>VS1</td>
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<td>Kono</td>
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<td>B6CBF1</td>
<td>Vitrification</td>
<td>VS1</td>
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<td>stepwise</td>
<td>75</td>
<td>48</td>
<td>59</td>
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<td>Wood</td>
<td>1993</td>
<td>B6CBF1</td>
<td>Vitrification</td>
<td>DMSO</td>
<td>6 M</td>
<td>stepwise</td>
<td>72</td>
<td>55</td>
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<td>Van der Elst</td>
<td>1993</td>
<td>B6CBF1</td>
<td>Rapid Freezing</td>
<td>DMSO</td>
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<td>single step</td>
<td>78</td>
<td>46</td>
<td>23</td>
<td>81</td>
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<td>Rayos</td>
<td>1994</td>
<td>B6CBF1</td>
<td>Rapid Freezing</td>
<td>EG</td>
<td>3 M</td>
<td>single step</td>
<td>77 and 71</td>
<td>53 and 50</td>
<td>44 and 41</td>
<td>87</td>
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<tr>
<td>Nowshari</td>
<td>1994</td>
<td>B6CBF1</td>
<td>Rapid Freezing</td>
<td>DMSO</td>
<td>4.5 M</td>
<td>stepwise</td>
<td>92</td>
<td>46</td>
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<tr>
<td>Nowshari</td>
<td>1994</td>
<td>B6CBF1</td>
<td>Slow cooling</td>
<td>DMSO or PG</td>
<td>1.5 M and 1.0 M, respectively</td>
<td>single step</td>
<td>single step for DMSO, stepwise for PG</td>
<td>60 and 32, 32 and 15, 18 and 9</td>
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</table>

Notes: The development numbers are for the respective CPAs.
### Table 3 (continued). Results from cryopreservation studies with MII mouse oocytes

<table>
<thead>
<tr>
<th>First Author</th>
<th>Year</th>
<th>Strain</th>
<th>Method of cryopreservation</th>
<th>Permeating cryoprotectant</th>
<th>Final concentration</th>
<th>CPA addition method</th>
<th>CPA removal</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Karlsson</td>
<td>1996</td>
<td>B6DBF1</td>
<td>Slow cooling</td>
<td>DMSO</td>
<td>1.5 M</td>
<td>single step</td>
<td></td>
<td>78</td>
<td>51</td>
<td>25</td>
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<td>Notes:</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Frozen using a theoretically-optimized protocol.</td>
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<tr>
<td>O’Neill</td>
<td>1997</td>
<td>B6CBF1</td>
<td>Vitrification</td>
<td>DMSO</td>
<td>6 M</td>
<td>stepwise</td>
<td>stepwise</td>
<td>95</td>
<td>86</td>
<td>54</td>
<td>65</td>
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<td>Notes:</td>
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<td></td>
<td>These results are for the oocytes frozen with PEG in the medium.</td>
</tr>
<tr>
<td>Van der Elst</td>
<td>1998</td>
<td>B6CBF1</td>
<td>Rapid Freezing</td>
<td>DMSO</td>
<td>3.5 M</td>
<td></td>
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<td>87</td>
<td>60</td>
<td>49</td>
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<tr>
<td>Lane</td>
<td>2001</td>
<td>B6CBF1</td>
<td>Vitrification</td>
<td>DMSO or EG</td>
<td>2.8 M each</td>
<td>stepwise</td>
<td>stepwise</td>
<td>99</td>
<td>69</td>
<td>67</td>
<td>70</td>
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<td>In the same study, only 26 % of oocytes frozen with a slow cooling procedure using 1.5 M PG in a choline chloride based medium developed to blastocysts.</td>
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<tr>
<td>Stachecki</td>
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<td>B6 x BALB/C</td>
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<td>PG</td>
<td>1.5 M</td>
<td>single step</td>
<td>stepwise</td>
<td>89</td>
<td>69</td>
<td>44</td>
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<td></td>
<td>Frozen using a choline chloride medium.</td>
</tr>
<tr>
<td>Kim</td>
<td>2006</td>
<td>B6DBF1</td>
<td>Vitrification</td>
<td>DMSO</td>
<td>5.5 M</td>
<td></td>
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<td>90</td>
<td>57</td>
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<td>Vitrified using EM grids.</td>
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</table>

* in vitro culture development data represents the percent of total cells frozen unless otherwise stated  
** VS1 medium contains 2.5 M DMSO, 2.36 M acetamide, 1.19 M PG, and 5.4 % polyethylene glycol
### Table 4. Results from cryopreservation studies with MII bovine oocytes

#### Table 4a: Equilibrium Cooling Methods

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>permeating cryoprotectant</th>
<th>final concentration</th>
<th>CPA addition method</th>
<th>CPA removal method</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent 8-cell</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
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<tbody>
<tr>
<td>Lim</td>
<td>1991</td>
<td>Glycerol</td>
<td>1 or 1.5 M</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>3 to 8% of those surviving freezing</td>
<td>3 to 8% of those surviving freezing</td>
<td>3 to 8% of those surviving freezing</td>
<td>3 to 8% of those surviving freezing</td>
<td>3 to 8% of those surviving freezing</td>
</tr>
<tr>
<td>Otoi</td>
<td>1992</td>
<td>PG</td>
<td>1.6 M</td>
<td>3-step</td>
<td>3-step</td>
<td>1 to 3</td>
<td>26</td>
<td>26</td>
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</tr>
<tr>
<td>Fuku</td>
<td>1992</td>
<td>PG</td>
<td>2 M</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>&lt; 1</td>
<td>4</td>
<td>4</td>
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<td>Notes:</td>
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<tr>
<td>Schellander</td>
<td>1994</td>
<td>Glycerol, PG, or DMSO</td>
<td>1 or 1.5 M</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>12 to 14</td>
<td>1 to 2</td>
<td>1 to 2</td>
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<tr>
<td>Otoi</td>
<td>1994</td>
<td>PG</td>
<td>1.6 M</td>
<td>3-step</td>
<td>3-step</td>
<td>10 to 15 (% of those surviving)</td>
<td>0.4 to 2.2 (of those surviving)</td>
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<tr>
<td>Otoi</td>
<td>1995</td>
<td>EG</td>
<td>1.8 M</td>
<td>10 to 15</td>
<td>1 to 5 (of those surviving)</td>
<td>0 to 1</td>
<td>31</td>
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<tr>
<td>Otoi</td>
<td>1997</td>
<td>PG or DMSO</td>
<td>1.5 M</td>
<td>3-step</td>
<td>stepwise w./ out sucrose</td>
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<tr>
<td>Otoi</td>
<td>1997</td>
<td>PG</td>
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<td>stepwise w./ out sucrose</td>
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Notes: 1.0M gave slightly better results than 1.5M. The authors tested 2 methods of CPA removal and neither was superior to the other.

Notes: The authors tested pre-treatment with hyaluronidase or trypsin and a control solution with no enzyme.

Notes: No apparent difference between CPAs used.

Notes: The authors tested 3 cooling rates after seeding, no difference noted.

Notes: The authors tested 3 concentrations of sucrose in the freezing medium (0, 0.1, or 0.2 M), no difference noted.

Notes: The effects of lipid centrifugation prior to cryopreservation. No difference was noted.
### Table 4a (continued): Equilibrium Cooling Methods

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>permeating cryoprotectant</th>
<th>final concentration</th>
<th>CPA addition method</th>
<th>CPA removal method</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent 8-cell blastocysts</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
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<tbody>
<tr>
<td>Kubota</td>
<td>1998</td>
<td>PG and EG</td>
<td>5% EG plus 6% PG</td>
<td>single-step</td>
<td>single-step</td>
<td>84</td>
<td>50</td>
<td>6</td>
<td>26</td>
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<tr>
<td>Lim</td>
<td>1999</td>
<td>Gly, PG, or DMSO</td>
<td>1 M</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>62, 86, and 83 for the respective CPAs</td>
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<td>0, 0, 3</td>
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<td>Saunders</td>
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<td>EG</td>
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<td>stepwise w./ galactose</td>
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<tr>
<td>Asada</td>
<td>2000</td>
<td>EG</td>
<td>1.8 M</td>
<td>single-step</td>
<td>single-step</td>
<td>16 and 23</td>
<td>0 and 1.5</td>
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</table>

* in vitro culture development data represents the percent of total cells frozen unless otherwise stated.

**Table 4b: Standard Vitrification Methods**

<table>
<thead>
<tr>
<th>Fuku</th>
<th>1992</th>
<th>DAP213</th>
<th>**</th>
<th>single-step</th>
<th>stepwise w./ sucrose</th>
<th>14</th>
<th>1</th>
<th>0</th>
<th>12</th>
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<tr>
<td>Hamano</td>
<td>1992</td>
<td>DAP213</td>
<td>**</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>0</td>
<td>9</td>
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<tr>
<td>Fuku</td>
<td>1995</td>
<td>DAP213</td>
<td>**</td>
<td>single-step</td>
<td>single-step or stepwise w./ out sucrose</td>
<td>67 and 70</td>
<td>5 and 7</td>
<td>0 and 0</td>
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</table>

* The authors tested a single-step CPA removal procedure to a 12-step procedure. The results are for each of these procedures, respectively.
### Table 4b (continued): Standard Vitrification Methods

<table>
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<tr>
<th>Author</th>
<th>Year</th>
<th>permeating cryoprotectant</th>
<th>final concentration</th>
<th>CPA addition method</th>
<th>CPA removal</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent 8-cell</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
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</thead>
<tbody>
<tr>
<td>Martino</td>
<td>1996</td>
<td>EG</td>
<td>5.5M</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>34</td>
<td>3</td>
<td>&lt;1</td>
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<tr>
<td>Notes:</td>
<td>The authors compared vitrification in straws vs. other methods. See table below for direct comparison.</td>
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</tr>
<tr>
<td>Obi</td>
<td>1998</td>
<td>EG</td>
<td>4 M</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>85</td>
<td>40</td>
<td>10</td>
<td>41</td>
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</tr>
<tr>
<td>Notes:</td>
<td>These results are for the best method described in this paper. See text for more details.</td>
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<tr>
<td>LeGal</td>
<td>1999</td>
<td>Glycerol plus EG</td>
<td>25 % of each</td>
<td>stepwise</td>
<td>stepwise w./ galactose</td>
<td>22 (of those surviving)</td>
<td>0</td>
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<tr>
<td>Notes:</td>
<td>These oocytes were frozen in straws. See table below for a direct comparison to oocytes frozen in microdrops of open-pulled straws.</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>** DAP213 contains 2 M DMSO, 1 M Acetamide, and 3 M PG</td>
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</tr>
</tbody>
</table>

### Table 4c: Vitrification Methods using "Ultra-Rapid" cooling approaches

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>permeating cryoprotectant</th>
<th>final concentration</th>
<th>CPA addition method</th>
<th>CPA removal</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent 8-cell</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Martino</td>
<td>1996</td>
<td>EG</td>
<td>5.5M</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>72 and 51</td>
<td>40 and 25</td>
<td>15 and 10</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>These results are for oocytes vitrified on EM grids in either liquid nitrogen or nitrogen slush, respectively. The differences reported for these 2 methods were not statistically significant.</td>
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<tr>
<td>Martino</td>
<td>1996</td>
<td>EG</td>
<td>4 or 5.5M</td>
<td>single-step</td>
<td>stepwise w./ sucrose</td>
<td>60</td>
<td>30</td>
<td>10 to 15</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>There was no effect of the different concentrations of EG on survival and development. These oocytes were also vitrified on EM grids, and in liquid nitrogen.</td>
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<tr>
<td>Vajta</td>
<td>1998</td>
<td>EG and DMSO</td>
<td>20 % of each</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>50</td>
<td>13</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>These oocytes were vitrified using OPS*.</td>
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<tr>
<td>LeGal</td>
<td>1999</td>
<td>Glycerol plus EG</td>
<td>25 % of each</td>
<td>stepwise</td>
<td>stepwise w./ galactose</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td>Notes:</td>
<td>These oocytes were vitrified using SSV.</td>
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<tr>
<td>LeGal</td>
<td>1999</td>
<td>Glycerol plus EG</td>
<td>20 % of each</td>
<td>stepwise</td>
<td>stepwise w./ galactose</td>
<td>11</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>These oocytes were vitrified using SSV.</td>
<td></td>
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<tr>
<td>Papis</td>
<td>2000</td>
<td>EG</td>
<td>5.5M</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>50 to 70</td>
<td>6 to 30</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notes:</td>
<td>These authors tested the effects of pre-equilibration in solutions of EG (0 to 6%) prior to exposing the cells to the final vitrification solution. The treatments had no significant difference on outcome. The numbers presented are those from all treatments. The oocytes were vitrified by dropping small volumes (6 microliters) of solution directly into LN2.</td>
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</tbody>
</table>
Table 4c (continued): Vitrification Methods using "Ultra-Rapid" cooling approaches

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>permeating cryoprotectant</th>
<th>final concentration</th>
<th>CPA addition method</th>
<th>CPA removal</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent 8-cell</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dinnyes</td>
<td>2000</td>
<td>EG</td>
<td>35%</td>
<td>stepwise</td>
<td>stepwise w./ trehalose</td>
<td>81 and 85</td>
<td>47 and 53</td>
<td>16 and 9</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Notes: These oocytes were frozen using SSV. The numbers represent cells either thawed immediately or stored in LN2 prior to thawing.</td>
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<tr>
<td>Hochi</td>
<td>2001</td>
<td></td>
<td></td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>87</td>
<td>16</td>
<td>2</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Asada</td>
<td>2002</td>
<td>EG</td>
<td>40%</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>87</td>
<td>16</td>
<td>2</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Notes: These oocytes were vitrified in a gel loading tip with 20% fetal calf serum. The authors also tested various concentrations of polyvinylpyrrolidone, with no apparent benefit.</td>
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<tr>
<td>Li</td>
<td>2002</td>
<td>EG and DMSO (for OPS), or EG (for SSV)</td>
<td>20% each or 35%, respectively</td>
<td>stepwise</td>
<td>stepwise w./ sucrose or trehalose, respectively</td>
<td>38 and 36</td>
<td>15 and 8</td>
<td>24</td>
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<tr>
<td>Notes: Results are for OPS and SSV, respectively. The differences in blastocyst development were not significantly different.</td>
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</tr>
<tr>
<td>Men</td>
<td>2002</td>
<td>EG and DMSO</td>
<td>20% of each</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>88</td>
<td>51</td>
<td>2</td>
<td>31</td>
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</tr>
<tr>
<td>Notes: These oocytes were vitrified using OPS. This method was essentially identical to that of Vajta (1998)</td>
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<tr>
<td>Rho</td>
<td>2002</td>
<td>EG and DMSO</td>
<td>3.2 M plus 2.36 M, respectively</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>42 and 39</td>
<td>7.7 and 8.4</td>
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<tr>
<td>Notes: The numbers represent those oocytes having been vitrified with either open-pulled glass capillaries or EM grids, respectively.</td>
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<tr>
<td>Mavridea</td>
<td>2002</td>
<td></td>
<td></td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>88</td>
<td>51</td>
<td>2</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Chian</td>
<td>2004</td>
<td>EG and DMSO, or EG and PG</td>
<td>15 % of each</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>92 and 98</td>
<td>69 and 70</td>
<td>1.7 and 7</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Notes: These oocytes were frozen with the cryotop device. The results are for the CPAs EG and DMSO and EG plus PG, respectively.</td>
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<tr>
<td>Albarracin</td>
<td>2005</td>
<td>EG or DMSO</td>
<td>20% of each</td>
<td>stepwise</td>
<td>stepwise w./ sucrose</td>
<td>28 and 13</td>
<td>3 and 0</td>
<td>33 and 8</td>
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</tr>
<tr>
<td>Notes: The authors tested the effects of oocytes from adult cows and pre-pubertal calves, and the numbers represent the results from these 2 groups, respectively. Vitrified in OPS.</td>
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</table>

OPS = Open-pulled straws; MD = microdrops; SSV = Solid surface vitrification.
Table 5. Results from cryopreservation studies with MII porcine oocytes

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Permeating CPA</th>
<th>Final concentration</th>
<th>CPA addition method</th>
<th>CPA removal method</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent 8-cell development</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagashima</td>
<td>1999</td>
<td>EG</td>
<td>40%</td>
<td>stepwise (see notes)</td>
<td>stepwise, in straw</td>
<td>56</td>
<td>6.3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Notes: The authors used a very unusual CPA treatment. The cells were initially exposed to 10% glycerol for 10 min, then 40% EG plus 1 M sucrose (no glycerol) for 1 minute prior to plunging the sample into liquid nitrogen. There was no observation of embryo development past the 8-cell to morula stage. Vitrified in 1/4 cc straws. These oocytes were fertilized using sub-zonal insemination.</td>
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<tr>
<td>Rojas</td>
<td>2004</td>
<td>EG</td>
<td>40%</td>
<td>stepwise (see notes)</td>
<td>stepwise</td>
<td>10</td>
<td>not given</td>
<td></td>
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</tr>
<tr>
<td>Notes: EG was added to the cells in 7 steps, from 5 to 30% in 5% increments, then to 40%, for 12.5 min total. Vitrified in OPS.</td>
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</tr>
<tr>
<td>Fujihira</td>
<td>2005</td>
<td>EG</td>
<td>30%</td>
<td>stepwise</td>
<td>stepwise</td>
<td>54 to 66%</td>
<td>30</td>
<td>4.5</td>
<td>22.5</td>
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</tr>
<tr>
<td>Notes: Vitrified with the cryotop method. Fertilized using ICSI.</td>
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<tr>
<td>Shi</td>
<td>2006</td>
<td>EG plus DMSO</td>
<td>20% of each</td>
<td>stepwise</td>
<td>stepwise</td>
<td>54</td>
<td>6</td>
<td>0</td>
<td>26</td>
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</tr>
<tr>
<td>Notes: Only 3.8% of oocytes exposed to the vitrification solution developed to blastocysts. Vitrified using Open-pulled Straws.</td>
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<tr>
<td>Gupta</td>
<td>2006</td>
<td>EG plus DMSO</td>
<td>17.5% of each</td>
<td>stepwise</td>
<td>stepwise</td>
<td>26</td>
<td>1</td>
<td>9</td>
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<tr>
<td>Notes: Vitrified with Solid Surface Vitrification Method.</td>
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</tr>
<tr>
<td>Shi</td>
<td>2006</td>
<td>EG plus DMSO</td>
<td>2.4M plus 1.77M, respectively</td>
<td>stepwise</td>
<td>stepwise</td>
<td>38</td>
<td>2.3</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notes: Vitrified in 1/4 cc straws. An additional experiment was conducted using ICSI with no major difference in development. Eleven percent of CPA exposure controls developed to blastocysts.</td>
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</tr>
<tr>
<td>Wu</td>
<td>2006</td>
<td>EG</td>
<td>40%</td>
<td>stepwise</td>
<td>stepwise</td>
<td>0</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Notes: Vitrified using OPS. Ultrastructural analysis showed that the cortical granules remained in the oocyte, suggesting that zona hardening was not the cause of developmental failure in this study.</td>
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</tbody>
</table>
Table 5 (continued). Results from cryopreservation studies with MII porcine oocytes

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Permeating CPA</th>
<th>Final concentration</th>
<th>CPA addition method</th>
<th>CPA removal method</th>
<th>Percent morphological survival</th>
<th>Percent undergoing cleavage*</th>
<th>Percent 8-cell development</th>
<th>Percent blastocysts of total oocytes frozen</th>
<th>Percent blastocysts of unfrozen control oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somfai</td>
<td>2006</td>
<td>EG</td>
<td>35%</td>
<td>stepwise</td>
<td>stepwise, with trehalose*</td>
<td>18</td>
<td>5</td>
<td>0.5</td>
<td>28</td>
<td></td>
</tr>
</tbody>
</table>

* in vitro culture development data represents the percent of total cells frozen unless otherwise stated

** all other studies used sucrose in the dilution medium

Vitrified with SSV. Twenty-five percent of CPA exposed oocytes developed to the blastocyst stage. These oocytes were artificially activated.
CHAPTER 2. IMMUNOCYTOCHEMICAL STAINING OF THE METAPHASE II SPINDLE IN MAMMALIAN OOCYTES

Introduction

Investigation into microtubule function in mature mammalian oocytes in relation to cryopreservation is one area of study for the present authors. An increasing number of couples are seeking the assistance of physicians for reproductive dysfunction, and cryopreservation of gametes and embryos plays a role in this type of therapy (http://www.cdc.gov/nccdphp/drh/ART99/index99.htm).

Unfortunately, the success of human oocyte cryopreservation is low. The metaphase II (MII) spindle in mature oocytes has been shown to be sensitive to the non-physiologic conditions imposed upon cells during cryopreservation, and it is believed that the disruption of the MII spindle is a principal cause of the loss of developmental potential in frozen-thawed human oocytes (Magistrini and Szollosi, 1980; Johnson and Pickering, 1987; Critser et al., 1997).

This chapter was published in the technical book series Methods in Molecular Biology. The full citation is as follows: Mullen SF and Critser JK (2004) Immunocytochemical Staining of the Metaphase II Spindle in Mammalian Oocytes. Methods Mol Biol 253, 201-214.
Using fluorescence immunochemistry with tubulin-specific antibodies allows one to visualize microtubular structures in cells. This technique has been applied in an effort to understand normal and abnormal microtubule structure and function in many of the reports cited in this chapter. Although we use this technique exclusively on oocytes, it is applicable to embryos and other cell types. In conjunction with confocal microscopy, this technique allows high resolution assessment of microtubules, which may provide a means of understanding their function, and in many cases malfunction.

**Microtubules**

Microtubules are one of the three principal components of the cytoskeleton. They can be very static structures, as those found in cilia or flagella, or dynamic structures such as those found in the cytoplasm. They have several functions, most involved with either movement of the cell or components therein. It is the structure of microtubules that confers their ability to facilitate movement, and this ability allows such important processes as protein and organelle translocation and DNA segregation (Hyams and Lloyd, 1994).

Microtubules are assemblies of subunits of α- and β-tubulin proteins. These subunits associate via non-covalent interactions to form tube-like structures with a diameter of approximately 24 nm. The αβ dimers can associate and disassociate rapidly causing the growth and shrinkage of microtubules, a process
termed dynamic instability (Mitchison and Kirschner, 1984). This dynamic behavior allows microtubules to “search” the 3-dimensional space inside of a cell for specific targets. As an example, this phenomenon has been visualized in living newt lung cells as a mechanism to capture kinetochores to the developing mitotic spindle (Hayden et al., 1990).

Movements of proteins, vesicles, and entire organelles within a cell are facilitated by microtubules in conjunction with microtubule motor proteins, of which kinesins and dyneins are two major families (Gelfand and Bershadsky, 1991). Microtubules have an inherent polarity, with both (+) and (-) ends dictated by the orientation of the tubulin dimers. Generally, kinesins are (+) end directed motors, moving toward the (+) end of the microtubule, while Dyneins translocate cargo toward the (-) end. There are, however, exceptions to this rule. Movements that rely upon microtubule motor proteins include the beating of cilia and flagella (dyneins) (Summers, 1974), positioning of the endoplasmic reticulum and golgi apparatus and movement of vesicles between (Kelly, 1990), and the migration of chromosomes during mitosis and meiosis (Wittmann et al., 2001). It is this last example that is of particular importance in developing oocytes.

