A. SPECIFIC AIDS.

In light of the benefit that the rat model system has provided to our basic understanding of the biology of human disease, and the importance of the rat to emerging fields of genomics and stem cell biology, the NIH initiated the Rat Genome Program and the Rat Genome Database. The NIH sponsored meeting on Rat Model Priorities recommended that a National Rat Genetic Resource Center be established to support research on germ-line modifications in the rat, including ear-marked funding for the study of rat sperm cryopreservation to alleviate the storage and transportation problems, and in vitro fertilization techniques in the rat. Unfortunately, the current state of the art in rat sperm cryopreservation is abysmal. An electronic search of the literature reveals only two papers published in the last 40 years that seeks to optimize the cryopreservation of rat sperm [1, 2]. This work establishes the feasibility of rat sperm cryopreservation but makes no attempt to optimize the technology, understand the damage caused to the sperm by cryopreservation, or to characterize the cryopreservation process in this species.

In this proposal, we will attempt to achieve three very important goals as it relates to sperm cryopreservation in general and rat sperm cryopreservation in particular. First, we will attempt to optimize rat sperm cryopreservation by optimizing the freezing rate based on careful characterization of the water transport properties of the sperm during freezing. Second, we will attempt to optimize the cryoprotective medium in which sperm are cryopreserved and to establish optimal sperm handling techniques prior to freezing. And third, we will determine the damage done to the sperm, at the molecular and structural level, caused by cryopreservation process, and design and test methods to minimize this damage based on our understanding of the mechanism. These goals are reflected in the following 3 specific aims:

Specific Aim 1: Determine the handling methods and CPA media most effective for rat sperm cryopreservation.
Specific Aim 2: Determine the biophysical water transport properties of rat sperm plasma membrane during freezing.
Specific Aim 3: Design and test strategies for protecting rat sperm from damage incurred by cryopreservation.

B. BACKGROUND AND SIGNIFICANCE.

INTRODUCTION. The first cryopreservation of mammalian sperm was reported from human sperm by Polge et al. in 1949 [3]. Since that time, cryopreservation has been used for preservation of sperm from many species [4]. Given the successes with sperm cryopreservation there has developed an impression that the problem of male gamete cryopreservation has been solved. This is certainly not the case. In many species of mammals, sperm cryopreservation remains a very difficult problem, with minimal success at cryopreserving sperm that recover the specialized function required for fertilization in vitro, much less the level of function required in the female reproductive tract. Sperm from important agricultural species such as the boar remain difficult to cryopreserve to a level useful to those working with this species [5, 6]. Even cryopreservation of human sperm is quite variable, with sperm donors selected on the basis of the ability of their sperm to survive cryopreservation. Mouse sperm is also difficult to cryopreserve, with great variation in cryopreservation survival depending on the strain of mouse [7]. The reality of the situation is that sperm cryopreservation remains a challenge to cryobiologists and true progress in this field is being hindered by a lack of understanding of the molecular and structural damage caused by the cryopreservation process.

The situation with rat sperm is even worse. A PubMed search of the literature reveals only two papers published in the last 40 years that achieve any success in the cryopreservation of rat sperm [1, 2]. These papers obtained live pups, albeit in small liters, from cryopreserved rat sperm by interuterine insemination. This work establishes the feasibility of rat sperm cryopreservation but falls far short of optimizing the technology, and it does not attempt to understand the damage caused to the sperm by cryopreservation or to characterize the cryopreservation process in this species.

In this proposal, we are attempting to achieve three very important goals in sperm cryopreservation. First, we will attempt to optimize the rat sperm cryopreservation by optimizing the freezing rate, based on our characterization of the water transport properties of the sperm during freezing. Second, we will try to optimize the methods by which rat sperm are treated during preparation for freezing, including optimizing the cryoprotective media. Finally, we will determine the damage done to the sperm, at the molecular and structural
level, caused by cryopreservation process, and design and test methods to minimize this damage based on our understanding of the mechanism.

Once the merit of this approach has been proven under the support of an R21 mechanism we will propose to optimize and standardize assisted reproductive approaches to creating offspring from cryopreserved rat sperm under an R01 mechanism.

IMPORTANCE OF RAT SPERM CRYOPRESERVATION. Although the mouse has led the way as the leading mammalian experimental model for genetic research, due primarily to the ability to manipulate the genome of this animal, the rat is now being recognized as a second model system. As a model, the rat presents key advantages over the mouse (for a summary see the report on the NIH Rat Model Workshop at <www.nhlbi.nih.gov/meetings/model>). Many of these advantages relate to the larger body size. For instance, the rat has been used extensively for transplant research and microdialysis. The rat is also the established model for toxicology studies, neurobehavioral studies and is a convenient model for hypertension. In addition to these classical areas of research, the importance of the rat to emerging fields of genomics and stem cell biology is now being recognized. In light of the benefit that the rat model system has provided to our basic understanding of the biology of human disease, the NIH initiated the Rat Genome Program in 1995 to completely map the rat genome followed by the Rat EST Program in 1997. All of this data will contribute to a Rat Genome Database (see <rgd.mcw.edu>).

In May of 1999, the NIH sponsored the meeting on Rat Model Priorities where issues concerning support of the rat as an experimental model were discussed. Recommendations of the meeting attendees, according to the published report of the meeting (<www.nhlbi.nih.gov/resources/docs/ratmtg.htm>), included establishment of a National Rat Genetic Resource Center and support for research on germ-line modifications in the rat, including ear-marked funding for the study of sperm cryopreservation, to alleviate the storage and transportation problems, and in vitro fertilization techniques in the rat. However, despite these recommendations and the imminent emergence of the rat as a genetic model of disease, no research on rat sperm cryopreservation is currently funded by the NIH (<crisp.cit.nih.gov>). The goals of this application go straight to this critical need for research on rat sperm cryopreservation.

SPERM PREPARATION: OPTIMAL HANDLING OF RAT SPERM. In preparation for cryopreservation, rat sperm must endure several manipulations, all of which can potentially be damaging to these cells. Sperm must be isolated from the epididymis, they must be equilibrated with an optimal cryopreservation media and they must be brought to a low suprzero temperature prior to freezing. Optimal sperm cryopreservation requires that sperm sensitivity to several factors be determined and conditions subsequently optimized. These factors include media components for sperm manipulation in vitro, sensitivity of the sperm to manipulations such as centrifugation, sensitivity to cryoprotective agents at concentrations required for cryo-protection, sensitivity to cooling to low supra-zero temperatures encountered during preparation for freezing, and sensitivity to osmotic excursions caused by the addition and removal of hypertonic CPA solutions to and from the sperm. These sensitivities are in most cases empirically determined, as are the steps taken to minimize that damage incurred by each of these factors.

For rat sperm, very little is known or reported concerning all of the above variables in the cryopreservation process. Some of what has been learned about cryopreservation of sperm in species may potentially apply to rat sperm but rat sperm are most likely to have unique sensitivities given that there are tremendous species to species differences in what sperm can tolerate relative to handling and cryopreservation. For instance, human sperm and equine sperm tolerate centrifugation while mouse and rat sperm quite sensitive to damage caused by centrifugation. Equine sperm are extremely sensitive to cold shock, the damage caused by cooling sperm too rapidly to ice temperatures, whereas mouse and human sperm are reasonably resistant [8, 9]. There are also sperm-specific sensitivities to osmotic excursions caused by the supra-physiological osmolalities common to most cryoprotective media. Given the nearly complete lack of specific information on the tolerance of rat sperm to various media and manipulations, this application will attempt to elucidate the optimal handling methods and CPA characteristics for rat sperm.