**Microtubules and the Spindle**

The separation of chromosomes during mitosis by the mitotic spindle is one of the earliest studied, and most fascinating, events occurring in cell biology
(Flemming, 1879). During the M-phase of the cell cycle, a highly choreographed series of events occurs, eventually culminating in cytokinesis (Nurse, 2000). Microtubules play an integral part in all of these events, and changes in microtubule dynamics and location occur during this period.

In mammalian cells, prior to the dismantling of the nuclear membrane in preparation for mitosis, the centrosome duplicates, and each daughter centrosome becomes located at opposite sides of the nucleus. Microtubules and kinesin motor proteins facilitate this movement, and along with the microtubule organizing centers (centrosomes) develop into the mitotic spindle (Karsenti and Vernos, 2001). Unlike G-phase microtubules, M-phase microtubules are restricted in location to the meiotic and mitotic spindle. Not only is the location of these microtubules modified, the dynamics of M-phase microtubules also changes rapidly. The rates of polymerization and depolymerization in spindle microtubules increases markedly compared to their G-phase counterparts (Saxton et al., 1984). The average lifetime of a mitotic microtubule is less than one minute, compared to approximately 10 minutes for G-phase microtubules. Because of this dynamic nature, the spindle is continuously changing in size and shape during mitosis.

Upon nuclear envelope breakdown, the kinetochore microtubules of the spindle probe the cytoplasm and "search" for the chromosomes (Hayden, Bowser and Rieder, 1990). It is believed that either the (+) end of the microtubule achieves a
direct hit on the kinetochore, or it passes the kinetochore and a lateral interaction occurs whereby the microtubule is captured and the chromosome is moved to the (+) end via kinesin motors. Either way, the kinetochore eventually caps the (+) end and stabilizes it, preventing further depolymerization. A microtubule from the opposite pole eventually attaches in a similar manner to the kinetochore of the sister chromatid and the sister pair migrate to the midpoint of the spindle. Once all of the chromatids have become aligned between the spindle poles, the proteins holding the sister chromatids together are cleaved and the chromatids migrate toward the spindle poles. Once all of the chromosomes have segregated to the respective spindle poles, cytokinesis commences and 2 daughter cells are formed which contain exact copies of the genome along with one compacted centrosome. Cytoplasmic microtubules are again nucleated from this centrosome, and the cell cycle begins anew.

**Microtubule Function in Mammalian Oogenesis and Fertilization**

In the mouse, meiosis is initiated as early as embryonic day 12 in the oogonia, transforming the cells into oocytes. Shortly after birth, all of the oocytes have reached the dictyate stage of meiotic prophase, where they will remain arrested until stimulated to resume meiosis at the time of ovulation (Wassarman and Albertini, 1994). This development of oocytes is similar in all vertebrate species,
and in those with a long reproductive span, such as humans, the oocytes can remain arrested at prophase I for decades.

Oocytes are recruited to resume growth and development during the life of the organism. They proceed through the stages of meiosis during maturation, and the pattern of microtubule activity resembles that described for somatic cells in the M-phase. Investigations of microtubule organizing center (MTOC) position and microtubule morphology in oocytes from mouse, bovine, porcine, and human have been reported and show very similar patterns during the stages of oogenesis (Messinger and Albertini, 1991; Kim et al., 1996; Kim et al., 1998; Kim et al., 2000).

At the time when oocytes are arrested at the first meiosis, only very short microtubules are present, and they are nucleated from the centrosome in mouse oocytes. Upon breakdown of the germinal vesicle and resumption of meiosis, a dramatic change in the arrangement of the centrosomes occurs, with one remaining near the chromatin and another migrating into the cytoplasm. In addition, microtubules begin to nucleate around the chromosomes. In many mammalian species, the centrosome lacks the pair of centrioles in the maturing oocyte, the centrioles having been lost during oocyte maturation. It is not until further embryonic development that centrioles reappear (Szöllösi, 1972). A bipolar spindle forms during this time as the number of microtubules increases. In addition, although remnants of the microtubule organizing centers in the cell
periphery still exist, they do not nucleate microtubules. Thus upon the onset of metaphase I, microtubules are only located within the spindle.

In the anaphase I stage, the pairs of sister chromatids are separated by the microtubule-based spindle and half of the DNA is extruded from the cell in the first polar body. The cell then enters Metaphase II (MII) and another spindle complex develops. As in metaphase I, microtubules are only localized in the spindle of MII oocytes (see Figure 1). At this stage the oocyte is arrested for a second time, with activation usually commencing upon interaction with a spermatozoon during fertilization.

Microtubules play a predominant role in the events which occur during fertilization (Schatten and Schatten, 1987). Initially, activation triggers the resumption of meiosis and results in the final meiotic division. The remaining sister chromatids are separated by the spindle and half are extruded into the second polar body. This results in the creation of a haploid set of maternal chromosomes to complement the haploid set of paternal chromosomes introduced by the spermatozoon. The sperm is dismantled during the early stages of fertilization, with the chromatin undergoing decondensation culminating in the formation of the male pronucleus. A pronucleus also develops around the female chromosomes at this time.
Upon activation in mice, cytoplasmic microtubules again form, radiating form the peripheral MTOC, and these microtubules eventually transport the male pronucleus from the cortex to the center of the egg cytoplasm. During this transport, the female pronucleus is also captured by the microtubules and both pronuclei migrate toward the same location. At the time when the pronuclei are in close apposition, a sheath of microtubules forms around each. After pronuclear dissolution and intermingling of the chromosomes from the two pronuclei, a process called syngamy, the chromosomes and microtubules develop into a spindle and the first embryonic cleavage division commences. A similar process occurs in fertilization from other mammalian species with an interesting exception. The focus of microtubule organization is the centrosome which develops from the centriole that is contributed by the spermatozoon. An MTOC develops from this organelle and nucleates microtubules which eventually cause the migration of the pronuclei and syngamy as in the mouse.

**Pathologies Associated with Microtubule Dysfunction in the Oocyte**

Given the importance of microtubules for gamete interaction and subsequent embryonic development, it is not surprising that microtubule dysfunction in oocytes and zygotes has serious repercussions. Incorrect chromosome segregation during meiosis and improper union of the 2 haploid sets of
chromosomes can lead to developmental failure of the embryo (Hewitson et al., 1997; Nasmyth, 2001).

There have been several reports on the correlations between male infertility and sperm centrosome failure. Navara et al. (1996) (Navara et al., 1996) investigated the correlation between fertility and centrosome organization and sperm aster morphology in bulls and found a strong correlation between those factors. The bull with the highest fertility demonstrated superior aster formation and morphology in comparison to bulls with lower fertility. In another study investigating 211 presumed failed to fertilize human oocytes (Asch et al., 1995), many of the oocytes had been penetrated by one or more sperm but had arrested development at very early stages. Although not rigorously tested, it was thought that some causes of this arrest were defects in the sperm centrosome. In a similar study (Van Blerkom, 1996), failed-fertilized and pronuclear-arrested oocytes derived from clinical cases where the males had severe oligoteratozoospermia were examined for microtubule patterns. Abnormal patterns were seen, and the conclusions were that centrosome dysfunction may have contributed to the developmental arrest.

Although as these studies show that male factor incidence of infertility can result from defects in microtubule organization and function, an even more common problem with fertility related to microtubule function in humans results from maternal aneuploidy. The estimated level of aneuploidy in humans, given that it
is nearly always lethal, is quite surprising. Although it is estimated that only 0.3 percent of newborns are aneuploid (Hassold et al., 1996) it is estimated that greater than 20 percent of human conceptions result in aneuploid embryos (Jamieson et al., 1994; Volarcik et al., 1998). It is believed that most aneuploidies are maternal in origin, and most of those arise from mis-segregation during the first meiotic division (Hassold and Hunt, 2001).

Although the precise mechanism or mechanisms which result in aneuploidy are unclear, a correlation between maternal age and aneuploid frequency has been known for decades (Penrose, 1933; Morton et al., 1988). The increase in incidence of trisomy in pregnancies in older women is striking, with the numbers of recognized pregnancies being trisomic rising from approximately 2 percent in women 25 years old to approximately 35 percent in women over 40 years old. The trend in delaying childbearing in developed countries is well documented (1989), as is the changes in microtubule patterns in aged mammalian oocytes (Battaglia et al., 1996; Kim, Moon, Prather and Day, 1996). A better understanding of the causes of microtubule alterations in ageing oocytes could assist in maintaining the fertility of those women who desire to have children yet choose to delay childbearing.
Materials

1. 35mm petri dishes.
2. Oocyte transfer pipettes.
3. Methanol with 5mM EGTA, pre-chilled in –20 degree freezer.
4. Thick glass slides with a concave well, for example Fisher Catalog Number 12-565A.
5. Immunochemistry Blocking Buffer: PBS, 0.2 percent (V/V) Triton X-100, 3mM sodium azide, 0.1% saponin (mass/V), 130 mM glycine, 2% bovine serum albumin (mass/V), and 5% Horse or Donkey serum (V/V) (see note 1).
7. Microscope slide coverslips #1.5 (see note 2).
8. Anti-Tubulin primary antibody, preferably clone DM1-A (see note 3).
9. Secondary Antibody labeled with fluorophore of choice. We use a donkey-anti-mouse antibody with a Texas Red® conjugate, from Jackson Immunoresearch (see note 4).
10. Sytox Green DNA stain from Molecular Probes, catalog # S7020 (see note 5).
11. ProLong Antifade reagent from Molecular Probes, catalog # P7418 (see note 6).
12. Waxy material for mounting coverslips to slides, to prevent the coverslips from squishing the cells upon mounting: 50% petroleum jelly and 50% paraffin wax (see note 7).
Methods

1. All treatments described here are performed at room temperature unless otherwise noted. However, since microtubules are cold labile, ensure that the cells are maintained at physiologic temperature prior to fixation.

2. Fix the cells by transferring them into cold methanol (-20 degrees C) for 6 minutes; transfer as little liquid with the cells as possible (see notes 8 & 9).

3. Evacuate the transfer pipette of all solution (see note 9).

4. After fixing the cells, transfer them to approximately 2 milliliters of blocking buffer in a 35 mm petri dish and hold them at 4 degrees C (see note 10).

5. Dilute the primary antibody in blocking buffer at a ratio of 1 microliter of antibody solution to 9 milliliters of blocking buffer. Keep the solution on ice until you are ready to use it. Mix it thoroughly by carefully inverting the tube at least 3 times, and transfer the cells to a 35mm dish containing 1.5 – 2 milliliters of the diluted primary antibody.

6. Maintain the cells in this solution for at least 1 hour at room temperature. If desired the cells may be held in this solution overnight at 4 degrees C (see note 11).

7. Transfer the cells to a 35mm dish containing 1.5 – 2 milliliters of blocking buffer as a wash. Transfer the cells with as little volume as necessary. They should be held in this solution for at least one hour. They may be washed for a few hours or overnight if desired (see note 12).
8. Dilute 5 microliters of the secondary antibody in 7.5 milliliters of the blocking buffer (see Note 4).

9. Transfer the cells to a 35mm dish containing 1.5 – 2 milliliters of the secondary antibody diluted as described above. Hold the cells in this solution for at least 1 hour, or overnight at 4 degrees C (see note 11).

10. Transfer the cells to a 35mm dish containing 1.5 - 2 milliliters of blocking buffer as a wash. They should be held in this solution for at least one hour (see note 13).

11. Get one each of the 2 types of tubes from the ProLong Antifade reagent box (see note 6). The liquid in the clear tube needs to be thawed. This can be done at room temperature or in a water bath. Carefully (see below) transfer 200 microliters of the clear solution into the amber tube. There is a crystalline substance in the bottom of the amber tube. Upon transfer, use the pipette tip to suspend the crystals in the liquid (with the action of a mortar and pestle). Once the crystals are suspended, let the solution sit for at least 15 minutes to allow the crystals to dissolve. If DNA counterstaining is desired, to the remainder of the liquid in the clear tube, add 0.25 microliters of the Sytox Green stock solution (see note 5). After 15 minutes, draw the solution in the amber tube into a clear pipette tip to make sure all of the crystals have dissolved. Return the 200 microliters of the solution from the amber tube to the clear tube. Mix thoroughly but carefully! This solution is very viscous and foams easily. If excessive foaming occurs, centrifuge the tube at maximum rpm in a benchtop microcentrifuge. Keep the tubes capped as often as possible.
12. Draw up 20 microliters of antifade reagent into a pipette and set the pipette down on the bench next to you. Using a dissecting microscope, transfer the cells to a microscope slide with as little liquid as possible. Suck up as much of the liquid as you can without removing the cells. Grab the pipette containing the antifade reagent and watch the remaining solution on the slide evaporate. When it has nearly all evaporated (this will facilitate the cells sticking to the glass), quickly but carefully add the antifade reagent to the cells. Adding more just makes a mess, adding less will create air bubbles in the solution as it dries. Once the cells are covered with the drop, add a small amount of the waxy substance to the corners of a coverslip, and carefully place the coverslip onto the slide, waxy side down, avoiding the creation of air bubbles in the antifade reagent. Carefully press down on the corners of the coverslip with a pipette tip just until you see the oocytes being squished. The coverslip will rebound slightly and the distance between the coverslip and oocytes will be minimal (see note 14).

13. Give the slides at least one hour to dry prior to looking at them under the fluorescence microscope. Although it may not be necessary, we keep the slides under aluminum foil during the drying process. Seal the coverslips with clear nail polish after the drying time. We recommend imaging the cells within 2 days to maximize the signal to noise ratio.
Notes

1. Serum from a species closely related to the host of the secondary antibody is important during the blocking step. We use horse serum since we are using a donkey antibody. Donkey serum is available from Jackson Immunoresearch, and either should work well for this application. The blocking buffer we use was derived empirically. Due to the presence of sodium azide, this material is considered toxic, and should be handled and disposed of appropriately. This buffer should be stored at 4 degrees C. Periodic filtration through a 0.45 micron filter can be performed if precipitates form over time. We make 1 liter volumes if we will be using all of it within approximately 30 days. If longer storage is expected, we make up a 1 liter volume of the solution without the BSA and Serum, and then make a 200 milliliter volume of working solution by adding BSA and serum to 200 milliliters of the stock solution.

2. Most high power microscope objectives are designed to work with microscope coverslips with a thickness of 0.17mm, which is equivalent to #1.5. Check the objectives on your microscope to verify this before mounting the cells, and use appropriate coverslips. Although this sounds trivial, it is important for getting good images. We use 18mm square coverslips. If larger coverslips are used, use a proportionately larger volume of antifade reagent when mounting the cells.
3. We use a primary antibody from Sigma (St. Louis, MO, USA, Catalog # T9026) without further purification. We also aliquot our stock solution in 5 microliter volumes and store these in a -80 degree freezer. Monoclonal antibody clone DM1-A has proven to be an excellent antibody for general use in labeling tubulin structures. The antibody dilutions discussed in this chapter are only relevant to the antibody sources we cite. Other sources of antibodies may work just as well, but the dilutions necessary to achieve a good signal-to-noise ratio will differ and will need to be determined empirically. If a different antibody is used, ensure that a secondary antibody is chosen that will bind to it (in other words, if the primary antibody is not from a mouse, do not choose a secondary antibody that binds to a mouse antibody).

4. The secondary antibody comes in the form of a lyophilized powder and we use the manufacturer's recommendations for resuspension. Add 400 microliters of quality room temperature water (DI or Milli-Q) to the powder and gently mix by pipetting. After about 10 minutes, draw the solution into a clear pipette to ensure complete dissolution. We either maintain this solution in a refrigerator if we expect it to be used within 2 months or we freeze aliquots diluted 50 percent in glycerol. To do this, add 400 microliters of glycerol to the stock solution and aliquot 10 microliter volumes of this dilution into 0.5 milliliter tubes and store the aliquots at -80 degrees C. To use this solution, thaw one tube and add the entire contents to 7.5 milliliters of blocking buffer. Add 1.5 to 2 milliliters of this solution to a 35mm petri dish for the final working solution. We use glycerol with these
antibodies for long-term storage but not for the primary antibody, only because that is what the manufacturer recommends. We believe that either way would work for both antibodies, but assume that the manufacturers have recommended the methods that work best for their products.

5. Sytox green from Molecular Probes is a superb DNA fluorophore. We aliquot the original product in 1 microliter volumes and dilute 0.25 microliters of this in 1 milliliter of the antifade reagent. We recommend it's use if it can be obtained.

6. The Prolong Antifade reagent has proven to be an exceptional antifade reagent. We have received positive comments from multiple core facilities on its photostable properties. The kit comes with multiple amber tubes each containing a crystalline precipitate, and two bottles of a transparent solution. We thaw these bottles and aliquot this solution in 1 milliliter volumes into transparent 1.5 milliliter microcentrifuge tubes. This way, a tube can be thawed for each procedure instead of having to repeatedly thaw and freeze the entire solution in the bottle. We use transparent tubes because it is easier to tell when the solution has been thoroughly mixed. The final solution is very viscous and foams easily, so handle it carefully. Be sure to mix the solution thoroughly prior to its use. We recommend using this reagent if it can be obtained.

7. The “waxy material” is a homemade concoction of half petroleum jelly (“Vaseline”) and half paraffin wax. To make the waxy material, take a 500 ml
beaker and put it on a hot plate. Add approximately an equal volume of paraffin wax (judge by the size of the blocks) and petroleum jelly. The wax and jelly can be purchased at any large grocery store. When the wax has fully melted, stir the two together to mix well, and draw the solution into 30 milliliter syringes (without needles). The material will solidify as it cools. Like many things in life, the precise ratio of the two materials is not critical. As long as it is solid but flows when pressure is put on the syringe plunger it will be fine.

8. We find that methanol fixation provides a quick, safe, and easy alternative to fixation in formalin. In addition, it seems to provide better access to intracellular antigens during immunocytochemistry. If a few oocytes are being fixed, we recommend using thick glass slides with a concave well. Adding about 4 drops of methanol to the well and transferring as little medium with the oocytes as possible has the best outcome. The oocytes are fragile while in methanol. Therefore, be gentle with them during fixation. Have the slides and methanol equilibrated in a freezer prior to fixation, and return the slides with the fixative and oocytes to the freezer during fixation. We keep the slides in a 60mm Petri dish during this procedure. If more oocytes are being fixed, 35 mm dishes containing methanol may be used. However, the oocytes tend to move around in the methanol during fixation thus making it more challenging to find and remove them after the procedure. It can be done with practice, however.
9. Because methanol precipitates proteins, if methanol and protein-containing
medium are mixed in the transfer pipette, it can become clogged with protein
precipitate, possibly leading to the loss of the oocytes. We find it essential to
evacuate the liquid out of the pipette before going into the methanol to retrieve
the fixed cells. Simply evacuate the pipette before moving into the methanol, then
allow the pipette to draw up methanol prior to picking up the cells. Keep the
oocytes near the end of the pipette during transfer from one medium to another.
If the oocytes are too far up into the pipette when fixed, they can rupture during
transfer into the blocking buffer.

10. Blocking buffer is used to lower the non-specific binding of antibodies in the
cells. Holding the cells in this buffer will prevent this and reduce the background
staining of the cells. We do not keep the dishes in a humidified chamber. Instead,
we check on the volume of the liquid and add additional medium if necessary to
prevent the all of the medium from evaporating. The holding time can range from
days to weeks, if desired. Just be sure to check on the volume of the liquid to
avoid complete evaporation. We have found that staining mouse oocytes with
this procedure produces high levels of background staining when compared to
oocytes from other species. This can be reduced by holding the cells in the
blocking buffer for a few days, however. For oocytes from other species, a few
hours of blocking prior to incubation with the antibodies usually will suffice.
11. The times in which the cells are held in the antibody solutions have been derived empirically. We have found that overnight incubations maximize the signal, and ensure full saturation of the cellular antigen. Shorter incubation times can be performed if time is of the essence, but we recommend at least 1 hour in all of the solutions (antibody and wash).

12. The precise time in the wash buffer is not critical, up to a point. At least one hour is recommended at room temperature. We have washed them for as long as overnight and found that the signal is still strong. Longer washes have shown to reduce the signal, thus we recommend washes no longer than overnight. We recommend the washes to be performed at room temperature, even if going overnight. This is especially true for mouse oocytes.

13. The time for the final wash can be as short as one hour. If control cells are used in the experiment (which we strongly recommend), they can be viewed under the microscope (briefly) to assess the level of background staining. If it is low after 1 hour, then the cells can be mounted on slides. We usually place the cells into the final wash buffer in the morning and then mount the cells in the afternoon. The signal should remain strong at least until the next morning. When the cells have been labeled with the secondary antibody, keep them protected from light until mounting. We cover the dishes with aluminum foil during the wash step.
14. This step requires some practice. It may be frustrating at first, but with experience it will become routine. Performing it properly, however, will result in high-quality imaging and less frustration in the long run. Since a small number of cells are usually being observed, it helps if they are all together on the slide. Thus, once the coverslip has been applied, allow the antifade solution to slowly spread out across the area under the coverslip. If the movement is too fast, the cells will detach from the glass and move with the flow. It is not a problem for imaging the cells, just more frustrating to find the cells during imaging. The cells don't always stick to the glass, so don't be surprised if they move. We have found that within 2 days the fluorescence signal begins to diminish, especially if the coverslips are not sealed with nail polish. Therefore we recommend imaging the cells within 2 days (1 day preferably). Although we do not store the slides in a freezer, reports in the literature suggest this to prevent loss of the signal. We recommend imaging with fluorescence microscopy as soon as possible. Sealing the edges of the coverslip with clear nail polish is recommended, but not necessary if the slides are handled carefully at this point. Give the samples at least 1 hour to dry before imaging. Overnight drying time is recommended. Be careful not to apply pressure to the coverslip before complete drying, which might cause the coverslip to move relative to the slide and destroy the sample.
References Cited


Figure 2.1. The microtubule pattern seen in a mature mouse oocyte.

Microtubules are located exclusively in the MII spindle at this stage of the cell cycle.
CHAPTER 3. THE EFFECT OF OSMOTIC STRESS ON THE METAPHASE II SPINDLE OF HUMAN OOCYTES, AND THE RELEVANCE TO CRYOPRESERVATION

Abstract

**BACKGROUND:** Knowing osmotic tolerance limits is important in the design of optimal cryopreservation procedures for cells.

**METHODS:** Mature human oocytes were exposed to anisomotic sucrose solutions at concentrations of 35, 75, 150, 600, 1200, or 2400 (± 5) milliosmolal (mOsm) at 37° C. A control treatment at 280 mOsm was also utilized. Oocytes were randomly allocated to each experimental treatment. After the treatment, the oocytes were cultured for 1 hour, then fixed in cold methanol.

Immunocytochemical staining and fluorescence microscopy were used to assess the morphology of the metaphase II (MII) spindle. Logistic regression was used to determine if media osmolality had a significant effect on spindle structure.

**RESULTS:** Osmolality was a significant predictor of spindle morphology. Hyposmotic effects at 35, 75, and 150 mOsm resulted in 100, 67, and 56 percent of oocytes having abnormal spindles, respectively. Hyperosmotic effects at 600, 1200, and 2400 mOsm resulted in 44, 44, and 100 percent of the spindles with abnormal structure, respectively.
CONCLUSIONS: Anisosmotic conditions lead to disruption of the MII spindle in human oocytes. Applying this fundamental knowledge to human oocyte cryopreservation should result in increased numbers of cells maintaining viability.

Key Words: cryopreservation/human/MII spindle/oocytes/osmotic tolerance
Introduction

Since the birth of the first human through assisted reproduction in 1978 (Steptoe and Edwards, 1978), the number of couples seeking infertility treatment has increased dramatically; over 80,000 cycles of clinical infertility treatment occur annually in the United States alone (http://www.cdc.gov/nccdphp/drh/art.htm). This is in part due to the increase in patient awareness and acceptability of fertility treatments. Additionally, technological improvements have led to a higher likelihood of a successful outcome. During routine clinical treatment, ovarian stimulation occurs to increase oocyte production. With increasing success rates have come increasing concerns regarding multiple gestational pregnancies (Collins and Graves, 2000; Derom and Bryan, 2000). This has led many clinicians to limit the number of embryos transferred during a cycle. It is common to cryopreserve the supernumerary embryos.

Cryopreservation of human spermatozoa and embryos has been conducted for several decades and contributes to the increased success of reproductive medicine (Bunge and Sherman, 1953; Trounson and Mohr, 1983). Unfortunately, human oocyte cryopreservation has proven much more challenging (Paynter, 2000). Having effective measures to cryopreserve oocytes would provide therapy for several patient groups. Patients facing potential iatrogenic sterility would have the option of cryopreserving oocytes, just as male patients can cryopreserve sperm prior to other medical treatments (Gosden, 2001; Posada et al., 2001).
Women who wish to delay childbearing may consider cryopreserving their oocytes for future clinical use. The establishment of oocyte banks could improve the safety of fertility treatments for women using oocyte donors by allowing improved screening of donors for potential transmittable diseases. Finally, patients who find gamete cryopreservation more acceptable than embryo cryopreservation could cryopreserve their oocytes, reducing the number of supernumerary embryos generated (Karow, 1997).

Similar to other cells, oocytes are susceptible to injury during cryopreservation (Van Blerkom and Davis, 1994). Mature mammalian oocytes have unique properties which make them difficult to cryopreserve. Several studies have documented the sensitivity of the MII spindle to various exposures experienced during cryopreservation. Included among these are sub-physiologic temperatures (mouse: (Magistrini and Szollosi, 1980); bovine: (Aman and Parks, 1994); human: (Pickering et al., 1990; Sathananthan et al., 1988)) and exposure to cryoprotective agents (CPAs) (mouse: (Johnson and Pickering, 1987; Van der Elst et al., 1988); bovine: (Saunders and Parks, 1999); human: (Gook et al., 1993; Sathananthan et al., 1988)). Of particular importance is how quickly spindle damage occurs. Two recent studies showed that within a matter of minutes, exposure to temperatures below 37°C caused disruption of the spindle in human oocytes, highlighting the challenge of preserving the integrity of this structure (Wang et al., 2001; Zenzes et al., 2001). It is noteworthy that previous studies (Eroglu et al., 1998; Pickering and Johnson, 1987) have documented the
ability of the MII spindle in mouse oocytes to undergo repair after disruption. This ability is diminished in human oocytes (Pickering et al., 1990; Wang et al., 2001). This difference likely contributes to the higher success of oocyte cryopreservation in the mouse compared to other species (Schroeder et al., 1990; Stachecki et al., 1998). Disruption of the MII spindle will result in failure of the final meiotic reduction division and the potential for the creation of an aneuploid embryo; a condition that is fatal in nearly all instances in human development (Hassold et al., 1996; Hassold and Hunt, 2001). Previous studies demonstrated the susceptibility of mature mammalian oocytes to failure in chromosome reduction after cryopreservation (Carroll et al., 1989; Kola et al., 1988). It is our thesis that understanding the effects of the various non-physiological conditions associated with cryopreservation on the MII spindle will facilitate oocyte cryopreservation.

While most reports on human oocyte cryopreservation have been based upon slow-cooling procedures, and some very recent reports have shown improvements in success compared to those from the past 2 decades (Boldt et al., 2003; Fosas et al., 2003; Quintans et al., 2002), the success rates remain sub-optimal, with highly variable fertilization rates (e.g. from 17 to 100% (Quintans et al., 2002) and 0 to 100% (Boldt et al., 2003)) as well as in vitro development rates (non-frozen cohort embryo data was not presented in these reports). Vitrification methods are a viable alternative to slow-cooling procedures (Kuleshova and Lopata, 2002), and improvements in human oocyte cryopreservation have been reported using such procedures (Katayama et al.,
While vitrification may afford protection against the chilling sensitivity of human oocytes, it has its associated challenges. To achieve vitrification, concentrations of cryoprotective agents need to be much higher than those used in slow cooling methods (~5-6 M vs 1.5M). The difficulties associated with these concentrations result from the potential toxic effects as well as the changes in osmotic pressure associated with their addition and removal (Fahy et al., 1984). During the addition and removal of CPAs, as well as during freezing and thawing, cell volume changes occur as CPAs and water enter and exit cells in response to osmotic and chemical gradients. These changes in cell volume associated with changes in osmotic pressure can impair a cell’s viability (Lovelock, 1953; Lovelock, 1957; Mazur and Schneider, 1986; Williams and Shaw, 1980).

Often times, the methods used for CPA addition and removal are empirically derived, and not based upon the cell’s tolerance to osmotic stress. Determining the osmotic tolerance limits for cells is a common approach to optimizing cryopreservation protocols, and has been performed on numerous cell types including mouse and bovine embryos (Mazur and Schneider, 1986); fertilized mouse ova (McWilliams et al., 1995; Oda et al., 1992) and unfertilized rhesus monkey (Songsasen et al., 2002), mouse (Pedro et al., 1997), and bovine oocytes (Agca et al., 2000); human (Gao et al., 1995), mouse (Willoughby et al., 1996), porcine (Gilmore et al., 1996), and bovine (Guthrie et al., 2002) spermatozoa, CD34+ cells derived from human placental/umbilical cord blood.
(Woods et al., 2000), canine pancreatic islets (Zieger et al., 1999); and rabbit corneal epithelial cells (Pegg et al., 1987). Knowing the osmotic sensitivity of the cell will allow one to engineer the CPA addition and removal techniques to keep a cell within its osmotic tolerance limits (Gilmore et al., 1997). Control of such volume changes can be achieved by stepwise addition and removal of CPAs and inclusion of non-permeating compounds in the freezing and thawing media (Gao et al., 1995; Leibo and Mazur, 1978). To determine an optimal method for CPA addition and removal, biophysical properties such as the cell’s permeability to water ($L_P$) and CPAs ($P_{CPA}$), the osmotically inactive cell volume ($V_b$), and the activation energies ($E_a$) of $L_P$ and $P_{CPA}$, must be known. These values have been determined in mature human oocytes for dimethylsulfoxide and 1,2-propanediol (Paynter et al., 1999; Paynter et al., 2001).

Although exposure to changes in osmotic pressure has been shown to decrease the viability of mouse and bovine oocytes as discussed above, to our knowledge, the effect of anisosmotic exposure on the MII spindle has not been investigated in any species. The purpose of the present experiment was to test the hypothesis that the probability of morphological disruption of the MII spindle in a human oocyte is related to the level of anisosmotic exposure. By understanding this relationship, improved CPA addition and removal methods can be designed which will prevent detrimental volume excursion as well as minimize the time to which the cells are exposed to the CPAs.
Materials and Methods

Chemicals

All chemicals were purchased from Sigma chemical company (St. Louis, MO, USA) unless otherwise indicated.

Source of Oocytes

The present study was approved by the Gwinnett Medical Institutional Review Board and the informed, written consent of patients was obtained. Patients underwent ovulation induction primarily using luteal phase leuprolide for down regulation followed by 2 amps of FSH combined with 2 mps of HMG daily in divided doses. A step down approach was utilized as the lead follicles reached the 14 mm level and hCG, 10,000 units, was given when two or more follicles reached 18 mm. Oocyte retrieval was carried out 35 hours after hCG administration. All manipulations were conducted in a manner to ensure the temperature of the oocytes remained near 37° C, preventing any detrimental effects of cooling on the spindle. Only oocytes having extruded the first polar body were used in these experiments.

Anisosmotic Solutions
Anisosmotic solutions consisted of sucrose as the extracellular, impermeable osmolyte supplemented with 0.23mM sodium pyruvate and 3 mg per ml bovine serum albumin, dissolved in Milli-Q water (18 MΩ; Millipore, Bedford MA, USA). Solution osmolality was determined using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT, USA). Treatment levels were 35, 75, 150, 600, 1200, and 2400 (±5) mOsm. A control treatment at 290 mOsm was also included (HEPES-HTF + 5% HSA: HEPES-HTF, Irvine Scientific, Santa Ana, CA, USA; HSA, InVitroCare, Inc., San Diego, CA, USA). An additional control treatment was utilized, using an isosmotic solution similar in composition to the treatment solutions, to test for effects of the non-physiological solution independent of osmolality.

**Oocyte Fixation and Immunocytochemistry**

Oocyte fixation was performed by transferring the cells to cold methanol (~ –10° C) containing 5mM EGTA for 5 minutes (Simerly and Schatten, 1993). They were then transferred to an immunocytochemical blocking buffer (PBS + 0.2% Triton X-100, 0.1% saponin, 130 mM glycine, 3mM sodium azide, 2% bovine serum albumin, and 5% horse serum (horse serum was obtained from Gibco BRL, Grand Island, NY, USA)) at 4° C and held overnight. The remaining procedures were carried out at room temperature. The following morning the cells were transferred to a solution of the blocking buffer containing an anti-α-tubulin mouse
monoclonal antibody (clone DM1A) diluted 1:4500, and held for 1 hour. They were subsequently washed in approximately 2 ml of blocking buffer without antibody for 1 hour, and then transferred to approximately 2 ml of blocking buffer which contained the secondary antibody (donkey anti-mouse IgG conjugated to Texas-Red®, Jackson Immunoresearch, West Grove, PA, USA) diluted 1:750 and held for 1 hour. Finally, the cells were transferred to approximately 2 ml of blocking buffer without antibody and washed for 2 to 4 hours. The oocytes were mounted on microscope slides using ProLong® antifade reagent containing either DAPI (1 μg/ml final concentration), or SYTOX® green (1μM final concentration) to counterstain the chromosomes. These products were obtained from Molecular Probes (Eugene, OR, USA).

**Assessment of the MII Spindle**

Assessing the structure of the spindle in fixed and stained cells is challenging using standard immunofluorescence techniques due to varied spindle orientation in relation to the focal plane. When the spindle poles are parallel to the plane of focus, the classic barrel shape is evident. However, if the orientation is not in this manner, the configuration of the chromosomes and spindle fibers are difficult to assess, and what may appear as a disorganized pattern of chromatin and tubulin may actually represent the 3-dimensional structure of a normal spindle. Confocal microscopy allows the collection of thin optical sections of cellular structures (Figure 1). Using appropriate software, a 3 dimensional interpretation of the
overall structure can be made. For the present study, optical sectioning of the spindles was performed using confocal microscopy, and the 3-dimensional structure was interpreted from these images. Spindle morphology was assessed using a BioRad Radiance 2000 laser scanning confocal microscope and LaserSharp 2000™ software (BioRad, Hercules, California, USA). A 60x UPlanApo (numerical aperture=1.25) objective was used for all experiments, and the zoom setting during image collection was 2.3. Confocal Assistant PC software (version 4.02) was used to perform 3-dimensional analysis of the spindle. Spindles with 2 anastral poles having the classic barrel shape of a human spindle, and lacking chromosome scattering were scored as normal. All other spindle morphologies were scored as abnormal.

**Experimental Design**

An incomplete randomized block design was employed for this study. Individual oocytes from each patient were randomly allocated to the experimental treatments for each replicate. Nine replicates were performed at all osmotic levels with the exception of the 35 mOsm level, where only 3 replicates were performed, and the isosmotic sucrose solution, where 6 replicates were performed. Oocytes were exposed to the treatment solution for 10 minutes, and subsequently returned to HEPES-HTF + 5% HSA for 10 minutes, which allowed the cells to return to their isosmotic volume. Subsequently, the cells were placed in HTF (Irvine Scientific, Santa Ana, CA, USA) + 5% HSA medium in a cell
culture incubator (Forma Scientific, Model 3130, Marietta, OH, USA) at 37° C with an atmosphere of 6.5 ± 0.5% CO₂ and air. The oocytes remained in the incubator for approximately 60 minutes prior to fixation.

**Statistical Analysis**

Spindles scored as normal or abnormal were coded as 0 or 1, respectively. Logistic regression was used to analyze the data (Hosmer and Lemeshow, 2000) using SAS (The SAS Institute, Cary, NC USA). The hypo- and hypertonic treatments were analyzed independently. The probability of a type-1 error (α) was chosen to be 0.05.
Results

Percentage of Cells Maintaining a Morphologically Normal Spindle

The numbers of cells with a normal spindle structure at each treatment level are shown in Figure 2. Less than half of the cells exposed to hypotonic levels were able to maintain a normal spindle, with increasing proportions of oocytes with disrupted spindles occurring as the solution concentrations decreased. A similar pattern was seen in the cells exposed to hypertonic solutions, with nearly half of the spindles exhibiting abnormal structure at the 600 and 1200 mOsm level; none of the spindles were normal in the cells exposed to 2400 mOsm. In contrast, all of the oocytes exposed to the control isotonic solutions (HTF, n=9 as well as sucrose, n=6) maintained a normal spindle structure.

Figure 3 shows representative images of the various spindle morphologies seen in the treated oocytes. In some cases, as in panels A & B, the morphology of the spindle appeared normal. Note the tubulin and chromosome staining associated with the first polar body in panel B to the lower right of the MII spindle. In other cases, such as the spindles shown in panels C - E, significant structural disturbances were evident. The image in panel E shows metaphase II chromosomes where microtubule staining was absent. In our experience, this pattern occurs in oocytes which lost the integrity of the oolemma during the
treatment. In one of the cells exposed to 150 mOsm, the microtubule pattern suggested that the cell was activated during the treatment and fixation occurred as the second polar body was being extruded (Inagaki et al., 1996). In the oocytes in which the spindle had a normal morphology, the chromosomes were aligned along the metaphase plate.

**Logistic Regression Analysis**

Osmolality was a significant predictor of loss of normal spindle structure for both the hypo- and hyperosmotic levels as assessed by the -2 Log Likelihood $\chi^2$ test ($p<0.001$). For the hyposmotic range, each 1 milliosmolal decrease in solution concentration increases the odds of spindle disruption by a factor of 1.006 to 1.038, with 95 percent confidence. For the hyperosmotic range, each 1 milliosmolal increase in solution concentration increases the odds of spindle disruption by a factor of 1.001 to 1.004 with 95 percent confidence.
Discussion

The current report documents the effects of volume changes associated with anisomotic exposure on the structure of the MII spindle in human oocytes. Our analysis allowed a determination of the overall structure of the spindle as well as the maintenance of the chromosomes at the metaphase plate. In those spindles classified as normal, the chromosomes were located at the metaphase plate, suggesting the bonds between the microtubules and kinetochores, as well as the sister chromatids, remained intact. We have shown that an increasing proportion of spindles exhibited abnormal morphology as the cell volume diverged from isotonic resulting from exposure to anisomotic solutions.

During the process of cryopreservation, cells are exposed to numerous non-physiological conditions. During the pre-freeze phase, the cells experience exposure to potentially toxic CPAs and cooling which can cause cold shock and disruption of cellular constituents. Thawing can also be detrimental with rehydration and the potential for the crystallization of previously vitrified cytoplasmic water (known as devitrification). The nature of these exposures is variable, depending on the approach one uses for cryopreservation. It has long been established that the presence of cryoprotective agents is necessary for cells to survive freezing (Polge et al., 1949). Exposing cells to CPAs causes transient volume changes due to osmotic pressure differences across the cell membrane, and these osmotically-induced volume changes can also have
detrimental effects on cell viability. The hyperosmotic tolerance of human oocytes, as measured using a vital dye assay, was previously assessed (McWilliams et al., 1995). While this study showed that human oocytes can tolerate anisosmotic exposure in a similar range of concentrations used in the present report (~ 600 – 3000 mOsm), our analysis suggests that the tolerance is more limited. The vital dye assay, while able to determine membrane integrity and enzymatic function, would have failed to detect any changes to the structure of the MII spindle, as shown in the present report.

The present study used a design to isolate the effects of osmolality, where sucrose was the primary extracellular, non-permeating compound, to avoid the potential confounding effect of high ionic concentrations (Gao et al., 1993). We included an additional control to test for effects of the brief absence of ionic conditions usually present in culture media. It was determined that there was not an effect of the composition of the treatment solutions independent of osmotic effects with this additional control. It should be noted that the present treatments did not include cryoprotective agents, which may have a transient effect on spindle stability during cryopreservation (George et al., 1996).

The spindle in human oocytes has been shown to be very sensitive to differing aspects of cryopreservation. Sathananthan et al. (1988) reported on the consequences of slow cooling oocytes in the presence or absence of DMSO to 0°C and holding them at this temperature for 20 or 60 minutes. All of the oocytes
from these treatments showed significant disruption of the meiotic spindle, while non-cooled oocytes displayed a normal microtubule pattern. Another report confirmed the temperature sensitivity of the spindle in human oocytes when the cells were cooled only to room temperature in the absence of CPAs (Pickering et al., 1990). In addition to highlighting the temperature sensitivity, this report demonstrated that the human MII spindle has a limited ability to undergo repair upon return to physiologic temperature. This property differs when compared with mouse oocytes (Pickering and Johnson, 1987). In more recent reports, the rate of human spindle disruption was assessed at different temperatures. Zenzes et al. (2001) documented this phenomenon at 0°C. They showed that within 2 minutes, noticeable spindle disruption had occurred. An additional two minutes caused complete loss of the microtubules. Wang et al. (Wang et al., 2001) reported on the effects of cooling between 37°C and 25°C on the spindle in live human oocytes using polarized light microscopy. They showed that spindle disassembly begins around 32°C. Although spindles that were disassembled for a brief period were able to recover upon return to 37°C, this was not the case when the spindles remained disassembled for 10 minutes. Zero of 5 oocytes held at 25°C failed to recover their spindle, and only 2 of 5 held at 28°C were capable of rebuilding their spindle. This is the first study to investigate the effects of osmotically-induced volume changes on the MII spindle in human oocytes. In our design, we allowed a 60 minute recovery period prior to fixation to allow for spindle repair. Data from this collection of studies reinforce the need to develop
methods capable of protecting the MII spindle from disassembly during cryopreservation.

Understanding the effect on human oocytes of all of the factors associated with cryopreservation is crucial to developing optimized procedures. The results from the present study can be used in a fundamental manner to optimize CPA addition and removal procedures for human MII oocytes, as has been done for human spermatozoa (Gilmore et al., 1997). Although spindle assessment was not undertaken, Fabbri et al. (Fabbri et al., 2001) demonstrated the beneficial effect of increasing the sucrose concentration to 0.3 molar in a freezing and thawing medium on the prevention of intracellular ice formation. Although this improvement is likely due to the reduction of intracellular water volume prior to freezing, increasing the sucrose concentration will impose greater osmotic stress and will increase the likelihood of causing damage to the spindle as shown by the present study. In an effort to reduce the osmotic stress on the oocyte during CPA addition and removal, the CPA addition was performed in 2 steps, and the CPA removal was performed in 4 steps. Such a procedure may have detrimental effects for reasons not associated with changes in osmotic pressure. The complexity of this procedure necessitates exposing the cells to suboptimal conditions for extended periods (approximately 30 minutes for CPA removal was required for this procedure). Considering this was performed at room temperature, the extended cooling of these oocytes may have had deleterious effects on the spindle. In a more recent report (Fosas et al., 2003), the
investigators used a similar approach for their cryopreservation procedure, but significantly reduced the amount of time for the CPA removal step, and found the outcome to be similar to the report by Fabbri et al. Minimizing the amount of time used to remove CPAs, thereby reducing the exposure to non-physiological conditions, is a logical approach to optimization of cryopreservation procedures. The results from the present study suggest that a single-step CPA removal could be accomplished without causing volume changes that would have deleterious effects on the spindle. Thus, having defined the osmotic tolerance limits for the human MII oocyte, one can engineer CPA addition and removal procedures for any cryopreservation procedure and minimize the exposure of the cells to suboptimal volume conditions.

With increasing numbers of human ART cycles being undertaken worldwide, developing improved cryopreservation procedures for human oocytes is essential. The results from the present study demonstrate that there is a relationship between anisomotic exposure and maintenance of a morphologically normal MII spindle in human oocytes. Understanding this relationship will allow the development of improved methods for CPA addition and removal procedures during human oocyte cryopreservation.
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References Cited


Figure 3.1. A three dimensional artistic reconstruction of a metaphase II spindle from a control human oocyte based on a representative oocyte from the present study. The focal planes approximate the optical sections through the spindle imaged with confocal microscopy. It is evident from the serial optical sections that the microtubule organization (red) is appropriate for a normal meiotic spindle in which the microtubules form from the two opposite poles as seen in sections a and d. Chromosomes (green) are aligned regularly at the metaphase plate located between the two half spindles. A two-dimensional analysis would not have allowed such an accurate interpretation.
Figure 3.2. The number of oocytes in each experimental group which had maintained a normal spindle structure after the osmotic treatment (values measured in milliosmolar, mOsm) and a 60 minute culture period is shown. The isosmotic exposure marked with an asterisk contained sucrose as the primary solute, similar to the other anisosmotic treatments. There were 6 oocytes exposed to this treatment, with all of them maintaining a normal morphology. The proportion of spindles in each treatment that maintained a normal structure decreased as the solution osmolality diverged from isosmotic. * n=6 for this isosmotic control treatment
Figure 3.3. Confocal microscopy images showing representative structures of MII spindles in oocytes exposed to different osmolalities. Examples of spindles with normal microtubule and chromosome organization are shown in panels A and B. Note the tubulin and chromatin staining associated with the first polar body located to the lower right of the spindle in panel B. Panels C-E represent microtubule organization that was abnormal for a spindle. Note the reduced spindle diameter and slight chromosome scattering in panel C. The spindle in Panel D has tubulin that is more dispersed lacking discrete spindle poles and chromosome scattering. The absence of tubulin staining in panel E is indicative of a cell that lysed during the treatment.
CHAPTER 4. MODELING THE PROBABILITY OF MII SPINDLE DISRUPTION IN BOVINE OOCYTES AS A FUNCTION OF TOTAL OSMOLALITY USING LOGISTIC REGRESSION, AND ITS APPLICATION TOWARD IMPROVED CPA ADDITION AND REMOVAL PROCEDURES

Abstract

During cryopreservation, cells undergo volume excursions as a result of exposure to anisosmotic solutions containing cryoprotective agents. Cryopreservation methods can be engineered to maintain cell volumes within their osmotic tolerance limits. In this study we investigated the osmotic tolerance limits of the bovine oocyte oolemma and metaphase II spindle. Oocytes were exposed to sucrose solutions (75 to 3000 milliosmolal) for 10 minutes, then cultured for 1 hour. Oolemma integrity was assessed visually prior to fixation. The structure of the metaphase II spindle was assessed using immunocytochemistry and laser scanning confocal microscopy. Logistic regression was used to model the probability of spindle disruption as a function
of solution osmolality. Few cells experienced oolemma rupture or spindle
disorganization between 200 and 1800 milliosmolal. Higher levels of osmotic
stress caused rupture of the oolemma and disruption of the spindle structure.
These results can be applied to improve methods for the addition and removal of
cryoprotective agents that will protect the spindle from osmotic damage.