CRYOPRESERVATION MEDIA: CYTOPROTECTION AND TOXICITY. Successful cryopreservation of cells usually involves the use of a medium containing one or more cryoprotective agents (CPAs). Although the exact mechanism by which CPAs protect cells has not been conclusively demonstrated, several mechanisms have been proposed including increasing the unfrozen fraction of the medium or protection of the cell membrane.
during the freeze-thaw process [10, 11]. While there is no question that CPAs are beneficial to the cryopreservation process, they can also damage the cells directly by intrinsic toxicity or by inducing osmotic excursions when added to the cells. For instance, glycerol, a very common permeating CPA is notoriously toxic to sperm [12]. And since all CPA solutions are hyperosmotic, exposure to these media can cause large cellular volume changes which are damaging to sperm cells. It has been shown in mouse sperm cells that >90% motility is maintained if the osmotic volume excursions are kept within 90% and 103% of the normal isotonic volume of the cell during shrinkage (upon addition of the permeating cryoprotectant, glycerol) and swelling (upon its removal), respectively [13]. Several reasons for the osmotic injury have been proposed, including a) mechanical rupture of the cell membrane in hyposmotic solutions (i.e. expansion lysis); b) frictional force between water and potential membrane 'pores' causing cell membrane damage [14]; c) resistance to cell shrinkage by cytoskeleton components [15, 16]); d) cell shrinkage causing irreversible membrane fusion changes, causing the cells to lyse when their normal volume is recovered after they return to isotonic conditions [17]; and e) hyperosmotic stress caused by leakage/influx of non-permeating solutes [18]). In summary, avoiding excessive variations in sperm volume is essential to reduce osmotic injury and obtain higher survival rates [13, 19]. Likewise, the shorter the time of exposure to the toxic compounds in CPA solutions, the higher the survival of the cells.

**BIOPHYSICAL EVENTS IN CRYOPRESERVATION: FUNDAMENTAL CRYOBIOLOGY.** All cells share two fundamental cryobiological responses to freezing; namely, dehydration and intracellular ice formation (IIF). An understanding of these two responses can be exploited to better understand and alleviate the specific problems of freezing in sperm [20]. When ice forms in a cellular suspension, electrolytes and proteins from the solution are excluded from the ice and create a highly solute-concentrated unfrozen liquid fraction. The cells, which remain unfrozen when ice first forms within the extracellular space, respond to the increased concentration of solutes in the unfrozen fraction by either dehydrating (transporting water out into the unfrozen liquid fraction), or by the formation of intracellular ice in the cytoplasm. Dehydration is the osmotic response of the cell to re-equilibrate the cytoplasmic concentration of solutes with the higher concentration in the extracellular unfrozen fraction. IIF occurs when the supercooling (temperature below the equilibrium phase change temperature) within the cytoplasm is sufficiently large to drive the nucleation and growth of an ice crystal within the cytoplasm of the cell. Both of these biophysical responses to freezing can damage the cell. Dehydration causes an increase in concentration of solutes both inside and outside of the cells and damages the cell by solute effects injury [21]. Intracellular ice formation is generally considered lethal if > 10-15% of the cellular water is involved [22]. These two biophysical processes compete during any cooling procedure to dominate the freezing history. The highest rates of cellular survival are typically found for cooling rates which are fast enough to minimize dehydration solute effects injury while still slow enough to preclude large amounts of intracellular ice and is a strategy used in the cryopreservation of cells in the presence or absence of cryoprotectants [23].

Water transport (dehydration) and IIF can be experimentally observed in most cell systems using a cryomicroscope to watch the cells as they freeze. Observations of cell dehydration and intracellular ice formation can then be fit to mathematical models which are used to predict the cell's response to freezing under arbitrary cooling conditions. This information, in turn, helps to develop better cryopreservation protocols. Although both water transport and IIF have been observed and modeled in many cell types during freezing, the small size of a sperm cell prevents either of these events from being observed in sperm using a cryomicroscope. Water transport can be experimentally determined in sperm cells using Differential Scanning Calorimetry (DSC), a technique pioneered in Dr. Bischof's laboratory and described in this proposal (see Preliminary Data). Once the water transport properties are known, it is possible to make extrapolations to the minimum amount of water that will be trapped as intracellular ice within a sperm cell. Thus, the biophysical portion of this proposal (SA 2) will be concerned with the experimental determination and mathematical modeling of water transport in rat sperm.

**POTENTIAL TARGETS OF CRYOINJURY: SPECIALIZED SPERM FUNCTION.** Sperm are endowed with several specialized structures and functions that are required for successful capacitation and fertilization. These include essential signaling cascades involved in the proper timing and progression through capacitation, including the controlled storage and release of intracellular calcium, the production of cyclic AMP and subsequent protein tyrosine phosphorylation events. It appears that the maintenance and proper
rearrangement of lipid microdomains in the sperm plasma membrane is also important for proper function in the female tract. And the sperm must maintain the integrity and fusibility of the acrosomal vesicle.

All of the specialized structures and functions of sperm are potential targets of damage during the cryopreservation process. It has been shown in several species that phosphorylation events caused by the cryopreservation process appear to mimic those observed during normal sperm capacitation [24-26]. This has given rise to the concept of cryo-induced capacitation which may cause the sperm to prematurely acrosome react, thus destroying their fertilization potential in the female reproductive tract. We are proposing experiments to determine if rat sperm undergo cryo-capacitation as a result of the cryopreservation process and to determine if the cryoprotective media can be altered in such a way that cryo-capacitation is prevented. We have recently shown that Crisp-1, an epididymal secreted protein, can suppress capacitation in vitro [27]. This protein may act to suppress cryo-capacitation if added to the cryopreservation medium. Likewise, eliminating calcium or bicarbonate ion from the cryopreservation medium may suppress cryo-capacitation given that these ions are required for the capacitation cascade. Finally, an exogenous source of cholesterol in the cryopreservation media may also block capacitation by blocking cholesterol efflux from the sperm membrane. All of these potential strategies will be employed in this application in an attempt to minimize the premature capacitation caused by the freezing process.

It has been shown in several cellular systems, as well as sperm, that the plasma membrane has lipid microdomains, or rafts, that harbor and segregate proteins [28, 29]. We have shown in rat sperm that these domains are disrupted by removing cholesterol from the membrane, suggesting that these microdomains are important for regulating sperm functions such as capacitation (see Preliminary Data). Perturbation of the plasma membrane by removal of cholesterol, a lipid known to stabilize these microdomains, leads to changes in molecular associations that result in the activation of signaling pathways. We have shown, using staining techniques for the glycosphingolipid GM1 present in lipid rafts, that these microdomains are disrupted by freezing (see Preliminary Data). We are proposing experiments to investigate the possibility that the cryopreservation process interrupts the organization of the sperm plasma membrane microdomains and testing methods to prevent such damage.

Freezing is known to cause a variable degree of damage to cell membranes which could effect the ability of sperm to normally sequester or take up Ca++. Given that Ca++ is an essential ion for normal sperm function, the inability to properly store and release Ca++ may be a cause of sperm dysfunction caused by cryopreservation. Likewise, cAMP is a critical second messenger in the sperm capacitation process and may play a role in sperm motility [30, 31]. The bicarbonate-sensitive soluble adenylate cyclase is the source of cAMP generation in sperm. Freezing damage to cells may include effects on membranes that alter the levels of ions such as bicarbonate, leading to insufficient bicarbonate levels. Finally, damage to the acrosome that results in the inability of the sperm to bind to and penetrate the zona pellucida will render the sperm unable to fertilize. In this application, we propose to determine the cAMP levels in cryopreserved rat sperm and determine if a potential point of sperm dysfunction post-thaw exists. We will also investigate direct damage to the acrosome caused by the cryopreservation process.

**Assisted Reproduction in the Rat.** Very few laboratories routinely perform in vitro fertilization in the rat. While it is relatively straight forward to obtain superovulation in the female rat, fertilization of these oocytes in vitro and embryo culture are not well established procedures. Studies using IVF to create rat embryos report that special conditions are required for fertilization and embryo culture is hindered by developmental blocks [32, 33]. Consequently, reports of rat pups created via IVF are scarce (we could find no such reports in the literature). There has been two reports of live births of rat pups from intracytoplasmic sperm injection (ICSI) in the rat [34, 35]. In these reports the embryos are cultured only to the two cell stage before transfer and one 1-3% of the fertilized oocytes resulted in live births. Clearly, much work remains to be done in area of rat assisted reproduction. Development of assisted reproductive technologies in the rat need to come together with advances in cryopreservation to facilitate consistent recovery of precious rat strains from frozen sperm. **Thus, once the principles of cryopreservation have been shown in the work proposed in this R21, we propose to pursue IVF and embryo culture in the rat with R01 support to facilitate reliable reconstitution of transgenic rat lines from frozen sperm using IVF.**
C. PRELIMINARY DATA.

Differential Scanning Calorimetry (DSC): We propose to use differential scanning calorimetry (DSC) to study the biophysics of water transport in rat sperm. The DSC is an instrument that continuously measures heat releases associated with phase change as a function of both time and temperature at controlled cooling rates. The DSC has been used in our laboratory to study water transport in a variety of biological systems including lymphocytes, rat liver tissue, Dunning AT-1 rat prostate tumor tissue, and liver tissue from the freeze-tolerant wood frog *Rana sylvatica* [36-40]. We have also used this technique to successfully measure the water transport properties of the plasma membrane in human, mouse and horse sperm [39, 41, 42]. The DSC technique is independent of the size and shape of the cells being observed and is therefore ideally suited to measure water transport out of sperm cells.

The technique is based on measuring the difference in integrated heat release between an *initial* freezing of intact cells or tissue (Curve A in Fig. 1) and a *final* heat release (Curve B in Fig. 1) from freezing of the lysed cells or tissue. This value, $\Delta q_{\text{tot}}$, has been shown to be proportional to the volume of osmotically active cell water in the sample prior to freezing. In addition, the temperature dependence of the integrated heat release difference $\Delta q(T)$ normalized by the total heat $\Delta q_{\text{tot}}$ has been shown to be proportional to the normalized amount of cell water which has left the cells down to a temperature $T$. This allows sperm cell volume to be calculated using the DSC measured heat releases shown in Fig. 1. The relationship of the cell volume with respect to temperature can be expressed as:

$$V(T) = V_0 - \left(\frac{\Delta q(T)}{\Delta q_{\text{tot}}}\right)(V_0 - V_b) \tag{1}$$

where $V_0$ is the initial cell volume and $V_b$ is the osmotically inactive cell volume or the end volume of the cell after water transport shuts off. Both of these volumes ($V_0$ and $V_b$) can be determined using a variety of techniques such as light microscopy, Coulter counter, electro-paramagnetic resonance (EPR) and electron spin resonance (ESR) [13, 43, 44]. Assuming that the sperm cell can be approximated to be a cylinder with a radius of 0.46 µm and length of 122 µm the isotonic cell volume (for mouse sperm), $V_0$, is calculated to be 81 µm$^3$ [43, 45]. The osmotically inactive cell volume, $V_b$, has been reported to equal 0.61$V_0$ [13]. Using these two values for $V_0$ and $V_b$ the DSC measured heat releases were translated to volumetric data at two different cooling rates of 5 and 20 °C per minute as shown in Figure 2 (for the case of mouse sperm cells in low CPA media). The experimentally determined water transport data obtained using the DSC technique can then be used to estimate the biophysical parameters of water transport, as has been described by our lab and others [46-48]. Determination of these biophysical parameters is discussed in the next section.

**Water Transport Modeling:** Kedem and Katchalsky proposed a model for water and solute transport in response to chemical potential gradients based on irreversible thermodynamics [49]. The Kedem and Katchalsky (KK) model consisted of two differential
equations which describe the water and CPA flux across the membrane. If the flux of CPA is negligible in comparison to the water flux, then the Kedem-Katchalsky model reduces to a model which assumes only water transport [50, 51]. Such a model was proposed by Mazur and later modified by Levin et al. [52, 53]. The various assumptions made in the development of Mazur’s model of water transport are discussed elsewhere [54, 55]. The water transport model of Mazur was further modified by Karlsson et al. [56, 57] to incorporate the presence of CPAs on the volumetric shrinkage response of cells during freezing and is given as,

\[
d\frac{V}{dT} = -\frac{L_p A_c RT}{B v_w} \left[ \ln \left( \frac{V_o - V_b - n_{cpa} v_{cpa}}{V_o - V_b - n_{cpa} v_{cpa}} + (\varphi_s n_s + n_{cpa}) \right) \right] - \frac{\Delta H_f v_{w} \rho}{R} \left( \frac{1}{T_R} - \frac{1}{T} \right)
\]

with \( L_p \), the sperm cell membrane permeability to water defined by Levin et al. [53] as,

\[
L_p = L_{pg}[cpa] \exp \left( -\frac{E_{LP}[cpa]}{R} \left( \frac{1}{T} - \frac{1}{T_R} \right) \right)
\]

where, \( L_{pg}[cpa] \) is the reference membrane permeability at a reference temperature, \( T_R (= 273.15 \text{ K}) \); \( E_{LP}[cpa] \) is the apparent activation energy (kJ/mol); \( V \) is the sperm cell volume at temperature, \( T \) (K); \( A_c \) is the effective membrane surface area for water transport, assumed to be constant during the freezing process; \( V_o \) and \( V_b \) are the isotonic (initial) and osmotically inactive sperm cell volumes, respectively; \( R \) is the universal gas constant (8.314 J/mol K); \( B \) is the constant cooling rate (K/min); \( n_{cpa} \) is the number of moles of CPA; \( v_{cpa} \) is the molar volume of CPA (73.3 x 10^{12} \mu m^3/mole for glycerol); \( v_w \) is the molar volume of water (18 x 10^{12} \mu m^3/mole); \( \varphi_s \) is the disassociation constant for salt (= 2); \( n_s \) is the number of moles of salt [\( = C_i \cdot (V_o - V_b) \), where \( C_i \) is the initial cell osmolality, 300 mOsm]; \( \Delta H_f \) is the latent heat of fusion of water (335 mJ/mg); \( \rho \) is the density of water (1000 kg/m^3). Also note that when \( n_{cpa} \) is zero (i.e. no CPA is present), Eqns. [2] and [3] reduce to the ‘water transport’ model as described by Mazur and Levin et al., and \( L_p \) is an Arrhenius function of \( L_{pg} \) and \( E_{LP} \). The two unknown biophysical parameters of the model, either \( L_{pg}[cpa] \) and \( E_{LP}[cpa] \) in the presence of CPA or \( L_{pg} \) and \( E_{LP} \) in the absence of CPA, are determined by curve-fitting the water transport model to experimentally obtained water transport data during freezing [52, 53].