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Introduction

Research conducted over the past two decades has demonstrated the susceptibility of mammalian oocytes to freezing injury (Critser et al., 1997). Upon final maturation, oocytes from most mammalian species are arrested at the metaphase stage of the second meiotic division, with the metaphase II (MII) spindle present in the ooplasm (Wassarman and Albertini, 1994). There are numerous reports in the literature which document the detrimental effects of cold exposure on the spindle structure in oocytes from various organisms including: human (Sathananthan et al., 1988), rhesus macaque (Songsasen et al., 2002), bovine (Aman and Parks, 1994), ovine (Moor and Crosby, 1985), caprine (De Smedt et al., 1992), porcine (Liu et al., 2003), and murine (Magistrini and Szöllösi, 1980). In addition to chilling, exposure to cryoprotective agents (CPAs) such as dimethylsulfoxide (DMSO), 1,2-propanediol (PG), and ethylene glycol (EG) has been shown to disrupt the spindle structure in mouse, bovine, and human oocytes (Johnson and Pickering, 1987; Van der Elst et al., 1988; Joly et al., 1992; Gook et al., 1993; Saunders and Parks, 1999). In the mouse oocyte it has been shown that this disruption is repairable to a high degree after return to normal culture conditions (Pickering and Johnson, 1987). Unfortunately, this is not the case for all species (Pickering et al., 1990; Aman and Parks, 1994). This characteristic may partially explain the ability to cryopreserve mouse oocytes at higher rates than oocytes from other species (Schroeder et al., 1990). This difference makes the mouse a relatively poor model for mammalian oocyte
cryobiology. The fidelity of chromosome reduction is essential to the
developmental potential of mammalian oocytes. For example, aneuploidy results
in developmental failure in the majority of instances in humans (Bond and
Chandley, 1983), and aneuploid embryos have been shown to result from
cryopreservation of mature mouse oocytes (Kola et al., 1988; Bos-Mikich et al.,
1995). Therefore, it is essential to maintain the integrity of this structure during
cryopreservation to preserve the developmental viability of the oocyte.

Damage resulting in loss of viability can occur at any stage in the
cryopreservation process, and often the cells are susceptible to different types of
damage during the various stages (Mazur, 1970). Damage as a result of
exposure to the CPAs can occur from the chemical nature of the compounds
(true chemical toxicity) as well as from the anisosmotic nature of the solutions
(Gao and Critser, 2000). In most instances, CPAs need to be present at high
concentrations, typically greater than 1 molar, to afford their protection. Thus the
concentration of solutes in these solutions (typically > 1500 milliosmolal (mOsm))
is much higher than in physiologic medium (approximately 290 mOsm). As a
consequence of exposure to such a solution, the cell will initially undergo
exosmosis and a concomitant reduction in volume as a result of the difference
between the intra- and extracellular total solute concentration. However, with a
permeating CPA such as DMSO, the cell volume will eventually increase as the
CPA enters the cell and water follows to maintain its chemical potential. A similar
volume change occurs during CPA removal, but with initial cell swelling as water
first enters the cell and subsequently CPA and water exits. The magnitude of the volume excursions is dependent upon the permeability of the cell membrane to water and the CPA and their activation energies (Agca et al., 1998).

Many cell types have limited tolerance for changes in cell volume, with increasing volume changes causing additional loss of cell viability (Mazur and Schneider, 1986; Pegg et al., 1987; Gao et al., 1993; Gilmore et al., 1996; Woods et al., 2000). Thus, knowing the tolerable volume excursions (often referred to as the "osmotic tolerance limits") is essential for optimizing cryopreservation procedures. Many investigations into cell cryopreservation use methods for CPA addition and removal that are empirically derived, best guess approaches. However, one can define the osmotic tolerance limits by assessing cell viability in relation to cell volume changes (Pegg et al., 1987; Gao et al., 1993; Gilmore et al., 1996; Woods et al., 2000; Agca et al., 2002; Guthrie et al., 2002) and use this information to design optimal methods for CPA addition and removal (Gao et al., 1995; Woods et al., 2003). Using such a fundamental approach, significant improvements in the cryopreservation of important cell types has been achieved (Gao et al., 1995; Woods et al., 2003).

In preliminary investigations it was determined that the MII spindle is more sensitive to anisosmotic exposure than the oolemma. Thus, the goal of the present study was to define the osmotic tolerance limits of bovine oocytes as measured by the maintenance of a morphologically normal MII spindle. To
achieve our goal, we designed an experiment to test the hypothesis that the proportion of oocytes which maintain a normal MII spindle structure is related to the concentration of hypo- or hyperosmotic non-permeating solute solutions to which they are exposed. We then developed a probability model for spindle disruption using the logistic regression analysis. Defining this relationship will allow the design of methods for CPA addition and removal which will reduce osmotic damage to the MII spindle in bovine oocytes and should lead to improvements in bovine oocyte cryopreservation.
Materials and Methods

Chemicals

All chemicals were purchased from Sigma chemical company (St. Louis, MO, USA) unless otherwise indicated.

Source and Handling of Oocytes

In vitro matured bovine oocytes were obtained commercially (BOMED, Inc. Madison, WI USA). Care was taken to keep all of the solutions near 39° C throughout the experiment. Upon completion of maturation (22-24 hours), the cumulus oocyte complexes (COCs) were transferred to a HEPES buffered Tyrodes Lactate solution (TL-HEPES) containing 3 mg per ml Bovine Serum Albumin (BSA) (Bavister et al., 1983) supplemented with hyaluronidase at a concentration of 5 mg (~5000 units) per ml. The COCs were held in this solution for 3 minutes, and subsequently vortexed for an additional 2 minutes to remove the cumulus cells. After vortexing, the oocytes were washed through three, 2 ml solutions of TL-HEPES. After the final wash, oocytes with a size nearly filling the space within the zona pellucida and possessing dark, evenly granulated cytoplasm were selected for inclusion into the experiment. During spindle assessment, maturation to the MII stage was confirmed by the presence of chromatin from the first polar body. Upon selection for inclusion into the
experiment, the oocytes were segregated from the remaining oocytes and held in TL-HEPES until the experiment commenced (within 10 minutes). The remaining oocytes were placed into a 200 µl drop of bovine IVF medium (Parrish et al., 1986) which had been equilibrated overnight in an incubator at 5% CO₂ and 39°C, and returned to the incubator for the duration of the experiment. These oocytes were subsequently fixed and used as positive controls for the staining assay.

**Anisosmotic Solutions**

Anisosmotic solutions consisted of sucrose as the extracellular, impermeable osmolyte supplemented with 0.23 mM sodium pyruvate, dissolved in commercial embryo culture grade water. Solution osmolality was determined using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT, USA). Solutions with osmolalities of 75, 150, 200, 600, 900, 1200, 1500, 1800, 2100, 2400, 2700, and 3000 (±5) mOsm were used. An isotonic treatment of 280 (±5) mOsm was also included (TL-HEPES). All solutions contained BSA at a concentration of 3 mg per ml.

**Oocyte Fixation and Immunocytochemistry**

Oocytes were fixed for 6-7 minutes in cold methanol (~ −20°C) containing 5mM EGTA (Simerly and Schatten, 1993). They were then transferred to an
immunocytochemical blocking buffer (PBS + 0.2% Triton X-100, 0.1% saponin, 130 mM glycine, 3mM sodium azide, 2% bovine serum albumin, and 5% horse serum (horse serum was obtained from Gibco BRL, Grand Island, NY, USA)) and held up to 7 days at 4°C prior to staining. The remaining procedures were conducted at room temperature unless otherwise noted. The cells were transferred to a solution of the blocking buffer containing an anti-α-tubulin monoclonal antibody (clone DM1A) at a final concentration of ~2.5µg/ml, and incubated overnight at 4°C. They were subsequently washed in approximately 2 ml of blocking buffer without antibody for 2 hours, and then transferred to approximately 2 ml of blocking buffer which contained the secondary antibody (donkey anti-mouse IgG conjugated to Texas-Red®, Jackson Immunoresearch, West Grove, PA, USA) at a final concentration of ~1µg/ml and held for 1-2 hours. Finally, the cells were transferred to approximately 2 ml of blocking buffer without antibody and washed for 30 – 60 minutes. Upon completion of the final wash, the oocytes were mounted on microscope slides using ProLong® antifade reagent which contained SYTOX® green (1µM final concentration) to counterstain the chromosomes (Molecular Probes, Eugene, OR, USA).

Assessment of the Oolemma and MII Spindle

Lysis of the oolemma was evident by a change in the color of the cytoplasm and loss of a sharply defined membrane boundary inside of the zona pellucida. Spindle assessment was performed using laser scanning confocal microscopy.
(Radiance 2000 system with LaserSharp 2000™ software, BioRad Inc. Hercules, California, USA). The Krypton Argon laser was used to excite the fluorophores. An HQ 515/30 and an E580LP emission filter were utilized for the green and red fluorophores, respectively. Spindles with only 2 anastral poles and chromosomes confined to the metaphase plate, having a size, shape, and estimated fluorescence intensity similar to the control oocytes’ spindles were scored as normal. All other spindle morphologies were scored as abnormal. Oocytes with the tubulin and chromatin staining displaying the structure of the telophase stage were scored as having been activated by the treatment. Even though the spindle in activated oocytes is normal from a functional standpoint, it is abnormal from the standpoint of preserving the cell in its pre-treated state. Hence we classified activated oocytes as having an abnormal spindle, since the spindle was not in the MII configuration.

**Experimental Design**

A completely randomized design was used for this study (Zolman, 1993). The first 6 replicates contained solutions with osmolalities between 75 and 2400 mOsm. Seven additional replicates were performed which included treatment levels of 2700 and 3000 mOsm since some oocytes from the initial replicates were able to tolerate exposure to 2400 mOsm as assessed by their normal oolemma and spindle structure. After selecting oocytes of acceptable quality for inclusion into the experiment as described above, individual oocytes were
randomly allocated to the experimental treatments for each replicate. Oocytes were transferred to, and held in the treatment solution for 10 minutes, then returned to TL-HEPES for 10 minutes, which allowed the cells to return to their isosmotic volume. Subsequently, the cells were placed in an incubator with an atmosphere of 5% CO₂ and air in bovine IVF medium at 39° C. The oocytes remained in culture for approximately 60 minutes prior to fixation. Prior to fixation, the integrity of the oolemma was recorded as intact or lysed as described above. The structure of the MII spindle was scored as normal or abnormal after immunofluorescence analysis.

**Statistical Analysis and Model Development**

Spindles scored as normal or abnormal were coded as 0 or 1 respectively. These data were analyzed by logistic regression (Hosmer and Lemeshow, 2000) using the SAS system (The SAS Institute, Cary, NC USA). The hypo- and hypertonic treatments were analyzed independently. An α value of 0.05 was used.

The logistic function used to relate the probability of the event being modeled and the experimental variable is described by:

\[ \pi = \frac{e^{\beta_0 + \beta_1 x}}{1 + e^{\beta_0 + \beta_1 x}} \]  \[1\]

where \( \pi \) is the probability of the event occurrence, in this case the loss of normal spindle morphology, \( \beta_0 \) is the coefficient for the model constant, \( \beta_1 \) the coefficient for the experimental parameter, and \( x \) is the value of the experimental variable,
extracellular concentration in mOsm in this instance. The coefficients are
determined by the logistic regression procedure using maximum likelihood
estimation.

Oocyte water and CPA volume and intracellular CPA concentration changes
were modeled using two coupled nonlinear differential equations as first
described by Kedem and Katchalsky (Kedem and Katchalsky, 1958):

\[
\frac{dV_{w+s}}{dt} = -L_p A R T \left( M_n^e - M_n^i \right) + \sigma \left( m_{CPA}^e - m_{CPA}^i \right); \quad [2]
\]

and

\[
\frac{dm_{CPA}^i}{dt} = \left( 1 + \frac{V_{CPA} m_{CPA}^i}{V - V_b} \right) \left\{ \frac{m_{CPA}^i}{1 - \sigma} - \frac{m_{CPA}^i}{1 + V_{CPA} m_{CPA}^i} \right\} \left( \frac{dV_{w+s}}{dt} + A P_{CPA} \left[ m_{CPA}^e - m_{CPA}^i \right] \right); \quad [3]
\]

where \( 0 \leq \sigma \leq 1 - \frac{P_{CPA} \overline{V}_{CPA}}{L_p R T} \). \quad [4]

\( V_{w+s} \) is the volume of water plus solvent, \( L_p \) is the cytoplasmic membrane water
permeability, \( A \) is the cell surface area, \( R \) is the gas constant, \( T \) is the absolute
temperature, \( M_n^i \) is the osmolality of internal salts, \( M_n^e \) is the osmolality of
external salts, \( \sigma \) is the reflection coefficient, \( m_{CPA}^i \) is the molality of the internal
CPA, \( m_{CPA}^e \) is the molality of the external CPA, \( \overline{V}_{CPA} \) is the partial molar volume of
CPA, $V$ is the cell volume, $V_b$ is the osmotically inactive cell volume, $\overline{m}_{CPA} = (m_{CPA}^e - m_{CPA}^i) / \ln (m_{CPA}^e / m_{CPA}^i)$, and $P_{CPA}$ is the CPA permeability.

The equilibrated cell water and CPA volumes were equated to the total cell volumes by the addition of $V_b$, and then related to the solution osmolality using the Boyle van’t Hoff relationship (Newton et al., 1999) as described by:

$$V = \left( \frac{M_{iso}}{M} \right) (V_{iso} - V_b) + V_b;$$

where $V$ is the normalized cell volume, $V_{iso}$ is the isosmotic cell volume, $M_{iso}$ is the isotonic osmolality, and $M$ is the solution osmolality. The appropriate cryobiological and biophysical parameters for mature bovine oocytes at 22° C have been previously reported (Ruffing et al., 1993; Agca, Liu, Peter, Critser and Critser, 1998; Agca et al., 1999).

Finally, the osmolality was related to the probability of spindle disruption using equation [1]. Since all of the oocytes at the treatment levels of 2700 and 3000 mOsm showed a disrupted spindle, the analysis for the hypertonic data were restricted to the treatments between 280 and 2700 mOsm.
Results

The effects of the treatments on the oolemma and spindle are shown in Table 1. A high proportion (91%) of oocytes maintained oolemma integrity after exposure to 150 through 2400 mOsm. However, many oocytes exposed to 150 and 2400 mOsm had a disrupted spindle, some (15%) as a result of oocyte activation. Nearly all of the oocytes exposed to 75, 2700, and 3000 mOsm exhibited abnormal spindles, most of these concomitant with oolemma lysis. In contrast, all of the cells in the isotonic treatment maintained an intact oolemma and had normal spindle morphologies.

Spindle structures representing those seen in bovine oocytes after anisosmotic exposure are shown in Figure 1. An example of a spindle with a normal structure after having been exposed to the isotonic treatment is shown in panel A. Only 3 of the 82 oocytes (4%) with spindles having a normal microtubule pattern showed evidence of chromosome displacement from the metaphase plate (panel B). Two were from the 2100 mOsm treatment and one was from the 600 mOsm treatment. In all three cases, the staining pattern suggested that there was a single sister chromatid located at each pole (arrows).

An interesting phenomenon occurred in two of the oocytes exposed to the 150 mOsm solution. Several clusters of chromosomes were present which were not confined to the metaphase plate, and each had an associated ‘mini-spindle’
We did not observe this occurrence in oocytes exposed to any of the other treatments. Occasionally, bovine spindles developed a third anastral pole after exposure to the anisosmotic treatment, as seen in panel D. When this occurred, some chromosomes became displaced from the metaphase plate and migrated toward the third pole as seen in this panel. Of the 39 abnormal spindles seen in the oocytes, 10 (26%) exhibited the pattern of a telophase spindle (panel E), where the sister chromatids had moved to opposite sides of the spindle and a spindle midbody was evident. When oolemma lysis occurred, microtubule staining was absent in 100% of the oocytes, as seen in panel F.

The solution concentration was a significant predictor of the loss of spindle morphology for both the hypo- and hyperosmotic treatment levels, with the p-value of the -2 Log Likelihood test statistic being less than 0.0001. Interpretation of the model suggests that the odds of spindle disruption increases by a factor of 1.016 to 1.052, with 95 percent confidence, for each 1 mOsm decrease in the hyposmotic range, and increases by a factor of 1.001 to 1.003, with 95 percent confidence, for each 1 mOsm increase in the hyperosmotic range. Using the logit equation [1], the probability of bovine oocytes experiencing irreversible disruption of the MII spindle as a function of the osmolality can be modeled as:

\[
\pi = \frac{e^{(6.3588-0.0344z)}}{1 + e^{(6.3588-0.0344z)}}
\]  

[6]

in hypotonic solutions, and

\[
\pi = \frac{e^{(-3.4282+0.00183z)}}{1 + e^{(-3.4282+0.00183z)}}
\]

[7]
in hypertonic solutions. By taking the inverse of these values, the probability of a
given oocyte maintaining a normal spindle structure as a function of the
osmolality can be determined. Graphic representations of these calculations are
presented in Figure 2 along with the experimentally determined probability at the
solution concentrations used for this study. As shown in the plots, the probability
decreases as the solution concentration diverges from isotonic. These models
can be used as guides in the development of procedures for the addition and
removal of CPAs for maintaining the integrity of the spindle structure at a chosen
probability. For example, to prevent osmotic damage to the MII spindle with a
probability of 0.9, one would have to use a CPA addition and removal procedure
which maintains the cells within a volume range of 1.1 to 0.52 times the isotonic
volume.
Discussion

Due to the generally low success of slow cooling as an approach to cryopreserve bovine oocytes, investigators have turned to ultra-rapid cooling approaches (>10,000º C min⁻¹) to overcome the difficulties inherent with these cells (Vajta et al., 1998). Two challenges associated with ultra-rapid cooling are the potentially toxic effects of the high concentrations (e.g. 5M) of CPAs required with this approach, and the osmotic effects associated with their addition and removal. The results from this study demonstrate that damage to the oolemma and MII spindle in bovine oocytes occurs at a higher frequency as the solution concentrations diverge from isosmotic. However, the tolerance of the bovine oolemma and MII spindle to hypertonic conditions is relatively high, with significant proportions of oocytes maintaining a normal spindle structure up to an 1800 mOsm exposure. With such a broad tolerance to hyperosmotic stress, CPA addition can be performed in relatively few steps. Using such an approach, the exposure times to these compounds prior to long-term storage can be minimized, which should reduce the associated CPA toxicity (Fuku et al., 1995). Critically, since the MII spindle was shown to be fairly sensitive to osmotic swelling, multi-step CPA removal procedures may need to be utilized, depending on the degree of dehydration prior to cryopreservation. A previous report from our group (Agca et al., 2000) analyzed the developmental potential of cumulus enclosed bovine oocytes after anisosmotic exposure, in a similar manner to the present investigation. In that study, fertilization and cleavage rates, normalized to the
controls, remained high for treatments ranging from 75 to 2400 mOsm. However, when in vitro development to the blastocyst stage was assessed, the rates were significantly reduced as the treatment levels diverged from isotonic.

**Potential Mechanisms for Osmotically-Induced Spindle Disruption**

There are several possible mechanisms by which exposure to an anisosmotic solution could alter the structure and association of microtubules and chromosomes in the meiotic spindle. With high osmotic and hydrostatic pressure, the hydration state of macromolecules can be altered; in extreme cases leading to changes in conformation and activity (Kornblatt and Kornblatt, 2002). The changes in solute concentrations within cells associated with volume changes may lead to alterations in protein-protein interactions due to changes in intracellular ionic strength. This effect is particularly applicable to microtubules, since the α- and β-tubulin molecules associate through non-covalent interactions, and changes in solute conditions have been shown to alter microtubule assembly characteristics (Olmsted and Borisy, 1973; Sackett et al., 1994). Either of these mechanisms could also cause disruption of the kinetochores or microtubule organizing centers (MTOC), both of which are multi-protein complexes. Several proteins associated with the MTOC in mitotic spindles have been shown to be necessary for structural maintenance, such as TPX2 (Wittmann et al., 2000) and NuMa (Merdes et al., 1996) in Xenopus and asp (do Carmo Avides and Glover,
1999) and dd4 (Barbosa et al., 2000) in drosophila. It is likely that similar proteins are responsible for maintaining spindle pole organization in bovine oocytes, and their disruption would likely lead to disorganized MTOCs as seen in many of the anisosmotically-treated spindles in the present study.

Maintaining the bonds between the spindle fiber proteins and the kinetochores as well as between the sister chromatids is critical for the proper function of the MII spindle during chromosome segregation (Nasmyth, 2001). It is interesting to note in our analysis that very few spindles with a normal microtubule pattern showed evidence of displacement of the chromatids from the metaphase plate (see table 1). In the few that showed such evidence, there was chromatin staining at both spindle poles (Figure 1, Panel B₁ and B₂), with the staining intensity suggesting that only one of the sister chromatids was present at each pole. Thus, it appeared that only the cohesin bonds between sister chromatids were affected. One common structural abnormality seen in this study, as well as in unpublished observations in our lab after exposing bovine oocytes to CPAs, is the appearance of an additional spindle pole with an associated focus of microtubule nucleating activity. When this occurs, several of the chromosomes appear to be "captured" by the microtubules associated with this new pole, and are displaced toward this new MTOC. Thus it seems that maintaining the association of the proteins of the MTOCs may be more critical than maintaining the bonds between the kinetochores and spindle fibers for preserving the overall spindle structure.
Due to the variety of potential mechanisms of damage, it is not surprising that a recent report (Rho et al., 2002) documented a high degree of disruption of the MII spindle structure in bovine oocytes after exposure to a vitrification solution. In this report, the effects of a two-step addition and a 3-step removal of a solution containing DMSO and EG resulted in disruption of the spindle structure in 31% of the cells (normalized to control). Because a multi-step procedure was employed, the results from the present study suggest that the effects seen resulted from a combination of osmotic as well as chemical damage. In their report, when vitrification was also performed with these CPA addition and removal procedures, 67% of the oocytes (normalized to controls) had a disrupted spindle structure after thawing. These results highlight the many challenges to stabilizing the spindle structure when ultra-rapid cooling procedures are employed.