**Biophysical Response in Different Media:** The dynamic volumetric shrinkage (water transport) data for mouse sperm were obtained using the DSC measured heat releases in Eqn. [1] at two different cooling rates 5 and 20 °C/min in three different media (D-PBS + 15% egg yolk; ‘low’ and ‘high’ CPA media). The biophysical parameters were also obtained after curve fitting the experimental data to the water transport equation (Eqns. [2] and [3]) as described above. The ‘combined best fit’ parameters which maximized the goodness of fit parameter for the 5 and 20 °C/min water transport data concurrently in the three media investigated are shown in Table 1. The fits generated by these parameters are shown in Figure 2 and the model simulated equilibrium cooling response (infinitely slow cooling) is also shown for reference (line without data points).

**Table 1:** A tabulated comparison of the ‘combined best fit’ biophysical parameters for mouse sperm obtained using the DSC water transport data at 5 and 20 °C/min in three different media in the presence of extracellular ice [39].

<table>
<thead>
<tr>
<th>Experimental Media</th>
<th>( L_{pg} ) or ( L_{pg}[cpa] ) ( \times 10^{15} ) m^3/Ns (µm/min-atm)</th>
<th>( E_{LP} ) or ( E_{LP}[cpa] ) kJ/mol (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-PBS</td>
<td>1.7 (0.01)</td>
<td>94.1 (22.5)</td>
</tr>
<tr>
<td>‘Low’ CPA media</td>
<td>1.7 (0.01)</td>
<td>122.2 (29.2)</td>
</tr>
<tr>
<td>‘High’ CPA media</td>
<td>0.68 (0.004)</td>
<td>63.6 (15.2)</td>
</tr>
</tbody>
</table>
Reconciliation of Experimental Data Using The DSC Technique: The suprazero permeability data (obtained in the absence of extracellular ice) suggest that mouse sperm cells should be able to dehydrate at rates up to 10,000 °C/min when in fact experiments show that the 'optimal cooling rate' for mouse sperm cells are between 10 - 40 °C/min depending on the concentrations of CPAs in the extracellular milieu [58-61]. One explanation for this discrepancy is that the values of biophysical parameters of water transport at subzero temperatures in the presence of extracellular ice are markedly different than those reported in the literature for suprazero temperatures. In particular, if $L_{pg}$ at subzero temperatures is markedly lower than $L_p$ at suprazero temperatures and $E_{LP}$ at subzero temperatures is markedly higher than the corresponding $E_a$ at suprazero temperatures, then the discrepancy between numerical simulations and experimental data can be reconciled [4]. The best fit parameters obtained in our work using the DSC water transport data during freezing of mouse sperm confirm that this is indeed the case; $L_{pg}$ ~ 0.7 to 1.7 x 10^{-15} m^3/Ns (0.0035 - 0.01 μm/min-atm) and $E_{LP}$ ~ 64.0 - 122 kJ/mole (15 - 30 kcal/mole).

Optimization of Cooling Rate: By performing water transport simulations using the 'combined best fit' parameters in Eqn. [2], the amount of intracellular water trapped inside the sperm cells can be determined for various cooling rates (5, 10, 20, 40, 50 and 100 °C/min) in a given media combination. The water transport simulations in Figure 3 show that for cooling rates of 20, 40, 50 and 100 °C/min, the trapped water volume was 1%, 21.3%, 30.8% and 51.8% of initial intracellular water volume, respectively in 'low' CPA media. An important observation from the water transport simulations shown in Figure 3 is that cooling rates above 25 °C/min will cause some intracellular water to be trapped within the mouse sperm cells and ultimately form intracellular ice on further cooling, depending on the concentrations of the CPAs in the media. Note that the simulations suggest that the optimal cooling rate (defined here as the cooling rate at which 5% of the initial cell water volume is trapped inside the cell after water transport ceases) in D-PBS, in 'low' and in 'high' cryomedia is 39, 26 and 44 °C/min, respectively.

Molecular Determinants of Rat Sperm Function; Potential Measures of Sperm Damage at the Molecular Level: Several key functions of the rat sperm must be maintained through the cryopreservation process in order for the process to be considered successful. Most studies in the past have used cell viability and/or motility as a gross assessment of cryodamage. While these are useful tools for demonstrating damage, they reveal very little about the nature of the damage to the sperm and, consequently, do not lend any insight into how to prevent this damage. In this application we will be looking at specific functions of the sperm using molecular and cellular techniques. The following data illustrate the techniques we will use and the sperm functional systems that they assess.

Sperm capacitation: Sperm must undergo the process of capacitation in the female reproductive tract in order to be capable of fertilization. Several studies in the literature suggest that sperm cryopreservation may have a capacitating effect on sperm [24-26, 62]. This could effect sperm function by causing a premature acrosome reaction or taking the sperm to a post-capacitate state where they can no longer fertilize. We will assess the cryo-induced capacitation using an assay for protein tyrosine phosphorylation which we have shown in the rat system to be indicative of true sperm capacitation. Figure 4 show the phosphorylation response...
of sperm under capacitating conditions and the lack of response when key capacitation requirements (BSA, Ca^{++} and bicarbonate ion) are omitted. We have carried out one preliminary experiment investigating the effect of a common CPA on protein tyrosine phosphorylation response in rat sperm. Figure 5 shows that exposing rat sperm in one step to cryopreservation media containing raffinose and skim milk at a total osmolality of 460 mOsm causes an immediate phosphorylation response. In Specific Aim 3 we will attempt to manipulate the cryopreservation protocol to minimize the capacitating effect of cryopreservation.

**Lipid microdomains:** One of the suspected mechanisms of damage in cryopreservation is alteration of the plasma membrane. We have shown that the rat sperm plasma membrane is highly organized with discrete lipid domains that appear to be lipid rafts and that removal of cholesterol from the plasma membrane disrupts these lipid domains (Figure 6A) [27]. This disruption of membrane domains correlates with sperm capacitation. The ganglioside GM_{1} is a lipid known to be present in many lipid rafts and can be easily monitored to determine if rafts are changing. Figure 6A shows that the very specific staining with the beta subunit of cholera toxin (βCT) over the equatorial segment and the head cap region observed immediately after isolation of sperm from the rat epididymis becomes diffuse over the entire sperm head and visible on the sperm tail after 5 hours of incubation with BSA. This redistribution of raft components correlates with sperm capacitation, implicating raft-associated signaling events in the capacitation process. Figure 6B shows GM_{1} staining on horse sperm before and after cryopreservation. Prior to cryopreservation GM_{1} is confined to the region over the acrosome, with a pronounced absence of staining in the equatorial region. However, after cryopreservation the staining is shifted to cover the entire head and extends further down the tail. These data suggest that sperm lipid domains are disrupted by the cryopreservation process. This will be investigated in specific aim 3.

**Natural inhibitors of sperm capacitation:** One of our hypotheses in this proposal is that cryopreservation induces membrane damage that leads to premature activation of signaling events associated with capacitation. We have recently shown that early events in the capacitation signaling pathway can be inhibited by the epididymal protein Crisp-1 [27]. Figure 7 illustrates that incubating sperm with Crisp-1 under capacitating conditions inhibits capacitation as measured by protein tyrosine phosphorylation and induced acrosome reaction. As part of this proposal, we will investigate the ability of Crisp-1 specifically, and epididymal fluid generally, to suppress premature activation of sperm signaling processes caused by the sperm cryopreservation process. The interaction between Crisp-1 and the sperm surface appears to be dynamic and reversible [27]. Moreover, the suppression of protein tyrosine phosphorylation by Crisp-1 can be reversed after several hours by removal of exogenous Crisp-1 [27]. Thus, if incubation with Crisp-1 during cryopreservation can inhibit premature signaling in the sperm, we expect that this inhibition can be reversed after the Crisp-1 is removed post-thaw.

**Calcium homeostasis in sperm:** The effect of cryopreservation on cellular membranes may also lead to an inability of the cell to sequester important ions, such as calcium, in the cell or intracellular compartments in the cell. Calcium is of particular interest because of its involvement in capacitation and the acrosome reaction. Thus, in this proposal we will investigate the uptake and storing of Ca^{++} during and after cryopreservation. As an initial step, we have shown that calcium-dependent fluorescence can be determined in rat sperm. Figure 8...
shows a rat sperm loaded with Fluo 4 AM before and after incubation in calcium ionophore A23187. The increase in calcium-dependent fluorescence is readily evident.

**D. EXPERIMENTAL DESIGN AND METHODS.**

**Specific Aim 1:** Determine the handling methods and CPA components most effective for rat sperm cryopreservation.

Much of cryopreservation science, particularly as it relates to the cryopreservation of sperm, is empirical in nature. That is, cellular behaviors crucial to cryopreservation cannot be predicted but must simply be tested in a systematic way. For sperm cryopreservation, these empirically-determined factors include media components for sperm manipulation *in vitro*, sensitivity to manipulations such as centrifugation, sensitivity cryoprotective agents at concentrations required for cryoprotection, sensitivity to supra-zero temperatures encountered during preparation for freezing, and sensitivity to osmotic excursions caused by the addition and removal of hypertonic CPA solutions.

For many cells, including sperm from numerous species, most of these variables are known. However, for rat sperm, all of the above variables in the cryopreservation process are currently unknown and/or unreported in the literature. It is known that there are species-specific differences in what sperm can tolerate relative to handling and cryopreservation. For instance, human and equine sperm can be centrifuged with minimal damage, as measured by loss of motility and membrane integrity, whereas mouse sperm and, to an even greater degree, rat sperm are quite sensitive to damage caused by centrifugation. Likewise, human and mouse sperm are somewhat sensitive to cold shock, the damage caused by cooling sperm too rapidly to near-ice temperatures, whereas equine sperm is extremely sensitive to cold shock and must be cooled very slowly to maintain viability. In terms of cryoprotective agents, mouse sperm can only tolerate minimal concentrations of the membrane-permeable CPA glycerol while bovine sperm survive cryopreservation optimally in CPA medium that contains high amounts of glycerol [7]. There are also sperm-specific sensitivities to osmotic excursions caused by nonphysiological osmolalities encountered in cryoprotective media.

Given the substantial differences between sperm from different species in their tolerance to various media and manipulations, and the relative lack of specific information in this regard concerning rat sperm, this specific aim focuses on elucidating optimal handling methods and CPA characteristics for rat sperm.

**Hypothesis:** Rat sperm have unique handling and medium composition requirements for optimal cryopreservation.

**SA1.1. What are the optimal cryopreservation media components for rat sperm?**

**Rationale.** Our approach to optimizing rat cryopreservation media is to begin with the Medium I and II described by Natatsukasa et al. (2001) [1]. Medium I will consist of 23% egg yolk, 8% lactose monohydrate and antibiotics (penicillin/streptomycin), pH 7.4, and be used for sperm isolation. Medium II will consist of medium I with 1.4% Equex Stem, also known as Orvus Paste, whose active ingredient is the anionic detergent sodium dodecyl sulfate (SDS), and with or without glycerol. Medium II will be added to the sperm suspension 1:1 after slow cooling to 4°C, just before packaging and freezing. The rationale for starting with these media is clear: the only successful cryopreservation of rat epididymal sperm that we are aware of (albeit minimal) was...
accomplished using these media. From these basic medium we will modify the solutions by testing the efficacy of 1) replacing the nonpermeant disaccharide lactose with trehalose in medium I and II, 2) replacing egg yolk with the LDL fraction of extracted egg yolk in both media, which has been shown to better protect sperm during cryopreservation than whole egg yolk [63], and 3) adding glycerol from 0-2% to medium II.

**Experiment.** Rat sperm will be isolated from cauda epididymides according to the method used in our recent paper (see Appendix 3) [27]. Briefly, rats will be killed by CO₂ asphyxiation and epididymides surgically removed. Radial slits will be made in each of the cauda epididymides followed by a 5 minute incubation in 1 ml of pre-warmed media on an orbital shaker to facilitate the swim out of sperm into the media. Sperm suspensions will be evenly dispersed, counted and an aliquot examined microscopically to determine percent motility and viability. Rat sperm will be isolated directly into medium I or into this media modified by the substitution of trehalose for lactose and/or by the substitution of whole egg yolk with the LDL fraction of egg yolk [63]. After slow-cooling to 4ºC, suspensions will be diluted 1:1 in medium II with or without glycerol and with Equex Stem. Sperm motility will be determined by CASA analysis as a measure of function, given that sperm motility is likely a more sensitive endpoint than is sperm viability. Motility will be assessed after isolation, cooling, loading CPA and again after subjecting the sperm to freezing in a controlled rate freezer, followed by plunging into liquid nitrogen and subsequent thawing.

**Potential results.** We expect this experiment to reveal the “optimal” media, given the components tested, for cryopreservation of rat sperm. Given our preliminary experiments, we expect that glycerol will be somewhat toxic to rat sperm, as it is to mouse sperm, and that trehalose will be a more optimal nonpermeating cryoprotectant than lactose. We also expect that the lipid extract of egg yolk will provide a further benefit during the entire process. The optimal concentrations of each component will be determined if an advantage is determined in initial experiments.

**Pitfalls.** The methods proposed in this experiment are all in routine use in our laboratory. We expect no technical difficulties. The optimal freezing rate will not be determined until the work of specific aim 2 is completed. Therefore, it is likely that the results from these experiments, freezing sperm at a fixed rate (~25°C/min), may be less than optimal and survival may improve once the correct freezing rate has been determined.

**SA1.2. What are the limits to centrifugation of rat sperm and the alternatives to centrifugation for medium changes?**

**Rationale.** Rat sperm do not tolerate centrifugation well. In our hands, rat sperm motility is reduced to near zero after spinning at 600 x g for 5 minutes. The objective of this experiment is to determine if there is any safe speed and time that rat sperm can be centrifuged with reasonable survival and motility. Since it may be necessary to remove sperm from the CPA prior to use in in vitro fertilization experiments or intrauterine insemination, a method for ‘washing’ the sperm and changing the medium is needed. Since it is possible that there is no safe centrifugation speed for rat sperm, we will also investigate the feasibility of changing media on rat sperm through a porous membrane.