Modeling the Probability of Spindle Disruption and Designing Theoretically Optimal Cryopreservation Procedures

Recent studies have used various approaches for the ultra-rapid cryopreservation for mature bovine oocytes, with higher rates of success compared to slow cooling methods (Martino et al., 1996; Vajta et al., 1998). In one of the experiments in the report by Martino et al. (Martino et al., 1996), the investigators applied a single step CPA addition of 4 M ethylene glycol (EG) plus 0.5 M sucrose in H-CZB medium, and utilized a multi-step removal as one of the treatments, achieving an average blastocyst developmental rate of approximately
14% (development rate of the controls was approximately 42%). This rate was a significant improvement compared to a slow cooling cryopreservation treatment used in the same study. When the volume change associated with this procedure was modeled with the approach previously described by Gao et al. (Gao et al., 1995), the greatest volume excursion would have occurred during the CPA addition, as it was performed in a single step. The probability of maintaining a normal spindle structure with this technique is approximately 0.88, according to the results from the present study. This high level is not surprising given the degree of tolerance of the MII spindle for hyperosmotic stress as we have shown. When the volume change associated with a single step dilution procedure was modeled using the single step EG addition as discussed above, the cell volume returned to near isotonic, with little cell swelling beyond isotonic volume. These results suggest that a single step CPA removal would be a more optimal procedure than the multi-step CPA removal scheme utilized in the original publication, with the estimated probability of spindle maintenance remaining at 0.88. The estimated volume changes associated with each of these approaches are plotted in figure 4. The top panel (A) shows the volume excursions associated with the original procedure described by Martino et al., (Martino, Songsasen and Leibo, 1996) while the bottom panel shows the volume changes associated with the theoretically improved procedure described above. While from an osmotic standpoint this method should be more optimal, there may be detrimental effects associated with the CPA concentration independent of the osmotic stress as discussed above. Future experiments will need to define
optimal CPA types and concentrations to further improve ultra-rapid cryopreservation of bovine oocytes. Using the results from this study, optimal procedures for CPA addition and removal can be designed in future experiments which prevent excessive damage to the MII spindle and should help to maintain the developmental competence of the cells after freezing.
References Cited


Table 4.1.
The Effects of Osmotic Stress on Bovine Oocytes

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<tr>
<th>Sample Size</th>
<th>Osmolality (mOsm)</th>
<th>Number of Cells with ruptured Oolemma</th>
<th>Number Activated*</th>
<th>Number with a Normal Spindle</th>
<th>Percent with a Normal Spindle</th>
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</table>

* Spindle having a telophase configuration
Figure 4.1. Examples of metaphase II spindle configurations seen after anisosmotic exposure. A normal spindle configuration is shown in panel A, while various abnormal configurations are shown in the remaining panels. Note the arrows pointing to chromatin located at opposite poles of the spindle in panels B₁ and B₂. These images are of the same spindle, with two different focal planes being shown in the sub-panels.
Figure 4.2. The experimental datapoints plotted along with the logistic model (line) described by equations 5 and 6 for the hypotonic (left side) and hypertonic (right side) conditions respectively. This model describes the relationship between solution concentrations and the probability of maintaining a morphologically normal spindle.
Figure 4.3. A comparison of the volume changes associated with a CPA addition and removal method described by Martino et al., 1996 (panel A) and a theoretically optimized method developed using the results from the current study (panel B). Not how rapidly the cell volume returns to near isotonic with the optimized method.
CHAPTER 5. THE EFFECT OF OSMOTIC STRESS ON THE CELL VOLUME, METAPHASE II SPINDLE AND DEVELOPMENTAL POTENTIAL OF IN VITRO MATURED PORCINE OOCYTES

Abstract

Porcine animal models are used to advance our understanding of human physiology. Current research is also directed at methods to produce transgenic pigs. Cryobanking gametes and embryos can facilitate the preservation of valuable genotypes, yet cryopreserving oocytes from pigs has proven very challenging. The current study was designed to understand the effects of anisotonic solutions on in vitro matured porcine oocytes as a first step toward designing improved cryopreservation procedures. We hypothesized that the proportion of oocytes demonstrating a normal spindle apparatus and in vitro developmental potential would be proportional to the solution osmolality. Oocytes were incubated for 10 minutes at 38 °C in various hypo- or hypertonic solutions, and an isotonic control solution and then assessed for these two parameters. Our results support the hypothesis, with an increasing proportion of spindles showing a disrupted structure as the levels of anisotonic exposure diverge from isotonic. Only about half of the oocytes maintained developmental potential after exposure
to anisotonic solutions compared to untreated controls. Oocyte volume displayed a linear response to anisotonic solutions as expected, with an estimated relative osmotically-inactive cell volume of 0.178. The results from this study provide initial biophysical data to characterize porcine oocytes. The results from future experiments designed to determine the membrane permeability to various cryoprotectants will allow predictive modeling of optimal cryopreservation procedures and provide a basis for designing improved cryopreservation procedures.

**Key words:** Porcine, Oocyte, Cryopreservation, Osmotic, Spindle, Blastocyst, Vitrification

This chapter has been accepted as a full paper in the journal *Cryobiology*. 
Introduction

Pigs are an important animal model for human physiology and pathology (Hau et al., 2004; Woodman et al., 2006). In some instances, they are a preferred model compared to other organisms (Turk and Laughlin, 2004). As with mice, transgenic technology is playing an increasingly important role in the development of genetically modified pigs (Prather et al., 2003) for diverse areas in biotechnology (Cozzi and White, 1995; Lai et al., 2002; Lindsay et al., 2004). Preservation of rare genotypes is important, but often costly (Critser and Russell, 2000). Germplasm cryopreservation can provide a cost-effective alternative to the maintenance of breeding colonies for the preservation of rare genotypes (Landel, 2005; Lyon, 1976) and can also provides a means to prevent the loss of such rare genotypes in the event of an unexpected catastrophe (Whittingham, 1974). The value and feasibility of genome resource banking for the preservation of rare genotypes has been recognized for many years (Whittingham et al., 1977). Having frozen oocyte banks could also improve research endeavors by providing freer access to oocytes and reduce the effects of seasonal variation in oocyte quality. In fact, it has been argued that “any improvements in the swine IVP (in vitro production) system would revolutionize not only the reproductive management of swine, but also increase the use of pigs for biotechnological and biomedical applications” (Wheeler et al., 2004).
Cryopreserving gametes and embryos from pigs has proven much more challenging compared to several other species (Bwanga, 1991; Dobrinsky 2002; Rojas et al., 2004). Porcine oocytes are very sensitive to cooling below physiologic temperatures (Liu et al., 2003; Yuge et al., 2003). The detrimental effects of cooling mammalian oocytes are manifested in many ways, including damage to the metaphase II spindle (Aman and Parks, 1994; Magistrini and Szöllösi, 1980; Zenzes et al., 2001), lipid phase changes in the oolemma (Arav et al., 1996; Ghetler et al., 2005) and developmental potential (Azambuja et al., 1998; Martino et al., 1996; Yuge et al., 2003). Because of the chilling sensitivity, rapid cooling methods have been the focus of attempts to preserve porcine oocytes (Fujihara et al., 2005; Nagashima et al., 1999); however, damage to the MII spindle and developmental potential remains a problem (Shi et al., 2006; Wu et al., 2006). In order to prevent intracellular ice formation at high cooling rates, a condition which is nearly always lethal (Mazur, 1977), high concentrations of permeable and non-permeable solutes need to be employed as cryoprotectants (Fahy et al., 1984). Exposing cells to solutions containing high concentrations of solutes can be damaging due to the direct chemical toxicity of these compounds and also as a result of the osmotic effects caused by the exposure. Fortunately, osmotic effects can be controlled by modifying the manner by which cells are exposed to the compounds. More specifically, high concentrations of permeable solutes can be loaded into cells in a stepwise manner, resulting in a reduced osmotic effect during each step (Paynter, 2005; Paynter et al., 2005). There are numerous reports in the literature describing the benefit of a stepwise approach.
to addition and/or removal of high concentrations of solutes compared to a single-step exposure (Bautista et al., 1998; Gao et al., 1995; Hamano et al., 1992; Nowshari et al., 1994; Otoi et al., 1998; Shaw et al., 1991). Thus, the optimal procedure for loading and unloading cryoprotectants will balance the chemical and osmotic effects by adding the cryoprotectants as quickly as possible (minimizing the chemical effects) but using a stepwise procedure to reduce the osmotic effects. Even though most reports on oocyte vitrification describe stepwise cryoprotectant (CPA) addition and removal procedures, the procedures employed are rarely designed to specifically take account of the osmotic tolerance and cell permeability characteristics of the cells. When these properties are taken into account, they can be utilized in a proactive manner to design theoretically optimal procedures for addition and removal of cryoprotectants (Fuller and Paynter, 2004). Such an approach was successfully demonstrated several years ago with human sperm by Gao and colleagues (Gao et al., 1995).

In order to use this approach, biophysical characteristics of cells need to be known. The current study was designed to characterize the relationship between cell volume and extracellular osmolality, as well as the osmotic tolerance of *in vitro* matured porcine oocytes as a first step toward developing optimal cryopreservation methods. Having this information will provide a foundation upon which CPA addition and removal procedures can be designed by setting osmotic
tolerance limits within which the cells should be maintained during CPA addition and removal.
Materials and Methods

Experimental Design

This study was designed to determine the effect of exposing mature porcine oocytes to solutions with varying osmolalities on: 1) the cell volume; 2) the morphology of the metaphase II (MII) spindle; and 3) the *in vitro* developmental ability as measured by *in vitro* fertilization and development to the blastocyst stage.

To determine the change in cell volume in response to the various solutions (see below for details of the solution compositions), photographs of the cells were taken after a 10 minute incubation period, and the total cell volume was estimated using computer image analysis techniques (Fovea Pro®, Reindeer Graphics, Asheville NC, USA, and Photoshop®, Adobe Systems, Inc. San Jose, CA, USA; see figure 1 for details). Only cells maintaining near spherical geometry were used for these calculations. The cell volume was expected to be a linear function of the inverse of the relative osmolality, and linear regression was used to make this assessment.

To determine the effect of the treatment on the morphology of the MII spindle, individual oocytes were randomly assigned to one of the osmotic treatments and incubated in these solutions for 10 minutes. The treatments were conducted at 38 ± 1 °C to avoid any confounding effects of sub-physiologic temperatures on
the MII spindle (Liu et al., 2003). The treatment solutions were prepared as described below and had osmolalities of 35, 75, 150, 225, 290, 700, 1100, 1500, 1900, 2300, 2700, and 3150 milliosmolal (mOsm). The cells were then returned to an isotonic solution for 10 minutes and allowed to recover their normal cell volume, and then put into culture medium (porcine maturation medium without gonadotropins (Fan and Sun, 2004)) in a culture incubator at 38.5 ± 0.5 °C with a 5 % CO₂ / air atmosphere for 1 hour prior to fixation. A complete randomized design was used for this experiment, with individual oocytes constituting the experimental units. The treatments were replicated within a day and also across several days. The oocyte morphology was scored as normal or abnormal based upon the spindle morphology in untreated porcine oocytes. The spindle of porcine oocytes is nearly spherical in shape, often with the pole-to-pole distance being slightly shorter than the distance across the metaphase plate. Spindles displaying any morphological differences to such a shape, including scattering of the chromosomes from the metaphase plate, were scored as abnormal. Logistic regression was used to analyze the data and determine the relationship between osmotic treatment and spindle morphology. Between 16 and 28 oocytes were assessed per treatment group (265 oocytes total). Approximately 10 % of the spindles were not able to be assessed due to the spindle being obscured by the intracellular lipids, which was merely a reflection of the orientation of the cell on the microscope slide.
To determine the effect of the treatment on the *in vitro* developmental potential, oocytes were randomly assigned to one of the osmotic treatments and incubated in these solutions for 10 minutes, also at 38 ± 1 °C. The treatment levels for this experiment were 75, 150, 225, 290, 460, 650, 880, 1410, and 2080 mOsm. The cells were then returned to an isotonic solution for 10 minutes and allowed to recover their normal cell volume, and then put into culture medium (porcine maturation medium II) in a culture incubator at 38.5 ± 0.5 °C with a 5 % CO₂ / air atmosphere for 1 hour. After this time, the cells were subjected to in vitro fertilization (IVF; see below for more details). A complete randomized design was used for this experiment, with groups of 10 to 25 oocytes constituting the experimental units (exact numbers depending upon the total number of acceptable quality oocytes on each day). The experiment was independently replicated on 7 days. Development was carried out to day 6 (the day of the experiment was day 0) and the proportion of oocytes undergoing development to blastocysts was determined. Analysis of variance with a Tukey correction for multiple comparisons was used to determine the relationship between osmotic treatment and developmental potential (Westfall *et al.*, 1999).

For all statistical tests, the α-value was chosen to be 0.05. Assignment of the experimental units to the treatments was conducted by randomizing the treatment order on each day with a random number table (Cochran and Cox, 1957). Statistical analyses were performed using the SAS system (Cary, NC USA).
Chemicals

Unless otherwise stated, the chemicals used in these experiments were purchased from Sigma-Aldrich (St. Louis, MO USA).

Source of Oocytes, *in vitro* Maturation (IVM), and *in vitro* Fertilization

Oocytes were purchased commercially (BOMED, Inc. Madison, WI, USA). The oocytes were shipped in a battery-powered and insulated incubator which held the oocytes at a temperature of 38.5 ± 0.5 °C during shipment to our laboratory on the day of collection from abattoir-derived ovaries. Immediately upon receipt, the tube containing the oocytes was transferred to a culture incubator which also was maintained at 38.5 ± 0.5 °C. Oocytes were shipped in maturation medium for 20 to 22 hours, and then transferred to maturation medium without gonadotropins in a culture incubator at 38.5 ± 0.5 °C with a 5 % CO₂ / air atmosphere for an additional 20 to 22 hours after reaching our laboratory. Each of these media was supplied by BOMED. After the final maturation time, the oocytes were incubated with approximately 500 µl of flushing and holding medium (FHM: Nagy *et al.*, 2003)) containing hyaluronidase at a final concentration of 1 mg/ml in a 1.5 ml Eppendorf tube. The cells were vortexed for 30 to 60 seconds to remove the cumulus cells and transferred through 3 dishes
containing ~ 2 ml of Hepes-buffered Tyrode Albumin Lactate Pyruvate (TALP-HEPES) medium (Bavister et al., 1983) containing 3 mg/ml of bovine serum albumin (Fraction V). The cells were inspected and any deteriorated oocytes or oocytes having undergone parthenogenetic activation (as assessed by cleavage) were discarded. The oocytes were then randomly assigned to the treatments. After the treatments and post-treatment holding periods, the oocytes were transferred to 50 µl drops of modified Tris-buffered medium (mTBM) and an equal volume of frozen and thawed porcine spermatozoa was added; final sperm concentration was ~ 0.5 x 10^6 total sperm per ml. The sperm and oocytes were allowed to incubate for 5 to 6 hours. After the IVF procedure, the oocytes were removed from the mTBM drop, washed through 3 volumes (~ 2 ml each) of TALP-HEPES, and transferred to North Carolina State University-23 (NCSU-23) medium for long-term culture in an incubator at 38.5 ± 0.5 °C with a 5 % CO₂ / air atmosphere. All oocyte manipulations were carried out in a laboratory room with an air temperature of 25 °C, and all manipulations were processed in a manner to maintain the cells at 38.5 ± 0.5 °C. The details of the maturation and fertilization procedures, including recipes for the solutions, can be found in (Fan and Sun, 2004).

**Treatment Solutions**

Dulbecco’s phosphate-buffered saline (DPBS) supplemented with glucose and pyruvate (Gibco # 14287-080) constituted the base isotonic solution (~290
mOsm). Hypotonic solutions were made by diluting the DPBS with embryo culture grade water. Hypertonic solutions were made by supplementing the DPBS with sucrose. The osmolality of the solutions was assessed using a vapor pressure osmometer (VAPRO 5520, Wescor Inc., Logan, UT, USA) and were made within ± 10 mOsm of the target osmolality. A 5 ml aliquot of these solutions was supplemented with bovine serum albumin (fraction V) at a final concentration of 1 mg/ml and sterile filtered before use. A volume of two ml of each solution was pipetted into a 35 mm Petri dish (Falcon 35-1008) and covered with sterile, embryo culture grade mineral oil, and held on a slide warmer to maintain the temperature at 38.5 ± 0.5 °C during the treatment.

**MII Spindle Staining and Analysis**

We have published our technique for immunostaining the MII spindle of mammalian oocytes in great detail (Mullen and Critser, 2004). Briefly, after the treatment and recovery period, the oocytes were fixed in cold methanol (-20 °C) for 5-6 minutes and held in a blocking buffer for at least 24 hours. A primary antibody (clone DM1-A) targeting alpha-tubulin was used by mixing the stock solution with blocking buffer at a final dilution of 1:9000. The cells were incubated in ~ 2 ml of this solution at 25 °C for ~ 60 minutes, or overnight at 4 °C. The cells were washed with ~3 ml of blocking buffer at 25 °C for ~ 60 minutes. The cells were then transferred to ~ 2 ml of blocking buffer containing the secondary antibody (donkey anti-mouse antibody conjugated with Texas Red®, purchased...
from Jackson Immunoresearch, West Grove, PA, USA) which was diluted by adding 5 µl to 7.5 ml of blocking buffer. This incubation also proceeded at 25 °C for ~ 60 minutes, or overnight at 4 °C. A final wash was conducted with ~ 3 ml of blocking buffer at 25 °C for 1-2 hours. Cells were then mounted on glass slides with ProLong antifade compound containing 1 microliter of SYTOX green DNA stain per ml of antifade. Each of these compounds was purchased from Molecular Probes (Invitrogen; Carlsbad, CA, USA). The antifade was allowed to set overnight. The spindle morphology was assessed using laser scanning confocal microscopy with a BIORAD 1024 confocal microscope equipped with a Krypton-Argon laser, at the University of Missouri’s Molecular Cytology Core Facility.
Results

Cell Volume and Osmolality Relationship

The volume of cells equilibrated in solutions with concentrations of 225, 290, 700, or 1100 mOsm was estimated as described above. The average volume of 31 oocytes held in the isotonic solution was $7.75 \pm 0.98 \times 10^5 \ \mu m^3$ (mean ± standard deviation). When the relationship between equilibrated cell volume and solution concentration was assessed, the cells were found to behave as ideal osmometers, with a linear change in volume as a function of the inverse of the relative osmolality (Figure 2; $R = 0.95$). In this instance, the linear relationship (usually referred to as a Boyle van’t Hoff relationship) is given by equation 1

$$Y = 0.824 X + 0.178$$  \hspace{1cm} [1]

Extrapolation of this relationship to infinite osmolality suggests that the osmotically-inactive cell volume ($V_b$) is 17.8 % of the isotonic volume (95 % confidence interval: 0.12 – 0.24).

MII Spindle Morphology

As the osmolality of the solution in which the oocytes were equilibrated diverged from isotonic, an increasing proportion of oocytes displayed an abnormal MII
spindle morphology (Fig. 3 and 4; P<0.05). The majority of oocytes were able to maintain a normal spindle structure when equilibrated in solutions ranging from 150 – 1900 mOsm (Fig. 4); concentrations beyond that range were more detrimental to the spindle structure. Two common anomalies in the spindle configuration were seen, as demonstrated by the images in panels E and F from Fig. 3. Reduced microtubule density with fibers projecting away from the chromosomes and into the cytoplasm was common (E). It was also common to see spindles which had an apparently normal density of microtubule fibers but an increase in the number of microtubule organizing centers, suggesting that the pericentriolar material was displaced from an organized focal point at the spindle poles. Apparently, the dislocation of the material did not affect its ability to act as a microtubule organizing center, as microtubules were focused at these different locations. Activation occurred to some oocytes (Panel D), and was more common in the oocytes having been exposed to extreme hypertonic solutions. Only 10 to 15 % of the oocytes were activated by treatments ranging from 35 to 2300 mOsm (except for oocytes exposed to 1500 mOsm, where about one third of them were activated). However, 2700 and 3150 mOsm solutions activated 26 and 63 % of the oocytes, respectively. Spindles with a telophase appearance were classified as abnormal spindles, as they deviated from the MII configuration.

**In vitro** Blastocyst Development
The success of the embryo production system used in the present study (ranging from 10 to 50 % blastocyst development across replicates for the control treatment) is consistent with other reports using a similar maturation and fertilization system (Sun et al., 2001); (reviewed in (Day, 2000)). Anisotonic exposure also affected oocyte developmental potential, as shown in Fig. 4. On average, 21% of the oocytes were able to develop to the blastocyst stage in culture after in vitro maturation without any anisotonic exposure. Treating oocytes with hypo- or hypertonic solutions caused about one-half of the oocytes to lose the ability to develop to blastocysts. However, as the level of hypotonic stress increased, the proportion of oocytes which lost the ability to develop to blastocysts in culture was not statistically different. While treatment with hypertonic solutions resulted in a reduction of the average developmental potential in an apparent concentration-dependent manner, the differences were not statistically significant.
Discussion

Developing effective methods to cryopreserve mammalian oocytes has proven very challenging, with those from domestic species, notably cattle and pigs, being especially sensitive to cryopreservation procedures. Many characteristics make mammalian oocytes particularly susceptible to cryoinjury, including the large amount of cytoplasmic lipids (in some taxa including swine (McEvoy et al., 2000; Szöllösi, 1972), large volume (Wassarman and Albertini, 1994), and the presence of the MII spindle (Simerly et al., 1995). Due to the inability of these cells to proliferate in culture like cells from standard cell lines, as well as the relatively small number of oocytes obtainable from a given individual, the proportion that survives cryopreservation is important. The present study was designed as a first step in characterizing the fundamental biophysical properties of porcine oocytes that are important for cryopreservation. We determined the isotonic cell volume and relative cell water volume, as well as the effect on the cells of exposure to anisotonic conditions. We have shown that the MII spindle structure can tolerate a fairly wide range of osmotic conditions. Developmental potential, on the other hand, was reduced by about 50% across all anisotonic conditions tested. This difference suggests that the MII spindle is less sensitive to osmotic stress in comparison to other cellular components in *in vitro* matured porcine oocytes.

Similar to other studies with mammalian oocytes (Newton *et al.*, 1999; Oda *et al.*, 1992; Ruffing *et al.*, 1993; Songsasen *et al.*, 2002), this study showed that
porcine oocytes act as ideal osmometers with a linear relationship between the cell volume and the inverse of the relative solution osmolality across the range of solutions tested. The estimate for $V_b$ in the present study (17.8 %) is similar to that for oocytes from other mammals (e.g. $V_b$ ranged from 19 to 25 % in the studies cited above).

The Boyle-van’t Hoff relationship can be used to relate the effects of solution osmolality on cell volume. The results from this study show that a high proportion (~ 80 %) of oocytes can maintain a normal spindle structure after exposure to solutions with osmolalities ranging from 150 to 1100 mOsm. The estimated cell volume range, as a proportion of the isotonic volume, is 1.77 to 0.4. On the contrary, developmental potential was reduced to about half that of the control oocytes in all of the solutions tested for that part of the study (75 to 2080 mOsm). The estimated cell volume range (relative to the isotonic volume) is 3.36 to 0.29. In these instances, the upper cell volume is an estimate based upon the Boyle van’t Hoff relationship. However, mammalian oocytes are confined within a glycoprotein matrix known as the zona pellucida. The zona restricts the amount of cell swelling; hydrostatic pressure will develop to compensate for the osmotic pressure difference. Using the same image analysis technique described in Figure 1, we determined that the cells could only swell to an average of ~ 1.54 times the isotonic volume in the 150 mOsm treatment solution, which is less than the expected volume using the Boyle-van’t Hoff estimation (~ 1.77). The discrepancy would be even greater at the 75 mOsm treatment level. This
restriction prevents the use of these data points to estimate the relationship between cell volume and solution osmolality. At hypertonic conditions > 1100 mOsm, aspherical shrinkage also restricted our ability to estimate the cell volume. However, when volume estimation techniques that do not rely upon cell shape have been applied to other cell types that do not have a restriction on cell swelling, a linear relationship between volume and osmolality has been shown to exist (Agca et al., 2004; Gao et al., 1998; Pegg et al., 1987; Woods et al., 2000), suggesting that it is acceptable to extrapolate beyond the osmotic range used to estimate the relationship between cell volume and solution osmolality in this study.

Previous investigations have established that mammalian oocytes from other taxa are also sensitive to anisotonic exposure. The osmotic tolerance of porcine oocytes as measured by the MII spindle structure was more similar to oocytes from cattle than humans (Mullen et al., 2004; Mullen et al., 2004), with a high proportion of porcine and bovine oocytes maintaining a normal spindle structure at osmolalities ranging from ~ 200 – 2000 mOsm. Given the unusually high incidence of aneuploidy in humans (Hassold and Hunt, 2001), it is not surprising that the spindle would be more sensitive to cellular perturbations in human oocytes compared to those from other taxa.