**Experiment 1.** A simple series of speeds and centrifugation times with rat sperm will be investigated. The motility of the rat sperm post-spin and the percentage recovery of the sperm in the pellet will be determined. For centrifugation to be a useful method for washing rat sperm, a 50% recovery of motile sperm after centrifugation must be attained.

**Experiment 2.** In this experiment, sperm will be eluted from epididymides and transferred to a 35 mm culture well lined with a 45 micron filter-bottom well. The media will then be exchanged by moving the filter bottom well, with sperm contained above the filter, and placing the filter well into a new 35 mm culture well containing the new medium. This process will be repeated after a 5-minute equilibration. The efficiency of the media change will be monitored by adding an inert dye, such as bromophenyl blue, to the initial culture media and monitoring the loss of dye. The motility and concentration of the sperm will be determined.

**Potential results.** We expect to determine unequivocally the speed and duration of centrifugation that rat sperm can functionally endure. The unknown question is whether or not there is any speed and time that will yield sufficient quantities of functional sperm. In the second experiment we hope to establish an alternative method for washing sperm and changing media that does not involve centrifugation. This may provide us the ability to manipulate rat sperm many times in order to prepare it for freezing or subsequent fertilization experiments.

**Pitfalls.** Neither of the proposed experiments will be difficult to carry out and we expect no technical difficulties.
SA1.3. What is the limit of medium osmolality that can be tolerated by rat sperm?

**Rationale.** All sperm have limits to the osmotic shock they can tolerate before losing specialized function such as motility. Many investigators have shown that increasing (or decreasing) osmolality can affect the motility and viability of sperm. Since the addition or removal of solutes to cryopreservation media will raise or lower the osmolality, it is imperative to know what the functional limits are for the sperm. This experiment will investigate the osmotic tolerance limits of rat sperm.

**Experiment.** Sperm will be isolated into BWW (285 mOsm), and basal motility will be determined. The osmolality of the solution will then be raised in 50 mOsm increments by adding predetermined amounts of sodium chloride from a concentrated solution. After equilibration at the higher osmolality (10 minutes), the sperm will be returned to iso-osmotic conditions and motility will be assessed. The degree of motility loss with increasing osmolality will be plotted and used as a guide in the creation of cryoprotective media.

**Potential results.** This experiment will establish the osmotic tolerance limits for rat sperm. There will likely be a gradient of effect on the population of rat sperm as the osmolality of the solution increases. In terms of the osmolality of optimal cryopreservation media, there may be some trade off between the osmotic damage caused by the CPA media and the protective effects of the components that are raising the osmolality. For this reason, it cannot be determined from the osmotic excursion injury data alone what the optimal osmolality of the cryopreservation media will ultimately be. However, knowing the functional behavior of sperm as the osmolality increases will provide guidance in the formulation of the CPA media.

**Pitfalls.** This experiment uses techniques that are well established in our laboratories. We expect no difficulties in carrying out these experiments.

SA1.4. What is the sensitivity of rat sperm to cold shock?

**Rationale.** Sperm from many species of animals, such as horses, pigs and mice, are sensitive to ‘cold shock’, the damage incurred by sperm when cooled to a near-ice temperature at a too rapid rate. The sensitivity of rat sperm to cold shock has not been determined but is likely an important consideration in the handling of rat sperm prior to cryopreservation.

**Experiment.** In this experiment we will investigate the cold shock response of rat sperm by cooling the sperm suspension to 4°C at three cooling rates. The fastest cooling rate (~20°C/min) will be accomplished by placing the suspension directly into an ice water bath; the slowest (~0.3°C/min) by placing the suspension in a Hamilton-Thorne Equitainer designed for the slow cooling of equine sperm. An intermediate rate (1-2°C/min) will be carried out in a controlled rate freezer. After holding at the 4°C for 10 minutes, the sperm will be quickly re-warmed by immersion in a 37°C water bath and motility measured by CASA. The cooling rate can then be optimized based on the sperm behavior at these three rates. In a second set of experiments, the sperm will be equilibrated in the cryopreservation media (SA 1.1) before cooling or at 4°C after controlled cooling. The motility of the sperm will be determined after re-warming the cells to 37°C.

**Potential results.** This experiment will elucidate the sensitivity of rat sperm to cold shock and reveal the optimal rate for cooling the sperm. This experiment will also determine whether it is better to equilibrate the sperm in cryopreservation media before or after the cooling has been completed.

**Pitfalls.** These experiments use techniques that are well established in our laboratories. We expect no difficulties in carrying out these experiments.

SA1.5. What is the effect of incubating rat sperm with seminal vesicle proteins on subsequent sensitivity to handling?

**Rationale.** Epididymal sperm are not fully mature in that they have not been exposed to proteins present in the ejaculate. Much of the volume of the ejaculate originates in the seminal vesicles. It has been demonstrated that proteins from the seminal vesicles become bound to ejaculated sperm and that some of these proteins effect sperm functions, such as capacitation [64, 65]. It is our hypothesis that one of the effects of exposure of sperm to seminal vesicle secretions is further maturation and stabilization of the sperm. If this hypothesis is true, it is possible that exposing epididymal sperm to seminal vesicle proteins may stabilize the sperm and facilitate survival during the cryopreservation process. Since it is not feasible to obtain large amounts of ejaculated rat sperm, we propose to incubate epididymal rat sperm in buffer containing seminal vesicle fluid and determine if this renders rat sperm more resistant to damage during the cryopreservation process.

**Experiment.** Seminal vesicles will be removed by cutting at the junction of the seminal vesicles and ejaculatory ducts. The fluid contained in the seminal vesicles will be diluted 1 to 1 with Medium 1 and sperm from the
epididymis will be eluted, as described in specific aim 1, directly into this media. Control sperm will be diluted into normal Medium 1. These sperm will be cryopreserved by optimal methods determined above. Motility and viability will be determined post-thaw.

*Potential results.* This experiment will reveal whether or not exposure of sperm to proteins and other fluid components from the seminal vesicles has a stabilizing effect on sperm that will protect these cells during the cryopreservation process. The results are expected to be a ‘yes’ or ‘no’ answer to this question. If such exposure does provide a stabilization of the sperm, it will be straightforward to incorporate this treatment into the routine isolation of epididymal sperm.

*Pitfalls.* We expect no difficulties in carrying out these experiments. It is simple to dissect and remove the seminal vesicles from the rat and extrusion of fluid is also easy. There is a tendency for the fluid from the seminal vesicles to become viscous after it is removed from the gland, which is why we have proposed to use a dilution of the fluid. If this remains a problem, further dilution may be required.

**Specific Aim 2:** Determine the biophysical water transport properties of rat sperm plasma membrane during freezing.

**Hypothesis:** Rat sperm have water transport properties that are unique to this species.

**SA2.1.** What are the water transport properties of the rat sperm plasma membrane in the presence and absence of cryoprotectants?

**Rationale.** Understanding the membrane permeability of rat sperm will allow us to determine the optimal cooling rate for cryopreservation. Understanding the permeability characteristics of the sperm plasma membrane to water in the presence of extracellular ice is critical to identifying the cooling rates during freezing which are optimal for sperm survival. As discussed above (Background and Preliminary Results sections), the optimal cooling rate is defined as the fastest rate at which water transport is still the dominant mechanism and IIF (intracellular ice formation) is minimized. Our preliminary results and our recent papers suggest that we have found this rate for ICR mouse sperm and human sperm in the presence and absence of CPAs [39, 42]. The purpose of this specific aim is to expand this methodology to determine the optimal cooling rate for rat sperm. The CPA solutions used for this aim will be determined from the results of Specific Aim 1.

**Experiment.** Cooling rate dependent biophysics of rat sperm in media with and without CPA will be characterized and optimized during freezing using the differential scanning calorimeter (DSC) technique and biophysical modeling. As mentioned earlier, the differential scanning calorimeter (DSC) is the only experimental technique which yields data on how sperm dehydrate during freezing in the presence of extracellular ice [39, 41, 42]. For DSC experiments, 5-20 x 10⁴ sperm in 10 µl of media are placed in a DSC sample pan. A small amount of *Pseudomonas syringae*, a natural ice nucleating agent, is added and the sample pan is sealed. The sample is then placed in the DSC (Perkin Elmer DSC Pyris 1, Perkin-Elmer Inc., Norwalk, CT). The sample is cooled at 5°C/min to nucleated ice in the extracellular space. After extracellular nucleation the sample is warmed very close to the phase change temperature of the media and allowed to equilibrate with the partially frozen extracellular medium. The sample is then cooled to -50 °C at a predetermined low cooling rate causing the cells to undergo extensive cellular dehydration. During this *initial* freeze, as the sample is cooled, the supercooled intracellular water permeates out of the cells and equilibrates with the extracellular ice by releasing the latent heat of fusion. The *initial* heat release measured by the DSC includes the latent heat release due to water transport and the heat released by the unfrozen fraction of the media. The DSC dynamically records this heat release as a function of temperature (Figure 1 - Curve A in Preliminary Data). The sample is then re-equilibrated at the phase change temperature by thawing at a very high warming rate (~100 °C/min). The sperm are then lysed by rapid cooling (~ 200 °C/min) to -50 °C and rapid re-equilibration to the phase change temperature. The sample is then cooled to -50 °C at the same predetermined low cooling rate during a *final* freeze to measure the heat released by the sample which is now composed of media mixed with cell and/or tissue debris (Figure 1 - Curve B in Preliminary Data). The difference in the initial and final heat release is Δqₜₒₜ. The temperature dependence of this difference in heat release between the initial and final cooling run, Δq(T), is then used in Eqn (2) to estimate volumetric shrinkage as previously characterized and described.
A nonlinear least squares curve fitting computer program is used to calculate the biophysical parameters ($L_{pg}$ and $E_{Lp}$) that best fit the volumetric shrinkage data. The optimal fit of Eqn. [2] and [3], to the experimental data will be obtained by selecting a set of parameters which minimizes the residual variance, $\chi^2$, and maximizes a goodness of fit parameter, $R^2$ [66]. In order to predict the biophysical parameters that produce a 'combined best fit' to the experimental water transport data at two or more cooling rates, a nonlinear curve fitting code has been created [39]. This method gives curve fitting results with an $R^2$ value greater than or equal to 0.9, indicating that there is a good agreement between the experimental data points and the fit calculated using the estimated biophysical parameters from this method. To simulate the biophysical response of a sperm cell under a variety of cooling rates the best fit parameters will be substituted in the water transport equation (Eqns. [2] and [3]). The equation is then numerically solved using a 4th order Runge-Kutta method using a FORTRAN code on a SGI (SGI, Mountain View, CA) workstation [39, 47].

Potential results. The expected results are the water transport response and corresponding biophysical parameters, $L_{pg[cpa]}$ and $E_{Lp[cpa]}$, as a function of [CPA], for every rat sperm in plain media and media containing CPAs. Our previous work has demonstrated that both volumetric response and parameters can be obtained from mouse, human and horse sperm in a variety of media and CPA solutions and hence the same is expected for rat sperm.

Pitfalls. Two potential problems exist. One is that the sperm are already destroyed before the water transport experiment can be performed, and the other is that the sperm are forming IIF during the DSC experiment instead of dehydrating. If the sperm have been destroyed by the CPA media or collection conditions prior to freezing the water transport response will be non-existent. In this case, the motility and viability endpoints from loading and unloading CPA will likely also bear evidence of the damage to the sperm. This will require either a different optimal cryomedia or a different unloading/loading procedure for the CPA. If the sperm are forming IIF during the DSC experiment, there is often no way to explicitly distinguish between a heat release due to water transport or IIF. Therefore, supplemental viability and motility information post-thaw will be used to help distinguish if substantial IIF has occurred. In our previous work, high viability and motility was achieved at a cooling rate of 20 °C/min which suggest that little or no IIF was present at this rate. Other empirical studies suggest that substantial IIF (indirectly assumed through motility and viability loss) occurs in some sperm cells cooled greater than 100 °C/min [59]. In any case, the combination of the DSC information with viability and motility will strongly suggest if IIF is present, in which case the cooling rate will be lowered to favor water transport.

Specific Aim 3: Design and test strategies for protecting rat sperm from damage incurred by cryopreservation.

Sperm are specialized cells with defined functions, including motility and the ability to capacitate and undergo an acrosome reaction, that must be maintained in order to carry out successful fertilization. Changes that occur at the molecular level during freezing must be minimized or repaired post-thaw. These molecular changes include tyrosine phosphorylation events, lipid microdomain organization, disruption of the acrosomal vesicle, Ca++ compartmentalization, and cAMP synthesis. The importance of each of these molecular events in sperm function is discussed in the Background and Significance section (B) in the section entitled Potential Targets of Cryoinjury: Specialized Sperm Function. In the following experiments, potential cryoprotective measures will be assessed for their ability to minimize damage to these sperm structures and functions at the molecular level.

Hypothesis: Protective measures can be implemented that will prevent or counteract some of the damage caused by cryopreservation.

SA3.1 Does the presence of exogenous cholesterol, or other lipids, prevent damage during freezing?

Rationale. Cholesterol is known to effect the permeability and fluidity of plasma membrane of sperm [67-69]. Thus, altering the cholesterol content of the plasma membrane should affect the behavior of the cell during the cryopreservation process. It has been shown that sperm with high cholesterol content have a greater resistance to cold shock than do sperm with lower cholesterol content in their plasma membranes [69]. Likewise, phosphatidylcholine, which is found in high amounts in egg yolk, has a stabilizing effect on the plasma membrane and can also protect sperm from cold shock damage [69]. In this experiment we will
determine if adding exogenous cholesterol and/or exogenous phosphatidylcholine has a protective effect against the damage caused to rat sperm by the cryopreservation process.

**Experiment.** Rat sperm will be isolated from the epididymis and cryopreserved according to the methods worked out in specific aims 1 and 2. Sources of undefined lipid content, such as egg yolk, will be excluded from the cryopreservation media for this experiment. The media will be supplemented with cholesterol saturated cyclodextran, or exogenous phosphatidylcholine, or both [68, 70, 71]. The sperm will then be cryopreserved. Upon thawing, aliquots of the sperm will be analyzed for tyrosine phosphorylation, evidence of cryo-capacitation, integrity of plasma membrane microdomains, loss of calcium homeostasis, damage or loss of the acrosome, and altered cAMP levels.