As in the present study with porcine oocytes, a detrimental effect of osmotic stress on the developmental potential of MII bovine oocytes has also been shown
(Agca et al., 2000). The results from these studies are qualitatively similar. In the study by Agca and colleagues (Agca et al., 2000), the developmental potential of bovine oocytes was reduced by approximately 50 % across the osmotic range used in the present study; however, a concentration effect was stronger with bovine oocytes in comparison to porcine oocytes. Anisotonic exposure has also been shown to negatively affect porcine spermatozoa and embryos. Boar sperm were determined to be very sensitive to anisotonic conditions, with 100 % motility loss beyond a range of 220 – 320 mOsm (Gilmore et al., 1998). An assessment of the osmotic tolerance of porcine blastocysts showed that both the morphology and cellular actin filament organization were disrupted by anisotonic exposure (Men et al., 2005). Morphological damage occurred in ~ 40 – 50 % of the blastocysts, and damage to the actin filaments occurred in ~ 60 to 70 % of the blastocysts at the extreme osmolalities (75 and 2400 mOsm).

Tolerance to anisotonic conditions is an important consideration for the development of cryopreservation procedures due to the near-universal requirement for cryoprotective compounds (Fuller, 2004). Permeating cryoprotectants (CPA), such as ethylene glycol (EG), dimethylsulfoxide (Me₂SO), and non-permeating CPA such as sucrose are typically used in combination and in multi-molar concentrations. Very high concentrations of CPA (~ 6-7 M) are typically needed to achieve vitrification (Fahy et al., 1984), which has become the standard approach for the cryopreservation of cattle and swine oocytes due to their sensitivity to prolonged exposure to cold temperatures (Martino et al., 1996).
It has been shown that just exposing porcine oocytes to a vitrification solution can be damaging. In one study (Shi et al., 2006), the proportion of oocytes with normal spindles was about 85% relative to untreated oocytes, whereas the proportion able to develop to blastocysts was reduced by about half compared to controls. These investigators used a 3-step CPA exposure procedure for their work, and the greatest degree of osmotic stress with their method would have been approximately equivalent to 1900 mOsm. On the contrary, a higher degree of spindle damage (~50% reduction compared to controls) and reduction of developmental potential (~15% of the control level) was reported by another group (Shi et al., 2006) after exposure to a different vitrification procedure. In this second report, the level of osmotic stress was higher (~3500 mOsm) due to greater CPA concentrations as well as the use of a 2-step addition procedure (each group used a 5-step dilution procedure). The results from our study reported here are consistent with the results from these previous reports, where intermediate levels of osmotic stress resulted in a high proportion of cells maintaining a normal spindle structure and about 50% developmental potential. The procedures used in the second report (Shi et al., 2006) exceeded the range of osmotic stress we imposed upon cells which underwent fertilization and development, which could explain the greater reduction in developmental potential than that seen in our study. Furthermore, a combination of osmotic stress and chemical toxicity of the cryoprotectants could account for the greater reduction in developmental potential seen in the report by Shi and colleagues (Shi et al., 2006).
Methods to cryopreserve oocytes have evolved to include a very short exposure to the final vitrification solution due to reports suggesting that oocytes can survive exposure to high solute concentrations for only brief periods of time (Bos-Mikich et al., 1995; Shaw et al., 1992). The results from such studies are usually interpreted to mean that the cells were damaged by the chemical toxicity of the solutions. In fact, results from such studies are often equivocal, as the chemical effects are confounded by osmotic effects. Several investigations into oocyte cryopreservation suggest that the osmotic effects may actually be more harmful than the chemical effects, as stepwise CPA addition and removal procedures are often better than a single-step exposure (Bautista et al., 1998; Kono et al., 1991; Shaw et al., 1991). This is fortunate because the osmotic effects can easily be controlled. Appropriate CPA addition and removal procedures can be designed a priori if the fundamental biophysical knowledge of the cells is taken into account. An illustrative example using human spermatozoa has been reported (Gao et al., 1995). This approach requires an understanding of the cell’s permeability to water and cryoprotectants, knowledge which is presently unavailable for porcine oocytes. Future studies will be directed at assessing the osmotic tolerance over a more narrow osmotic range near isotonic conditions to determine the range of osmolalities which a high proportion of porcine oocytes can tolerate, as well as determining the membrane permeability of porcine oocytes to water and permeable CPA. With this information, fundamental principles will be applied to
develop and test optimal methods to vitrify porcine oocytes in an effort to improve the survival of these important cell types.

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References Cited


matured oocytes to ambient temperature on in vitro fertilization and development of the oocytes. Cryobiology 47, 102-108.

Figure 5.1. The steps in the procedure used in image analysis are shown from left to right. The first panel shows an original photograph. A threshold process, with the oolemma intensity chosen as the threshold level, was applied to the original image, isolating pixels based upon their grayscale value. The result is shown in the second panel. The next process involved filling in areas that were completely surrounded by black pixels (panel 3). Finally, all objects smaller than a minimum size (chosen as 4000 pixels) were rejected from the image, leaving only the area originally occupied by the oocyte (panel 4). The radius of a circle containing an equivalent area was computed by the software, as shown. The volume of the oocyte was calculated using this value, assuming spherical geometry.
Figure 5.2. The relationship between the cell volume of *in vitro* matured porcine oocytes and the solution osmolality (Miso/M) is shown. Data points represent the mean ± SEM.
Figure 5.3. Various morphologies of MII spindles are shown. Normal spindle morphology is represented by the images in panels A and B, with near spherical shaped spindles and chromosomes aligned along the metaphase plate. Panel C shows a spindle which has chromosomes dislocated from the metaphase plate, with some located near one of the spindle poles. Panel D shows a spindle after oocyte activation, with the chromosomes in a late-anaphase configuration, being located at opposite sides of the spindle. The images in Panels E and F show abnormal spindle configurations. The spindle in Panel E was highly disrupted, with reduced microtubule density as well as displaced chromatin. The spindle in Panel F shows the development of multiple spindle poles after treatment, with chromosomes being displaced toward these multiple poles.
Figure 5.4. The proportions of oocytes having a normal spindle configuration (with exact 95% confidence intervals; left panel) and *in vitro* developmental potential (± SEM; right panel) after equilibration with solutions of various osmolalities are shown.
CHAPTER 6. HUMAN OOCYTE VITRIFICATION: THE
PERMEABILITY OF METAPHASE II OOCYTES TO
WATER AND ETHYLENE GLYCOL AND THE APPLIANCE
TOWARD VITRIFICATION

Abstract

OBJECTIVES: To determine the permeability of human metaphase II (MII) oocytes to ethylene glycol and water in the presence of ethylene glycol to use this information to develop a method to vitrify human oocytes.

DESIGN: An incomplete randomized block design was used for this study.

SETTING: A University-affiliated assisted reproductive center.

PATIENTS: Women undergoing assisted reproduction in the Center for Reproductive Medicine at Shandong University.

INTERVENTIONS: Oocytes were exposed to 1.0 molar ethylene glycol (EG) in a single step, and photographed during subsequent volume excursions.

MAIN OUTCOME MEASURES: A 2-parameter model was employed to estimate the permeability to water and EG.
RESULTS: Water permeability ranged from 0.15 to 1.17 μm/(min·atm), and EG mobility ranged from 6.58 × 10⁻¹⁷ to 1.18 × 10⁻¹⁵ moles/(μm²·min·atm) between 7 °C at 36 °C. The activation energy for water permeability and EG mobility were 14.42 Kcal/mol and 21.20 Kcal/mol respectively.

CONCLUSIONS: Despite the lower permeability of human MII oocytes to ethylene glycol compared to previously published values for propanediol and dimethylsulfoxide, methods to add and remove human oocytes with a vitrifiable concentration of EG can be designed which prevent excessive osmotic stress and minimize exposure to high concentrations of this compound.

Key Words: Vitrification, ethylene glycol, human, oocytes, permeability, computer modeling

This chapter is currently under consideration for publication as a full paper in the journal Fertility and Sterility.
Introduction

Cryopreservation of gametes and gonadal tissue offers a means to preserve fertility among individuals undergoing cancer therapy (ASRM Practice Committee, 2004; Sonmezer and Oktay, 2004). Oocyte cryopreservation may also obviate cryopreservation of preimplantation embryos – a practice which is forbidden in some locales – when ovarian stimulation procedures are utilized and result in the generation of supernumerary oocytes (Fugger, 1989; Benagiano and Gianaroli, 2004; Bankowski et al., 2005). Having effective means to cryopreserve oocytes would also facilitate the development of oocyte banks, making therapy for patients more effective by eliminating the need for cycle synchrony between donor and patient; it would also allow more effective screening of donors for transmittable diseases, as is currently practiced for sperm donors (Karow, 1997; Hornicek et al., 2002).

Progress in improving human oocyte cryopreservation has been made in recent years (recently reviewed in (Leibo, 2004; Smith and Silva, 2004; Stachecki and Cohen, 2004). Following successful reports in the mid-1990s (Gook et al., 1993; Gook et al., 1994; Gook et al., 1995; Kazem et al., 1995), an equilibrium freezing method using propylene glycol (PG) as the penetrating cryoprotective agent (CPA) has become the most utilized method to freeze human oocytes (Koutlaki et al., 2006). Fabbri and colleagues (Fabbri et al., 2001) demonstrated a significant improvement in survival when the concentration of sucrose in the
freezing medium was increased to 0.3 mol L⁻¹; reports of live births using this method or slight variations followed (Quintans et al., 2002; Boldt et al., 2003; Fosas et al., 2003; Borini et al., 2004; Chen et al., 2005). Despite these promising results, very recent reports, some using large cohorts of patients, suggest that the current methods are still sub-optimal (Borini et al., 2006; La Sala et al., 2006; Levi Setti et al., 2006). It continues to be argued that caution should be taken when applying oocyte cryopreservation in a therapeutic setting (ASRM Practice Committee, 2004; Stachecki and Cohen, 2004; Gosden, 2005). In recent years, vitrification (see (Shaw and Jones, 2003) for a definition of terms associated with vitrification procedures) has been applied to human oocyte cryopreservation as an alternative to equilibrium freezing (Yoon et al., 2003; Kuwayama et al., 2005; Kyono et al., 2005; Lucena et al., 2006; Antinori et al., 2007); and some of these outcomes have been very promising. With further progress in applying vitrification to human oocytes, this approach may eventually surpass equilibrium freezing as the method of choice (Kuleshova and Lopata, 2002). Vitrification in the context of reproductive medicine, and the associated challenges, were recently reviewed (Pegg, 2005).

Cryopreservation procedures constitute several steps, including CPA addition to and removal from cells, and cooling and warming. Vitrification methods are particularly challenging in regards to the application of CPA, as high concentrations of these compounds are needed to assure that a vitreous state is attained during cooling and maintained during warming (Fahy et al., 1984). When
developing a vitrification procedure, primary considerations include: (1) determining the appropriate solution to use; and (2) determining the manner in which it is to be used. Regarding the consideration of the appropriate solution, one method to reduce potential chemical toxicity is to replace permeating solutes with non-permeating solutes. However, the glass-forming, or vitrification properties of solutes are not identical, and the proportion of each type of solute in a solution affects the overall vitrification properties of a solution (Fahy et al., 1987). Another consideration regarding the appropriate solution is the total amount of solutes that will maintain a vitreous state during cooling and warming. When these considerations are taken together, designing a vitrification solution requires one to know the appropriate proportions of each solute in relation to the total concentration of all solutes necessary to prevent ice formation during cooling and warming. Regarding the second consideration mentioned above – the manner in which a solution is to be used – adding CPAs to cells and removing them from cells can be osmotically damaging. Fortunately, osmotic damage can be alleviated by using stepwise addition and removal procedures (Shaw et al., 1991; Fuku et al., 1995; Gao et al., 1995; Papis et al., 2000; Songsasen et al., 2002; Rojas et al., 2004).

Ethylene glycol (EG) is frequently used as a permeating CPA for vitrification of mammalian embryos and oocytes (Bautista and Kanagawa, 1998), particularly due to its lower toxicity compared to other CPAs (Fahy et al., 2004). It has been used in most studies with human oocytes, either alone (Yoon et al., 2000;
Kuwayama, 2007) or in conjunction with dimethylsulfoxide (DMSO) (Kyono et al., 2005; Lucena et al., 2006; Antinori et al., 2007). Quantitative permeability values of MII human oocytes to PG and DMSO have been determined (Paynter et al., 1999; Paynter et al., 2001). While quantitative permeability values for EG have been reported for oocytes from some mammals (Agca et al., 1998; Paynter et al., 1999; Songsasen et al., 2002), these values for human MII oocytes have only been investigated qualitatively (Coticchio et al., 1994). However, in order to design CPA addition and removal procedures in a rational manner, the quantitative membrane permeability values need to be known (cf. Fuller and Paynter, 2004 for a discussion of these concepts).

The present study was designed to address some of these gaps in our present knowledge. First, we determined the quantitative permeability of mature human oocytes to EG and water in the presence of EG at different temperatures. Second, we assessed the relationship between the amount of EG and sucrose in saline necessary to maintain an ice-free state when cooling to and warming from cryogenic temperatures. Finally, we used computer modeling to investigate vitrification methods based upon the experimental results from the present study.
Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO USA) unless otherwise stated.

Experiment 1: Oocyte Permeability to Ethylene Glycol

Oocyte collection

This study design was approved by the Shandong Provincial Hospital Institutional Review Board. Informed, written consent was obtained from all patients who donated oocytes to this study. The average age of the oocyte donors was 28 years (range: 22 – 33 years; n=19). Cumulus-oocyte complexes (COCs) derived from smaller follicles of the total follicle cohort were used in this study. Only oocytes having a normal appearance and a visible first polar body were used in this experiment. The ovarian stimulation protocol commenced with 150 IU human menopausal gonadotropin (HMG; lebaode, lizhu, China) by muscle injection from menstrual cycle day 5. Thirty-six hours after an injection of 10,000 IU human chorionic gonadotropin (hCG; lizhu, China), COCs were aspirated transvaginally. The COCs selected for the study were separated from the others and placed into 4-well dishes containing human tubal fluid medium (HTF; Irvine Scientific) + 10%
serum substitute supplement (SSS; Irvine Scientific) in a culture incubator at 37 °C with a 5% CO$_2$/air atmosphere. The cumulus cells were removed approximately 3-4 hours after collection using hyaluronidase (Sigma-Aldrich, St Louis MO USA; 80IU/ml) and gentle pipetting. Oocytes were returned to HTF after cumulus stripping. The oocytes remained in culture for no more than 5-6 hours prior to use in the experiment.

**Oocyte perfusion and image acquisition**

Measurements of oocyte permeability were conducted at different temperatures on an inverted microscope (Leica DMIRB, China). Perfusion of the oocytes was performed as previously described and validated in our laboratory (Gao *et al.*, 1994). Oocytes were placed in 20 μl drops of Hepes-HTF (Irvine Scientific) with 10% SSS in a petri dish and covered in mineral oil (Vitrolife; Englewood, CO, USA) to prevent evaporation. An initial photograph was taken of the oocyte in order to calculate the initial volume. Then, approximately 3.5 ml of the same medium supplemented with EG at a final concentration of 1.0 M was added to the dish. When the perfusate merged with the 20 μl drop of base medium, photographs of the oocytes were taken at regular intervals. The oocytes were held in place during the experiment using a glass holding pipette (Humagen Holding MPH-MED-30, I.D.-20μm, O.D.-95~120μm) and micromanipulators (#RI MODEL SAS11/2-E, Integra Research Instruments, UK). For temperatures lower than ambient, a 35mm Petri dish (Falcon 35-1008) was placed in an aluminum
chamber that was cooled with circulating liquid via a cooling bath (Fisher model 9109) and pumped through the aluminum chamber using polymer tubing. The bottom of the dish was wrapped in aluminum foil and a square hole was cut in the foil to facilitate viewing the oocyte. This was performed so the dish would fit snugly in the chamber to facilitate heat transfer from the chamber to the dish. The temperature in the cooling bath was set to either -2 °C or +7 °C for the 2 cold temperatures. The actual temperature of the media in the dish was warmer than this due to heat exchange of the fluid as it circulated through the tubing and chamber. For the third treatment, oocyte permeability was measured at ambient temperature (~25 °C) where the perfusion took place in a 50mm Petri dish (Falcon 35-1006) placed on the microscope stage with no heating or cooling. Finally, the warmest temperature was attained in a similar fashion but the temperature of the media was raised by using a microscope stage warmer set to 39 °C. Variations in temperature of the media occurred due to day-to-day fluctuations in temperature in the laboratory. However, the actual temperature of the media during the experiment was measured using a type-T (copper-constantan) thermocouple and an electronic thermometer (model 51 II, Fluke Corporation, Everett, WA USA). After the experimental run, the oocyte was released from the holding pipette on the micromanipulator and the temperature of the medium was measured such that the thermocouple was visible in the microscope field of view, ensuring that the temperature was recorded at the exact location of the oocyte. On several occasions the media was measured both during and after the experiment, and the initial and final temperatures did not
vary by more than 0.5 °C. The media was either pre-warmed or pre-cooled prior to perfusion, depending upon the experimental conditions. For the experiments using the warm stage, photographs were taken every 3 seconds for the duration of the oocyte volume excursion. For the experiments at ambient temperature, photos were taken every 5 seconds. For the experiments when the cooling bath was set to 7 °C, photographs were taken every 5 seconds initially, and after the oocyte reached a nadir in volume and began to swell, the time duration was changed to 30 seconds. For the coldest temperature, photos were taken every 10 seconds initially, and every 60 seconds during swelling. The exact time of these transitions was recorded during each experiment and was accounted for during the calculations to estimate the permeability parameters (please see below).

**Image analysis, parameter estimation, and cell dynamic modeling**

Only those oocytes remaining nearly spherical during the volume excursions were used to estimate the permeability parameters. Forty three of 72 oocytes fit this criterion (~60%). In several of the instances where the oocytes were rejected for analysis, the cells shrunk close to spherical, but folds in the membranes prevented accurate determinations of cell volume. Image analysis was performed using image analysis software (Fovea Pro®, Reindeer Graphics, Asheville NC, USA, and Photoshop®, Adobe Systems, Inc. San Jose, CA, USA). To determine the cell volume in each image, the following process was performed (see Fig 1).
Initially, the image was thresholded by grey scale to isolate the oolemma, as it is noticeably darker than the area in its immediate surroundings (Fig 1A and 1B). Imperfections in this step were manually corrected. The next step involved filling in areas completely surrounded by black pixels (Fig 1C). The next step involved isolating the oocyte from the remainder of the features in the image by deleting objects other than the oocyte (1D). Finally, the area of the oocyte was calculated by counting the number of black pixels, and the diameter of a circle with this equivalent area was calculated. All of these processes were performed by the software, and the software was calibrated with an image of a stage micrometer. Details of these processes can be found in (Russ, 2002). The volume of the oocyte was calculated from this diameter assuming spherical geometry.

For this study, we used a 2-parameter model to describe the cell dynamics (Kleinhans, 1998). We adopted the 2-parameter model (vs. the 3-parameter model) as it has been argued that this model is more parsimonious and many previous investigations into membrane permeability of cells including oocytes have shown that the interaction factor ($\sigma$) is insignificant (Agca et al., 1999; Paynter et al., 1999). This model uses a pair of coupled, linear ordinary differential equations to describe the change in cell water volume and moles of intracellular permeating solute (e.g. EG). Equation 1 describes the change in cell water volume ($V_w$) over time ($t$) as a function of the hydraulic conductivity ($L_p$), surface area ($A$), gas constant ($R: 0.082 \text{ L Atm mol}^{-1} \text{ K}^{-1}$), temperature ($T$, in K), intracellular permeating ($m^e_s$) and non-permeating ($m^e_n$) solute concentration in
osmoles, and the extracellular permeating and non-permeating solute concentration (osmoles of solute ($n^i_s$ and $n^i_n$ respectively) / cell water volume ($V_w$)):

\[
\frac{dV_w}{dt} = -L_p A R T \left( m^e_s + m^e_n - \frac{n^i_s}{V_w} - \frac{n^i_n}{V_w} \right) .
\]  

[1]

Equation 2 describes the change in intracellular moles of permeating solute ($n^i_s$) over time as a function of the permeating solute mobility ($\omega$):

\[
\frac{dn^i_s}{dt} = \omega A R T \left( m^e_s - \frac{n^i_s}{V_w} \right) .
\]  

[2]

All other terms are equivalent to those in equation 1.

The solute permeability ($P_s$) is the permeability term commonly reported in the literature. In equation 2, the terms $\omega A R T$ are equivalent to $P_s$ (Mazur, 1990). However, as $P_s$ is linearly dependent on the temperature, it is more appropriate to use the solute mobility when assessing the effect of temperature on the movement of a permeable solute through a membrane by the use of the Arrhenius relationship (Igor Katkov, personal communication). Therefore, we have assessed the permeability of EG in terms of $\omega$ for the initial experiment, and
have converted the value of $\omega$ to $P_s$ for further discussion to be consistent with units presented in previous investigations. A spreadsheet was created to estimate the permeability parameters using the Solver tool in Microsoft Excel® (Microsoft, Redmond WA, USA). Briefly, for each experimental run, the volume change data from the image analysis was imported into the spreadsheet and compared to the theoretical model of volume change data calculated from the above equations. The initial volume of cell water in the oocyte was determined by subtracting the osmotically inactive fraction of the cell volume (0.19x isotonic volume; from (Newton et al., 1999)) from the total cell volume estimated from the initial image of the oocyte. Similarly, the total cell volume from the model calculations was determined by summing the cell water volume calculated from equation 1, the volume of EG in the cell (calculated as the product of the partial molar volume of EG (0.056 L mol$^{-1}$) and the intracellular moles of EG from equation 2) and the osmotically inactive cell volume as described above. The solver tool in Excel® was used to estimate the permeability parameter values by minimizing the sum of squared errors between the experimental cell volume and the volume calculated from equations 1 and 2 for each run (Billo, 2001). Once these parameters have been estimated, they can be applied in equations 1 and 2 to calculate the change in volume and intracellular CPA concentration an oocyte will undergo for any CPA addition and removal procedure (Mazur, 2004).
The activation energy for \( L_p \) and \( \omega \) was determined assuming an Arrhenius relationship between the parameter and temperature (Mazur et al., 1984). This relationship can be described as in Equation 3 for \( L_p \):

\[
L_p = L_{p0} \cdot e^{-\frac{E_a}{R(T-T_0)}},
\]

where \( L_{p0} \) and \( T_0 \) are reference parameters (e.g. \( L_p \) at a specific temperature \( T_0 \)). When \( 1000/RT \) (on the abscissa) is plotted against \( \ln L_p \) (on the ordinate), the slope of the linear regression through these data gives the value for \( E_a \). In this instance, the appropriate value for \( R \) is 1.987 cal \text{ mol}^{-1} \text{ K}^{-1}. The equation describing \( E_a \) of \( \omega \) is similar, with \( \omega \) substituted for \( L_p \) in (3).

**Experimental Design and Statistical Analysis**

An incomplete randomized block design was used in this experiment (Zolman, 1993), the blocking factor being a patient. The order of the four temperatures on each day was randomized for each replicate using the random number generator in Excel®. Linear regression analysis was performed with the statistical analysis system (SAS®, Cary NC, USA).
Experiment 2: Assessing the Amount of EG Necessary to Achieve and Maintain a Vitreous State during Cooling and Warming for Different Concentrations of Sucrose in Saline

This experiment was designed to determine the amount of EG necessary to maintain an ice-free (i.e. vitreous) state during cooling to cryogenic temperatures (~ 160 °C; below the glass transition temperature of these solutions) and warming, when the sucrose concentration ranged from 0.1 to 1.1 molal (m; equivalent to 0.053 to 0.53 molar (M)). In studies of the physical properties of solutions, components of the solutions are usually measured by weight, not volume. A primary reason for this is because weight (in comparison to volume) does not change with temperature. Furthermore, concentrations are usually reported in weight fractions (weight percent (w/w, or wt %)). We will use these conventions here and make references to the equivalent molar concentrations when comparing the results to previously published studies.

A Diamond Differential Scanning Calorimeter (DSC; Perkin-Elmer Waltham, MA, USA) outfitted with an autosampler and CryoFill liquid nitrogen cooling system was employed for the thermal analysis. The solutions were composed of water (5 g) and NaCl (0.0045 g) to which various amounts of sucrose and EG were added. For this experiment, spectrophotometric grade (>99%) EG was utilized. All components were weighed on an analytical balance. Errors between the target and actual weight percent were less than 0.1 % in all instances. To test the
vitrification properties of the solutions, 5 μl (~ 0.0055 grams) was loaded into standard 10 μl aluminum DSC pans (Perkin-Elmer Part # BO14-3015), and the pans were hermetically sealed. Embryo-grade water and HPLC-grade (> 99.9% purity) cyclohexane were also run as calibration standards. Analyses were conducted at a cooling rate of 100 °C/min and a warming rate of 10 °C/min to be consistent with previous studies (Kuleshova et al., 1999; Baudot and Odagescu, 2004). In instances when crystallization and melting peaks were not clearly evident on the curves, plots of the heat flow as a function of temperature were analyzed by fitting a polynomial curve to the data and determining if a crystallization or melting peak was evident above the random noise of the signal. Random noise was estimated by determining the standard deviation of the actual signal from the expected value from the polynomial fit. When an apparent crystallization peak followed by a melting peak in the expected temperature range was noted, and the maximum point on the melting peak was beyond 3 standard deviations from the expected value, it was classified as a thermal event (Draper and Smith, 1966). Only when three independent solutions confirmed the absence of crystallization and melting was the solution classified as having achieved and maintained a vitreous state throughout the cooling and warming procedure.

**Experiment 3: Computer Modeling Toward an Optimal Vitrification Method for Human Oocytes**
Using the experimental data from the present study and osmotic tolerance data from our previous study (Mullen et al., 2004), we have investigated methods to vitrify human oocytes using computer modeling. Our goal in the present work was to develop a method which should ensure ice-free cryopreservation (i.e. achieve vitrification and preclude devitrification during cooling and warming) and prevent osmotic damage to the cell during the CPA addition and removal processes using an optimal combination of sucrose and EG.

The first step was to determine the appropriate composition of a vitrification solution using EG and sucrose as the cryoprotectants. There were two criteria used to make this determination: (1) the total solute concentration should be high enough to preclude ice formation during cooling and warming at rates applicable to devices used for cryopreservation currently in practice; and (2) the sucrose concentration should be as high as possible (high enough just to reach the oocyte osmotic tolerance threshold, which will allow the lowest amount of EG to be used, reducing the chemically-toxic properties of the solution).

It is known that the concentration of solutes necessary to achieve vitrification and avoid devitrification decreases with increasing cooling and warming rates (MacFarlane, 1986; MacFarlane, 1987). Therefore, it was necessary to account for this property when estimating the appropriate solution composition. Baudot and Odagescu (Baudot and Odagescu, 2004) analyzed cooling and warming rates necessary for vitrification of solutions containing EG. Their analysis
confirmed that warming rates are more critical than cooling rates when trying to maintain a vitreous state. Therefore, we focused on the warming rates necessary to avoid devitrification from this point forward (for a discussion on why this is the case, please see reviews on vitrification (Fahy et al., 1984; Pegg, 2005)). In their analysis, Baudot and Odagescu (Baudot and Odagescu, 2004) determined that a solution containing 50 wt % EG should maintain a vitreous state when the warming rate is on the order of $1 \times 10^3$ °C/min, and 48 wt % when the warming rate is on the order of $1 \times 10^4$ °C/min. It has previously been shown that 59 wt % EG is necessary to maintain a vitreous state during cooling and warming at the rates that we used in our DSC analysis (Kuleshova et al., 1999). Cooling and warming rates using standard ¼ cc straws and the so-called ultra-rapid cooling devices (e.g. cryotops, open-pulled straws) can achieve cooling and warming rates on the order of $1 \times 10^3$ and $1 \times 10^4$ °C/min, respectively (Rall et al., 1987; Kuwayama et al., 2005). Because the difference in wt % necessary to avoid devitrification with a warming rate of $\sim 1 \times 10^4$ °C/min compared to $1 \times 10^3$ °C/min is relatively minor (48 vs. 50 wt %), we focused on the lower warming rate, applicable to ¼ cc straws for the remainder of our analysis (please see below for a more complete discussion of this choice). This analysis suggests that the weight percent of solutes can be reduced by 15% (59 vs. 50 wt %) when the warming rates are increased from $\sim 1 \times 10^1$ to $\sim 1 \times 10^3$ °C/min. Therefore, for our investigation of an ideal vitrification solution, we focused on solutions across the range of sucrose molalities used in experiment 2, yet having a total solute composition reduced by 15 wt %.
For the next step, we calculated the degree to which solutions containing the various concentrations of EG and sucrose from the previous step will affect the equilibrium volume of a human oocyte. This was done by solving equations 1 and 2 using the appropriate values of the variables for each solution. For the modeling work reported here, we assumed that the oocyte would have properties of an average human oocyte, namely a cell radius of 63 μm, a permeability to water of 0.69 μm/min/atm, and a permeability to EG of 9.16 μm/min (average values at 25 °C from the present work).

From this analysis we could determine a solution that would have the optimal combination of EG and sucrose for vitrification, meaning that it would have the maximum amount of sucrose, and hence the minimum amount of EG, yet would not result in the oocyte exceeding the estimated osmotic tolerance. Osmotic tolerance data for human oocytes are relatively scarce compared to data for oocytes from other taxa (Agca et al., 2000; Adams et al., 2003). To our knowledge, our previous report on the osmotic tolerance of human oocytes using MII spindle morphology as an endpoint (Mullen et al., 2004) is the most comprehensive published data set. Therefore, we used this information as a guide for estimating the osmotic tolerance. In that study, we determined the relationship between osmolality and the morphology of the MII spindle as an experimental endpoint using a logistic regression analysis. For the present work, we used those data and calculated the 95 % confidence interval for the mean
using the logistic regression model (Hosmer and Lemeshow, 2000). We defined the osmotic tolerance as the range of osmolalities which 90% of oocytes should tolerate, and then estimated the corresponding cell volume utilizing the previously published Boyle van’t Hoff relationship for human oocytes (Newton et al., 1999). The results from this analysis suggest that 90% of human oocytes should tolerate changes in cell volume ranging from 57% to 154% of their isotonic volume. Thus, of the solutions analyzed, we chose the one which contained a combination of sucrose and EG such that the cell volume would be reduced to 57% of the isotonic volume upon equilibration.

Finally, after determining the optimal solution using these criteria, we proceeded to model EG addition and removal procedures which will keep the oocytes within the osmotic tolerance limits defined above. Cell dynamic modeling was performed using Mathematica® (Wolfram Research, Champaign IL, USA) with a program written by one of the authors (SFM).
Results

Experiment 1: Oocyte Permeability to Ethylene Glycol

When the oocytes were abruptly exposed to a solution containing 1.0 M EG, they responded osmotically, first by shrinking, then swelling, as expected. Furthermore, the degree of shrinkage and time for swelling was influenced by the temperature of the solution (Fig 2). This response is dictated by the permeability of the oolemma to both water and EG, as described by equations 1 and 2.

Figure 3 shows the relationship between temperature and the two permeability parameters for the oocytes in the present study. As expected, temperature and permeability were strongly correlated ($r = -0.97$ for both $L_p$ and $\omega$). The values of $L_p$ differed by an order of magnitude across the temperature range (from 0.15 μm/(min·atm) at 6.7 °C to 1.17 μm/(min·atm) at 35.7 °C). For $L_p$, the activation energy was 14.42 Kcal/mol (95% confidence interval: 13.19 to 15.65 Kcal/mol). The values of $\omega$ differed by two orders of magnitude across the temperature range in the present study (from $6.58 \times 10^{-17}$ moles/(μm²·min·atm) at 6.7 °C to $1.18 \times 10^{-15}$ moles/(μm²·min·atm) at 35.7 °C). The corresponding values of $P_s$ are 1.51 and 29.98 μm/min. For $\omega$, the activation energy was 21.20 Kcal/mol (95% confidence interval: 19.49 to 22.91 Kcal/mol). Linear regression allows the calculation of the expected value of these parameters at any temperature $T$ (in K). In this instance, $L_p$ can be calculated by solving the following equation:
Exp \[-14420 / (1.987 \, T) + 23.983\] ; \quad (4)

and \(\omega\) can be calculated by solving the following equation:

\[
\text{Exp} \left[ -21196 / (1.987 \, T) + 0.276 \right]. \quad (5)
\]

**Experiment 2: Assessing the Amount of EG Necessary to Achieve and Maintain a Vitreous State during Cooling and Warming for Different Concentrations of Sucrose**

Figure 4 shows examples of DSC thermograms during warming for solutions containing 0.3 m sucrose with varying total solute concentrations. The thermal transition on the far left of the curve, near -130 °C represents the glass transition. Two other transitions are evident as the solution warms and approaches -60 °C. The first change, when the heat flow decreases, represents the heat of fusion of water as ice crystallization occurs. Approximately 20 °C warmer, another transition occurs, as the ice crystals melt. It is evident that the magnitude of these later transitions becomes smaller as the total weight percent increases, until no evidence for crystallization or melting is apparent. In all of the solutions tested, there was no evidence for crystallization during cooling (data not shown). The
total concentration of the solution necessary to avoid crystallization during warming increased as the sucrose concentration increased from 0.1 to 1.1 m; solutions containing 0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 m sucrose required 59, 59, 59, 60, 61, and 61 wt %, respectively.

**Experiment 3: Computer Modeling Toward an Optimal Vitrification Method**

Having established the permeability of human oocytes to EG and water in the presence of EG in experiment 1 and the appropriate proportions of EG and sucrose to include in vitrification solutions in experiment 2, we analyzed the effect of solutions with the same proportions, yet with a total concentration reduced by 15 %, on the equilibrium volume of human oocytes. As expected, the volumes that human oocytes attained upon equilibration were reduced in direct proportion to the sucrose concentration (Figure 5). From this analysis it was determined that a solution containing 0.75 m (0.40 M) sucrose would dehydrate the cell just to the point of the osmotic tolerance threshold (57 % of the isosmotic volume).

According to the results from the second experiment, the concentration of EG necessary to maintain a vitreous state during cooling and warming in a solution containing 0.75 m sucrose is 12.49 m (6.72 M). Thus, we chose this combination as the optimal solution for further analysis.
We proceeded to determine an appropriate stepwise method to expose human oocytes to EG with this optimal solution as the final target, in preparation for vitrification. Sucrose is only necessary in the final vitrification solution; in fact, having sucrose in the initial solutions would be less efficient because it would cause osmotic shrinkage of the cell and reduce the amount of EG in the initial solutions to which oocytes can safely be exposed (safely in this context refers to preventing osmotic damage). Using equations 1 and 2 described above, and the osmotic tolerance limits discussed, we determined an optimal EG addition and removal procedure for human oocytes. The procedure requires a 4-step CPA addition (5 minutes for the first 3 steps) and a 2-step CPA removal. The details of this procedure can be found in Table 1.
Discussion

Equilibrium freezing (i.e. slow-cooling (Mazur, 1990)) is the predominant method in practice for human oocyte cryopreservation (Koutlaki et al., 2006). Despite approximately twenty years of effort, the results from this approach remain highly variable (cf. (Paynter, 2005)), and human oocyte cryopreservation is still considered an experimental procedure (ASRM Practice Committee, 2004). The relatively poor performance of equilibrium freezing has been highlighted in recently published large trials (e.g. (Borini et al., 2006; La Sala et al., 2006; Levi Setti et al., 2006)). In recent years, vitrification methods have been applied to cryopreserve human oocytes to determine if improvements can be made (Yoon et al., 2003; Kuwayama et al., 2005; Kyono et al., 2005; Lucena et al., 2006; Antinori et al., 2007). Some of these investigations have demonstrated remarkable success (Kuwayama et al., 2005), but, to date, too few reports on vitrification have been published to reach definitive conclusions.

Ethylene glycol permeability and its use for vitrification

Ethylene glycol is one of the primary permeating cryoprotectants used in vitrification methods, principally due to its relatively low toxicity compared to other compounds (Ali and Shelton, 1993). Because vitrification procedures necessitate the use of high solute concentrations, making toxic and osmotic damage more likely, it is somewhat surprising that the quantitative permeability values for EG
have yet to be determined. The results from the present study fill in this important gap in the human oocyte cryopreservation literature.

Concern about osmotic damage is particularly important when using EG, as mammalian oocytes generally have a lower permeability to EG relative to other permeating CPAs. For example, it has been shown that the oolemma in mouse oocytes has a lower permeability to EG relative to PG and DMSO (Paynter et al., 1997; Paynter et al., 1999; Pedro et al., 2005). In the study by Paynter et al., the average permeability to EG was 0.24 µm/s at 30 °C (Paynter et al., 1999). This value is very similar to the estimated average permeability of human oocytes at this temperature from the present study (0.27 µm/s). In contrast, the permeability of Rhesus macaque oocytes to EG at this temperature was reported to be much lower (0.14 µm s⁻¹; (Songsasen et al., 2002)). Although quantitative permeability values were not determined in the study by Pedro et al., of the 5 cryoprotectants tested, the volumetric response of the oocytes suggests that only glycerol had a permeability value lower than EG.

The results from the present study also show that MII human oocytes have a lower permeability to EG in comparison to PG and DMSO. At 24 °C, the average permeability values for the three CPAs are 8.2, 15.0, and 16.8 µm/min, respectively (Paynter et al., 1999; Paynter et al., 2001). The effect of this difference on the volume response of human oocytes to cryoprotectant solutions can be calculated using equations 1 and 2 from above. For example, a single-
step exposure to 1.5M PG, DMSO, and EG at 24 °C will result in the reduction of cell volume to approximately 67 %, 61 %, and 53 % of the isosmotic volume for the respective cryoprotectants. Although the difference in cell volume response suggests that EG may be an inferior permeating agent for cryopreservation of human oocytes, the volume changes associated with permeating cryoprotectants are only one of many important factors to consider when designing cryopreservation procedures. In fact, because volume changes can be modulated by changing the method used to expose the cells to such compounds, the inherent toxicity of the permeating cryoprotectant may be a more important consideration.

Osmotic stress, and the resulting cell volume excursions, is one of the primary theories of injury during cryopreservation (Meryman, 1970; Meryman, 1971). Previous studies have shown that mammalian oocytes are sensitive to solutions with high osmolalities. Agca et al. (Agca et al., 2000) documented the effects on bovine oocytes of exposures to anisosmotic solutions, and determined that exposure to concentrations of 1200 milliosmolal or greater (equivalent to a reduction in volume to ~ 40 % of the isotonic volume (determined from the analysis of (Ruffing et al., 1993))) reduced the potential to develop to blastocysts by ~50 % relative to untreated cells. Rhesus macaque oocytes suffered membrane damage after exposure to concentrations of EG at 3 M or higher using a single-step addition and removal (Songsasen et al., 2002), although it was not determined if this damage occurred specifically as a result of the volume
excursions, or from a chemical effect of the EG, or an interaction between these 2 factors. While it has been shown that human oocytes can tolerate exposure to fairly high concentrations of mono- and disaccharides (up to 1.5 M; equivalent to a reduction in volume to ~35 % of isotonic (Newton et al., 1999)) as measured by immediate viability using membrane integrity and enzyme activity (McWilliams et al., 1995), a reduced tolerance was reported as measured by MII spindle morphology (Mullen et al., 2004). To our knowledge, viability after osmotic stress, as measured by developmental potential, has not been reported for MII human oocytes.

Identifying optimal combinations of EG and sucrose for vitrification

When designing a vitrification procedure, one consideration is whether the cryoprotectant solution can form and maintain a stable glass during cooling and warming. Additionally, the lowest concentration of solutes necessary to meet this condition should be used to minimize the potential toxicity of the solution. However, a review of the literature suggests that these criteria are rarely used in an explicit manner when different vitrification methods have been tested. Because solutes have different physical properties, the relative amount of each solute in a solution will affect the ability of a solution to vitrify (Fahy et al., 1987). Ethylene glycol and sucrose are commonly used as permeating and non-permeating solutes for vitrification of mammalian oocytes, yet few investigations have been undertaken to determine the glass-forming properties of aqueous
solutions containing these solutes. Kuleshova and colleagues conducted a similar analysis to the one undertaken in this study, but with fewer concentrations of sucrose (0, 0.1, 0.5, and 1.0 m) (Kuleshova et al., 1999). The results from these two studies are similar. Kuleshova et al. determined that the solutions required 59, 60, 61, and 65 wt % at the respective sucrose concentrations to maintain a vitreous state during cooling and warming. In both studies, the total weight percent necessary to avoid crystallization increased as EG was replaced with sucrose. This is expected, as aqueous solutions containing only sucrose as the solute require higher concentrations (> 70 wt %; (Ablett et al., 1992)) to maintain a vitreous state during slow warming compared to solutions containing EG as the only solute (~ 59 wt %).

**Investigating methods for vitrification using computer modeling based upon fundamental principles**

Cellular damage can occur during any one of the steps in a cryopreservation procedure. Optimizing such a multi-step procedure through purely empirical means would require a very large experiment – the number of treatment combinations could easily be in the thousands. By using fundamental principles and computer modeling, estimates of optimal methods can be arrived at through rational analysis, with the potential to save a significant amount of resources by narrowing the choices to test via empirical methods. Such an approach is particularly appealing for human oocyte cryopreservation, where experimental
material is scarce, and experimental design is significantly influenced by ethical considerations. In the present study we have used such principles to make an initial prediction of an optimal vitrification method for human oocytes using EG and sucrose as cryoprotectants. We based these predictions on several criteria, including the necessity of attaining a stable vitreous state during cooling and warming, using the minimum amount of permeating cryoprotectant, and designing the method to reduce the potential for osmotic damage to the cell.

The reasons for moving away from equilibrium freezing methods and toward the use of vitrification include reducing cell damage associated with chilling injury and ice formation. If the later is a true goal, then one should use a solution that maintains a stable vitreous state during cooling and warming. However, using solutions with higher concentrations of solutes than is necessary to maintain a vitreous state exposes cells to unnecessary risks of chemical and osmotic damage. In our modeling, we used the measured vitrification properties of solutions from the second experiment as a guide for determining the appropriate concentration of the various solutes. If solutions are used that are not true vitrification solutions, the degree to which ice forms and the size of the resulting crystals are difficult to control. Having such little control over an important variable such as ice formation is likely to add to the variability of a method (Shaw et al., 1992).
Vitrification methods for mammalian oocytes have evolved toward the use of devices to achieve so-called “ultra-rapid cooling” following their application with bovine oocytes (Martino et al., 1996; Vajta et al., 1998). Because of the results from these and similar studies, an implicit assumption seems to have developed in the field of human oocyte cryopreservation that successful vitrification requires cooling rates which can only be attained with such devices. In fact, most of the reports on human oocyte vitrification have used such devices, including open-pulled straws (Kuleshova et al., 1999), electron microscope grids (Yoon et al., 2003), cryoloops (Liebermann and Tucker, 2002) and cryotops (Kuwayama et al., 2005; Kyono et al., 2005; Lucena et al., 2006; Antinori et al., 2007). However, this assumption may not be valid. Firstly, the use of different devices in an experimental comparison introduces potentially confounding variables other than cooling and warming rates (e.g. the time for which the cells are exposed to the vitrification solution prior to plunging into liquid nitrogen). Secondly, in particular reference to human oocytes, very high cooling rates may not be necessary because previous studies have suggested that human oocytes are more tolerant to chilling than bovine oocytes (Bernard et al., 1992). One possible reason for this is the relatively low level of intracellular lipids in human oocytes compared to oocytes from other domestic animals (e.g. cattle and swine), a property which makes these oocytes chilling sensitive (Nagashima et al., 1994).

Current vitrification methods have also evolved to rely upon a very brief exposure to the final vitrification solution, and it is often assumed that the damage from
longer exposures is due to chemical toxicity of the solutes. However, such data are equivocal, as the chemical effect is completely confounded by the osmotic effect. Several reports with mammalian oocytes have shown that cryopreservation outcomes can be improved if the osmotic effect of exposure to the solutions is modulated by prolonged CPA addition and/or removal (Hamano et al., 1992; Wood et al., 1993; Nowshari et al., 1994; Rayos et al., 1994; Bautista et al., 1998; Otoi et al., 1998) suggesting that consideration of the osmotic effects are at least as important as the chemical effects.

We focused our modeling on the use of a solution that could maintain a stable vitreous state during cooling and warming when using a standard ¼ cc freezing straw for several reasons. First, there are years of experience in the clinical IVF community with the use of these devices for cryopreservation of other samples. The use of some of the ultra-rapid cooling devices has been reported to be cumbersome, potentially increasing the likelihood of damage to the oocytes during their use, losing oocytes during handling, and requiring extensive technical training periods (Antinori et al., 2007). In addition, unlike many of the other devices used, standard freezing straws can be safely sealed, thus avoiding the potential for contamination through direct contact with liquid nitrogen (Bielanski et al., 2003; Rall, 2003). We acknowledge that, more recently, some of the ultra-rapid cooling devices have been modified to be fully sealed systems and should alleviate this problem; however, to our knowledge no reports of the use of these devices for human oocytes have been published. Furthermore, the design
of some of these sealed systems makes it likely that the cooling and warming rates will not be as fast as the open systems. Although seemingly paradoxical, the larger thermal mass of a ¼ cc straw may have benefits. It is well known that devitrification and recrystallization are serious risks when vitrification solutions are used that are near the threshold of thermodynamic stability (Pegg, 2005). For a device with a low thermal mass, accidental warming, and the concomitant risk of devitrification, is higher than a device with a higher thermal mass. Finally, it is often argued (frequently without supporting evidence) that the higher cooling rates attainable with ultra-rapid cooling devices allow a significant reduction in the concentration of cryoprotectant necessary to maintain stable vitrification (up to 30 % according to one recent report (Vajta and Nagy, 2006)). However, the analysis by Baudot and Odagescu (2004), based upon measured physical properties of solutions, suggests that the actual reduction is quite small (~ 2 %), at least for EG, given the difference in cooling rates between standard freezing straws and the other devices.

Since the work by Martino et al. in 1996 (Martino et al., 1996), few investigations of bovine oocyte vitrification using standard freezing straws have been published, presumably because of the assumption stated above. However, even for bovine oocytes the necessity of ultra-rapid cooling has been successfully challenged. Otoi and colleagues attempted to refine methods for bovine oocyte vitrification using ¼ cc straws to determine if success rates comparable to those achieved by ultra-rapid cooling methods could be attained (Otoi et al., 1998). The best results
they achieved rivaled those originally achieved with EM grids (Martino et al., 1996) and open-pulled straws (Vajta et al., 1998). The method by which Otoi and colleagues were able to achieve their success is strikingly similar to what we have proposed to be an optimal method based upon computer modeling. Otoi et al. achieved the best results using a solution they called ES40. This solution contains EG at a concentration of 7.15 M whereas our proposed optimal solution contains EG at 6.72 M. The sucrose concentration in ES40 is 0.35 M whereas in our solution the sucrose concentration is 0.4 M. Otoi et al. were also able to achieve a significant improvement in survival by changing the method for CPA addition to include a prolonged addition procedure (3 steps total vs. 1 or 2 steps). This is very similar to the conclusions from our analysis. Overall, the results by Otoi et al. support our argument that osmotic stress is a significant concern when vitrifying mammalian oocytes yet can be overcome by changing the CPA addition and removal procedures.

To our knowledge, the report by Kuwayama and colleagues is the only publication where an experiment was conducted to test different vitrification methods directly on human oocytes (Kuwayama et al., 2005). These investigators tested 2 solutions with the cryotop method, one contained 6.8 M EG and the other contained 5.0 M EG. Each solution also contained sucrose at 1.0 M. The proportion of oocytes that showed normal morphology and blastocyst formation was higher in the solution containing the lower concentration of EG. Our proposed optimal solution contains EG at 6.72 M, which at first suggests that
it will perform poorly compared to a solution with a lower concentration. However, our solution also contains considerably less sucrose (0.4 M), and this, coupled with our proposed method for CPA addition and removal, should reduce the damaging osmotic effects associated with its use compared to the method used in the study by Kuwayama et al (Kuwayama et al., 2005).

There are several aspects of our modeling that may influence the predictive accuracy. We have focused much of the model restrictions on the estimates of osmotic tolerance based upon MII spindle morphology. We recognize that the developmental potential of human oocytes may be a more sensitive indicator of osmotic tolerance than spindle morphology, as has been shown with bovine oocytes (Agca et al., 2000; Mullen et al., 2004). However, to date, for human oocytes this is the best estimate of osmotic tolerance we have. In addition, our previous experiment on the effect of osmotic stress on human oocytes was conducted in the absence of intracellular cryoprotectants which may have an effect on the spindle microtubules, and hence the estimates of osmotic tolerance (Vincent and Johnson, 1992). One of the assumptions in our model is that achieving and maintaining a true vitreous state is a prerequisite for a robust procedure. It is known that cells can survive freezing in the presence of ice (both extracellular and intracellular). Therefore, solutions with lower solute concentrations than the one we propose may allow successful cryopreservation, despite not being able to vitrify. However, the tolerance of cells to ice formation (particularly intracellular ice formation) is not well understood. Furthermore,
controlling ice formation and growth is difficult. Therefore, we believe that, if true vitrification can be achieved and the potential damage from the solution used to achieve vitrification can be managed, elimination of ice during cooling and warming is preferable.

The present modeling has focused on a vitrification method using a single permeating cryoprotectant (EG). As was stated above, EG is generally less toxic than other compounds. Bautista and colleagues showed that mouse oocyte developmental potential was reduced by only 30% after exposure to 7 M EG in a 2-step manner (Bautista et al., 1998). Hotamisligil and colleagues showed that mouse oocytes could tolerate 8 M EG fairly well if the exposure time was less than 1 minute (mean blastocyst development rate of ~ 50% vs. ~ 62% for controls), and exposure to 6 M EG for 5 minutes had no effect on development compared to untreated oocytes (Hotamisligil et al., 1996). Martino et al. showed a significant reduction in bovine oocyte developmental potential when the cells were exposed to 4 or 5.5 M EG in a single step (Martino et al., 1996). However, because the exposure was conducted in a one-step manner, osmotic effects could account for much of the damage. Our procedure would expose human oocytes to less than 4 M EG throughout the EG loading steps up until transfer to the vitrification solution. The oocytes should reach near equilibrium with the EG after exposure to the final solution within 12 seconds. Thus, the cells needn’t be exposed to the final solution for long before transfer to liquid nitrogen.
In summary, the present research was conducted to examine fundamental cryobiological properties of human oocytes and vitrification solutions in a first step to optimize vitrification methods. The results from our analysis suggest that successful vitrification in standard freezing straws can be achieved, as the resulting theoretically-optimized method is similar to one shown to improve vitrification with bovine oocytes. Future work should be directed at testing this method with other methods currently in practice and further research into the fundamental cryobiological properties of human oocytes is warranted.

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References Cited


Kuleshova LL, MacFarlane DR, Trounson AO and Shaw JM (1999) Sugars exert a major influence on the vitrification properties of ethylene glycol-based solutions and have low toxicity to embryos and oocytes. Cryobiology 38, 119-130.


Meryman HT (1970) The exceeding of a minimum tolerable cell volume in hypertonic suspensions as a cause of freezing injury. In Wolstenholme, GEW and O’Connor, M (eds) The exceeding of a minimum tolerable cell volume in


Table 6.1a. The solution parameters for the proposed 4-step addition of EG to MII human oocytes and intracellular EG concentration values are shown. Each of the first 3 steps should proceed for 5 minutes at 25 °C.

<table>
<thead>
<tr>
<th>Solution</th>
<th>EG addition step</th>
<th>Hepes-HTF mass (g)</th>
<th>Water supplement mass (g)</th>
<th>EG mass (g)</th>
<th>Sucrose mass (g)</th>
<th>Final intracellular EG concentration (molarity/molality)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>58.3</td>
<td>41.7</td>
<td>9.2562</td>
<td>0</td>
<td>0.96 / 1.34</td>
<td></td>
</tr>
<tr>
<td>Solution 2</td>
<td>58.3</td>
<td>41.7</td>
<td>23.4490</td>
<td>0</td>
<td>2.23 / 3.54</td>
<td></td>
</tr>
<tr>
<td>Solution 3</td>
<td>58.3</td>
<td>41.7</td>
<td>47.8236</td>
<td>0</td>
<td>3.90 / 7.36</td>
<td></td>
</tr>
<tr>
<td>Solution 4</td>
<td>58.3</td>
<td>41.7</td>
<td>77.1349</td>
<td>25.5227</td>
<td>5.02 / 12.5 b</td>
<td></td>
</tr>
</tbody>
</table>

* A lower concentration of non-permeating components (i.e. salts) was utilized as a means to allow an increase in the total concentration of EG exposure during each step while keeping the cells within their osmotic tolerance limits. Such a reduced concentration of non-permeating solutes has been shown to be tolerated by human MII oocytes (Mullen et al., 2004a).

b Values after exposure for 12 seconds, after which the straw could be plunged into liquid nitrogen.
Table 6.1b. The solution parameters for the CPA removal steps are shown. Step 1 should proceed for 4 minutes at 25 °C.

<table>
<thead>
<tr>
<th>EG removal step</th>
<th>Hepes- HTF mass (g)</th>
<th>Sucrose mass (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>100</td>
<td>50.8316</td>
</tr>
<tr>
<td>Solution 2</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 6.1. The steps in the procedure used for image analysis are shown. Panel A shows an original photograph. Notice that the oolemma is darker than its surroundings. A threshold process was applied to the original image, isolating pixels based upon their grayscale value. The result is shown in Panel B. The next process involved filling in areas that are completely surrounded by black pixels (Panel C). Finally, all objects smaller than a minimum size (chosen as 3000 pixels) and those touching an edge of the image were rejected, leaving only the oocyte (Panel D). The area of this object was determined by summing the total number of pixels and multiplying this value by the area per pixel. From this area, the total volume of the oocyte was determined assuming spherical geometry.
Figure 6.2. Examples of the volume changes that representative oocytes experienced during equilibration with 1.0M EG at 4 different temperatures are shown. Notice that lower temperatures are associated with a greater total volume loss and a slower return to isotonic volume.
Figure 6.3. Arrenhius plots of the relationship between temperature and $L_p$ (Panel A) and $\omega$ (Panel B) is shown along with the linear regression lines.
Figure 6.4. Examples of DSC thermograms during warming for solutions containing 0.3 molal sucrose, EG and saline, with a total solute concentration ranging from 55 to 59 wt %. The primary thermal transitions, including the glass transition, crystallization, and melting are noted on the top graph. Notice how the magnitude of the crystallization and melting transitions diminish as the concentration increases from 55 to 59 wt %.
Figure 6.5. This plot demonstrates the effect on the relative cell volume of human oocytes of vitrification solutions containing varying compositions of EG and sucrose in saline (black circles). As the sucrose concentration is increased, the amount of EG in the solution necessary to achieve and maintain a vitreous state during cooling and warming decreases. However, as the sucrose concentration increases, a greater effect on the cell volume will occur. For our choice of an optimal solution, we wanted a combination of EG and sucrose such that the solution would have the maximum amount of sucrose (reducing the EG concentration) yet would not result in excessive cell shrinkage. The osmotic tolerance we chose for this analysis would result in the cell shrinking to 57% of its isosmotic volume (horizontal line). Therefore, the optimal solution will occur where the curve intersects the horizontal line. This occurs when the sucrose concentration is 0.75 molal (0.40 M). The corresponding EG concentration is 12.5 molal (6.72 M).
CHAPTER 7. GENERAL DISCUSSION AND FUTURE DIRECTIONS

The research described in this dissertation was conducted in order to gain further insight into the fundamental cryobiological properties of metaphase II (MII) oocytes from humans, cows, and pigs. When this work began, the state of knowledge on the cryobiology of mammalian oocytes varied. Little was known about porcine oocyte cryobiology; significantly more work had been done with oocytes from cows and humans.

As described throughout the earlier chapters of this dissertation, cryopreservation procedures consist of several steps, including cryoprotectant addition, cooling, storage, warming, and cryoprotectant removal. Each of these steps can be conducted in many ways. For example, cooling rates can range from less than 1°C/min to tens, hundreds, or even thousands of degrees per minute. Cryoprotectants can be of several classes (e.g. those which can freely permeate the cell or those which are unable to cross a lipid bilayer; both are typically used) and of several specific types. Cryoprotectants can be added to and removed from cells in many ways, including in a single-step or multiple steps, with equal or unequal concentration differences across steps. The same can be said for cryoprotectant removal. When one considers all of the possible permutations with these steps, the number of possible cryopreservation methods that can be
applied becomes overwhelming; therefore, an important question is: which method should be employed for a given cell type?

This question is important because cell damage can occur at any step throughout this process, and successful methods require all sources of damage to be minimized. One commonly-used approach to determine a combination of parameters to test is to simply try a method that has been employed for other cell types. Frequently, the results from such an approach are “good enough” for the needs of the end-user. However, it is also common that such an approach leads to failure, and in this situation the causes for the failure are usually obscure. A different approach involves investigating the effects of the different stresses involved in cryopreservation on cells, and then developing and testing methods which take this knowledge into account. The second approach described above can be considered an investigational methodology termed fundamental cryobiology. It is this methodology which formed the philosophical foundation for the choice of studies which are described in this dissertation.

The early investigations described in this dissertation focused on the sensitivity of the MII spindle to osmotic perturbation. The spindle was a primary focal point because the spindle structure has been shown to be more easily disrupted from cryopreservation processes. As discussed, spindle disruption and malfunction of normal chromosome segregation can result in the creation of aneuploid embryos, leading to developmental failure in most cases. Furthermore, the spindle in
mouse oocytes is better able to undergo repair compared to oocytes from other taxa, providing a potential mechanism to explain why mouse oocytes are more tolerant to cryopreservation compared to oocytes from cattle, pigs, and humans.

Osmotic tolerance was a focal point for several reasons: (1) MII oocyte cryopreservation has evolved toward the use of vitrification methods due to the chilling sensitivity of oocytes from several species including pigs and cows (Martino et al., 1996; Azambuja et al., 1998; Yuge et al., 2003), and osmotic stress is a particular concern when using vitrification due to the high solute concentrations typically employed (Fahy et al., 1984; MacFarlane, 1987); (2) osmotic stress is considered one of the primary mechanisms by which cells are damaged during cryopreservation (Meryman, 1971); and (3) osmotic stress can be modulated by altering the cryoprotectant addition and removal procedures (Gao et al., 1995).

The results from these studies showed that osmotic stress does result in spindle disruption, with the fraction of affected oocytes being proportional to the level of that stress. A comparison of the results of the osmotic tolerance across the three taxa suggests that the MII spindle in human oocytes is more sensitive to hyperosmotic stress compared to the oocytes from cows or pigs (Figure 1). Nearly half of the human oocytes showed an abnormal spindle structure after the 600 and 1200 milliosmolal (mOsm) treatments, whereas a higher proportion of oocytes from cows and pigs were normal. In fact, the pattern of spindle disruption was very similar for oocytes
Figure 7.1. The proportions of oocytes from humans, cows, and pigs that maintained a normal MII spindle after treatment with solutions of various osmolalities are shown.
from cows and pigs, at least up ~ 2400 mOsm. Human oocytes had an intermediate tolerance for hyposmotic stress, however.

In the study with porcine oocytes (Chapter 5), the effect of osmotic stress on the potential to develop to blastocysts *in vitro* was also investigated. The comparison of the results from the spindle assessment and the development assessment suggests that the developmental failure can not be attributed solely to spindle damage, as loss of developmental potential occurred to approximately half (on average) of all of the oocytes across anisosmotic treatments. Considering osmotic stress has the potential to affect numerous cellular processes (Parsegiian *et al.*, 1995; Kultz and Burg, 1998; Dmitrieva *et al.*, 2001), this result is not surprising. The pattern of osmotic damage to the developmental potential of porcine oocytes shown by our study is similar to that previously shown for bovine oocytes (Agca *et al.*, 2000) (Figure 2).

In the discussion in Chapter 4, an example of the application of the osmotic tolerance data was presented. In this example, it was shown how a modification to the cryoprotectant removal procedure in comparison to the procedure originally published in the report by Martino *et al.* could be applied (Martino *et al.*, 1996). Using computer modeling and the osmotic tolerance data from our report, we showed how a modified method for cryoprotectant removal could be applied
Figure 7.2. The proportions of blastocysts derived from porcine (data from Chapter 5) and bovine (Data from (Agca et al., 2000)) oocytes equilibrated with solutions containing nonpermeating solutes at the indicated concentrations are shown.
which would result in a more rapid return of the cells to isosmotic volume yet keep the cells from exceeding their osmotic tolerance range. In order to use computer modeling in the manner in which we have demonstrated in Chapter 4 and Chapter 6, several biophysical cell parameters must be known, including the osmotically-inactive water volume and membrane permeability to cryoprotectants. Before we initiated the study described in Chapter 5, none of these values for porcine oocytes were known. In that study we determined the osmotic tolerance for \textit{in vitro} matured porcine oocytes, as well as the osmotically-inactive cell volume, which can be used to calculate the isosmotic water volume, as initial data on porcine oocytes that can be used for further investigations into porcine oocyte cryobiology. Future studies will be designed to further characterize the fundamental cryobiology of these cell types, including measurements of the osmotic tolerance in a narrower range of osmolalities near 290 mOsm, and oolemma permeability characteristics to ethylene glycol and water in the presence of EG. After conducting these characterization studies, predictive modeling will be employed to develop and test a theoretically-optimal approach for porcine oocyte vitrification as we described for the human oocyte in Chapter 6.

Before we initiated the study described in Chapter 6, the quantitative permeability values for MII human oocytes to EG were not known. We chose to pursue the research described in Chapter 6 because EG has been shown to have relatively
low chemically-toxic properties compared to other permeating cryoprotectants. The toxic effect seen with mouse oocytes after exposure to propylene glycol in an unpublished study in our laboratory (cf. Figure 1.8) was identical to that also seen with human oocytes in our collaborative project with the Atlanta Center for Reproductive Medicine (unpublished data). It was these similarities that lead us to shift our focus from using propylene glycol to ethylene glycol in future work with human oocytes.

As our long-term goal is to develop an efficacious vitrification method for human oocytes, and we prefer to develop cryopreservation methods via a fundamental approach, we felt it necessary to conduct the experiment described in Chapter 6. As an additional component of the work designed toward developing efficacious vitrification procedures, we also conducted a study to evaluate the vitrification properties of solutions containing EG and sucrose. Determining the EG permeability allowed us to move forward with computer modeling as the osmotically-inactive volume for human oocytes had been determined in a previous study (Newton et al., 1999) and our earlier study provided an estimate of the osmotic tolerance of MII human oocytes. Our predicted optimal vitrification method described in Chapter 6 was very similar to an optimal method determined in a previous study using bovine oocytes (Otoi et al., 1998). We are currently in the process of determining the feasibility of developing clinical trials to assess the success of this method with our collaborators at the Reproductive Medical Center of Shandong University.
Despite the progress on mammalian oocyte cryobiology described in the studies presented in this dissertation, numerous outstanding questions remain. As we showed directly in the work on porcine oocytes in Chapter 5, the loss of developmental potential occurs to a higher proportion of oocytes compared to the loss of the MII spindle structure. While strictly from an applied perspective, one could simply accept that some type of damage occurs and use the more sensitive measure of osmotic tolerance for improving cryopreservation methods. However, a more basic study designed to understand the nature of the osmotic damage could be more meaningful. Having such fundamental information may provide insight into methods that may counter the effects of the stress, allowing an improvement in the ability to cryopreserve oocytes. The same could be said for any of the other damaging mechanisms associated with attempts at cryopreservation.

An additional interest would be to determine the tolerable level of permeating cryoprotectants to oocytes. As described in the introductory chapter, several studies have investigated the effects of exposing oocytes to permeating cryoprotectants with the goal of determining the effects on viability. Unfortunately, it is rare that these studies are designed to control for osmotic effects. As just described, we have shown that propylene glycol at a relatively low concentration (relative to vitrifiable concentrations) is toxic to mouse oocytes, and this is due to a chemical effect ad not an osmotic effect. Having such knowledge will provide a
basis for further improvement in vitrification methods. Our initial attempt at developing an optimal vitrification procedure via computer modeling was based upon the use of a solution with a single permeating solute. Having a better understanding of the chemically-toxic concentrations of cryoprotectants will allow improved vitrification procedures to be designed by keeping the concentration of the compounds below their toxic levels. If it is determined that oocytes can not tolerate a level of a single permeating solute necessary to maintain a vitreous state during cooling and warming, solutions containing multiple permeating solutes, each at a lower concentration may need to be employed. Models do exist to describe the osmotic behavior of cells to solutions containing multiple permeating solutes (Katkov, 2000). Applying a multi-solute osmotic model should allow cryoprotectant addition and removal procedures to be designed as we have shown for solutions containing a single permeating solute in Chapter 6.

Some of the properties of oocytes (e.g. large size, spherical shape) make them good model cell types for further basic studies in fundamental cryobiology. Some simplifying assumptions are usually inherent in the physical-chemical modeling utilized in cryobiology studies, such as assuming that the solutions (extra- and intracellular are ideal and dilute (Dick, 1979). However, we know that this assumption may introduce substantial errors (Elliott et al., 2007). A practical example in the field of cryobiology which could have a significant implication in the design of optimal cryopreservation procedures is in the determination of the vitrification properties of the cytoplasm. Due to the high macromolecule
concentration in cells, the concentration of permeating cryoprotectant within the
cytoplasm necessary to achieve and maintain a vitreous state should be lower
that the concentration necessary in a typical vitrification solution (e.g. one without
substantial macromolecular supplementation; cf. (Fahy, MacFarlane, Angell and
Meryman, 1984)). To make progress in improving cryobiological models, our
laboratory has been investigating the physical properties (e.g. melting
temperature, devitrification temperature) of oocyte cytoplasm in the presence of
permeating cryoprotectants using techniques such as cryomicroscopy and micro-
scale calorimetry. Results from these studies should provide a more accurate
representation of the properties of cytoplasm, and may also be used as a model
for other cell types.

One of the limitations of the proposed method for human oocyte vitrification
described in Chapter 6 is that it is based upon a model which uses population
average values for the cell parameters such as the membrane permeability to EG
and water, and osmotically-inactive cell volume. Creating more complex models
to account for the population variation of these parameters may allow even better
methods for human oocyte vitrification to be developed because the models
should be able to predict the proportion of oocytes in the population that will
survive a given procedure, thereby allowing a choice of methods which will
predict a high proportion of oocyte survival.
Despite all of the progress that has taken place in the field of oocyte cryopreservation during the past few decades, the current status of success suggests that there remains room for improvement, particularly for human, bovine, and porcine oocytes (cf. Chapter 1, Table 2). It could be argued that the demand for efficacious oocyte cryopreservation methods is higher now than ever before. The number of assisted reproduction cycles initiated per year continues to increase (Figure 3). In proportion to the total population size, the number of assisted reproduction cycles initiated in 2001 in Europe was approximately twice that in the US (414 cycles vs. 829 cycles per million of population; (Gleicher et al., 2006). In some countries, legal restrictions preclude embryo cryopreservation, which necessitates the cryopreservation of supernumerary oocytes (Benagiano and Gianaroli, 2004). If the recent literature is a good indication, the numbers of oocytes being cryopreserved in Italy has increased substantially over the past 4 years (Borini et al., 2004; Bianchi et al., 2005; Chamayou et al., 2006; Levi Setti et al., 2006; Bianchi et al., 2007; De Santis et al., 2007). As discussed throughout this dissertation, human oocyte cryopreservation remains highly variable and an efficacious method remains to be developed. Oocyte cryopreservation from other domestic animals is also important, as described, as one means to preserve rare genotypes, and also as a potential means to increase the efficiency of basic research. Despite the struggles over the past decades to develop effective means to cryopreserve mammalian oocytes, the work completed as part of this dissertation should contribute to the attainment of this goal.
Figure 7.3. The total number of assisted reproduction cycles initiated in the United States in each of the indicated years is presented. These data were compiled by the US Centers for Disease Control and Prevention and obtained from their website (www.cdc.gov/ART/index.htm).
References Cited


Bianchi V, Coticchio G, Distratis V, Di Giusto N, Flamigni C and Borini A (2007) Differential sucrose concentration during dehydration (0.2 mol/l) and rehydration (0.3 mol/l) increases the implantation rate of frozen human oocytes. Reprod Biomed Online 14, 64-71.


aspiration on meiotic competence of porcine oocytes and of exposing in vitro matured oocytes to ambient temperature on in vitro fertilization and development of the oocytes. Cryobiology 47, 102-108.
Steven Francis Mullen was born on 18 July 1968 in St. Louis Park, MN, and grew up in Maple Grove, MN. He graduated from Osseo High School in 1986, and the University of Minnesota (B.S.) in 1995.

Steve has always had a keen interest in the natural world. He spent many days during his youth in the fields and forests near Madelia, MN on the farm where his mother was raised. During this time, his father and other relatives introduced him to small game hunting, a pastime for which he is still passionate. It was during these years that he developed an appreciation for the power of human society to shape the natural world, and this appreciation guided him to make the choices that he has made.

When it came time to determine the path he would choose toward achieving a graduate education, he decided to follow his heart. As a younger man, Steve seriously considered the field of conservation biology. During his time as an undergraduate, he became fascinated with cellular and molecular biology. Because of these areas of interest, he decided to investigate areas of science for his career which had the potential to combine cell biology with aspects of conservation biology, particularly wildlife conservation. During this time he came
across a text co-edited by Dr. John K. Critser titled *Reproductive Tissue Banking*. Within this book was a chapter written by David Wildt of the Smithsonian’s National Zoological Gardens. This chapter described the application of reproductive science to endangered species conservation, with a particular emphasis on the potential of genome resource banking. This field appeared to be exactly what Steve was looking for. After discussing his interests with Dave Wildt on the phone, and additional consultation with William Rall of the National Institutes of Health, it was concluded that Professor Critser was the best candidate as a dissertation advisor. After mustering up the courage to discuss his interests with John, Steve was offered a position in his laboratory to begin pursuit of a Ph.D. degree.