Tyrosine phosphorylation, lipid microdomains, and acrosomal integrity will be measured by methods described in our recent paper (Roberts et al., 2003; see Appendix 3). For determination of intracellular Ca**, sperm will be loaded with Fluo-4/AM by incubation in a 4 µM solution for 30 minutes in the dark. Extracellular Fluo-4/AM will be removed by changing the medium and Ca**-dependent fluorescence will be read from aliquots of the sperm. For imaging cellular calcium in sperm, cells will be loaded and washed as described above. After the experimental manipulation, cells will be viewed and photographed on our microscope equipped for fluorescence. Steady state levels of cAMP in the sperm will be determined using the Cyclic AMP Immunoassay (R&D Systems, Inc. Minneapolis, MN). Briefly, sperm will be harvested and lysed with 0.1M HCl. The supernatant will be used in a competitive binding assay with a rabbit anti-cAMP polyclonal antibody and an alkaline phosphatase-labeled cAMP standard. After incubation and washing to remove unbound material, absorbance will be read at 405 nm in a microplate reader.

**Potential results.** This experiment will reveal whether or not exogenous cholesterol or phosphatidylcholine have a protective effect against the damage caused by cryopreservation process. The ultimate benefit of this experiment will be to better understand why additives like egg yolk, which are high in both cholesterol and phosphatidylcholine, have a protective effect on sperm during freezing. It may also allow us to optimize the concentrations of these additives for the cryopreservation process.

**Pitfalls.** For the protein tyrosine phosphorylation experiments we are assuming that enough rat sperm will survive the cryopreservation process to generate a signal in these assays and that dead sperm do not interfere with the assays. If these assumptions prove false, we can perform the protein tyrosine phosphorylation assay by immunocytochemistry and eliminating dead cells by counterstaining with a vital dye. The pitfalls associated with staining sperm for lipid microdomains are minimal given our experience with this technique. Likewise, the coomassie staining method for assessing the acrosome is very sensitive and can determine damage that causes complete loss of the acrosome, as well as damage that causes changes to the acrosome. However, it is possible that there can be damage done to the acrosome that will not be detected by this method and that will, as a result, be missed by these experiments. The techniques for measuring Ca** flux are well established in the literature and the required reagents are readily available from commercial sources. Therefore, we expect no technical hindrances to the successful completion of these experiments. Finally, the measurement of cAMP by ELISA assay is a straight forward technique and we expect no difficulty with it. The main potential problem is sensitivity of the assay. If it proves necessary, the sensitivity of the cAMP ELISA assay can be increased by acetylation of the samples and the standards. This modification of the procedure is included with the cAMP ELISA assay kit.

**SA3.2 Does the presence of surfactants in the CPA medium prevent damage during freezing thawing processes of cryopreservation?**

**Rationale.** Since damage during cryopreservation includes damage to cellular membranes that can result in leakage of cellular materials, such as important ions as well as proteins, it is possible that inclusion of a surfactant that will facilitate the closure of holes in membranes will protect against cryopreservation damage. In this experiment we will investigate the usefulness of Poloxamer 188, a triblock copolymer of polyethylene oxide and polypropylene oxide, in the cryopreservation media to prevent damage to sperm caused by persistent membrane permeability after thawing. Data in the literature suggest that Poloxamer 188 inserts preferentially into damaged portions of membranes and is effectively excluded from the membrane after the membrane integrity is restored [72, 73]. This property of Poloxamer 188 may make it more suitable for post-thaw fertilization using sperm exposed to this surfactant.
**Experiment.** Poloxamer 188 will be added to the cryopreservation media at a concentration of 50 $\mu$M, a concentration below the critical micelle concentration. Rat sperm will be cryopreserved in this medium according to the methods worked out in specific aims 1 and 2. Upon thawing, aliquots of the sperm will be analyzed for the integrity of the acrosome, lipid microdomains, and calcium homeostasis.

**Potential results.** If persistent membrane leakage is a major damage mechanism in rat sperm cryopreservation, and if poloxamer 188 is effective as a surfactant to reduce or prevent membrane leakage, we expect that inclusion of the surfactant will result in decreased damage to sperm. If this proves to be true, the concentration of surfactant can be further optimized and other surfactants can be tried in our rat sperm cryopreservation system.

**Pitfalls.** The damage assays proposed in this experiment are the same as those of SA3.1 and subject to the same pitfalls.

SA3.3 Does inclusion of antioxidants in the cryopreservation medium help prevent damage to sperm during the cryopreservation process?

**Rationale.** Reactive oxygen species (ROS) are a known mechanism of damage to cells caused by cryopreservation. ROS have also been shown to be involved in inducing capacitation in sperm [74-76]. It stands to reason that, in addition to damage caused by lipid peroxidation and other established mechanisms of ROS damage, that ROS may be the cause of premature capacitation observed in sperm as a result of the cryopreservation process. This hypothesis will be tested by this experiment.

**Experiment.** The following antioxidants will be added to the cryopreservation media individually or in combination: superoxide dismutase (SOD), catalase, EDTA (a metal chelator), oxyrase, and ascorbic acid (vitamin C). Rat sperm will be cryopreserved with these additives according to the methods worked out in specific aims 1 and 2. Upon thawing, aliquots of the sperm will be analyzed for protein tyrosine phosphorylation as well as lipid domain integrity and calcium homeostasis. The sperm will also be analyzed for other damage indicators including damage or loss of the acrosome and decreased cAMP.

**Potential results.** If ROS are a predominant mechanism of damage to the sperm during cryopreservation according to our protocol, this experiment will delineate this damage and determine the protective effect that can be afforded the sperm by the inclusion of antioxidant enzymes and molecules.

**Pitfalls.** The damage assays proposed in this experiment are the same as those of SA3.1 and subject to the same pitfalls.

SA3.4 Does elimination of required capacitation factors from the CPA medium or inclusion of Crisp-1, a proven inhibitor of capacitation in rat epididymal sperm, prevent premature capacitation-like changes in sperm during freezing?

**Rationale.** One of the potential reasons that cryopreserved sperm do not function optimally is premature capacitation caused by the cryopreservation process. If this is true, preventing premature capacitation during the cryopreservation process should result in more functional capacity of the sperm post-thaw. In this experiment we will test this hypothesis by cryopreserving sperm in medium that are deficient in calcium ion or bicarbonate ion, both of which are required components for sperm capacitation. Additionally, we will cryopreserve sperm in the presence of exogenous Crisp-1 protein, which is an epididymal protein that has been shown by our laboratory to be a natural inhibitor of the capacitation process [27].

**Experiment.** Rat sperm will be cryopreserved in medium devoid of Ca$^{++}$ or/and HCO$_3^-$, and in medium containing 400 mg/ml purified Crisp-1 (see Appendix 3) [27]. Upon thawing, aliquots of the sperm will be analyzed for protein tyrosine phosphorylation and calcium homeostasis.

**Potential results.** The results of this experiment will be very informative. If any of these media prevent protein tyrosine phosphorylation then we will have gained insight into the mechanism and requirements of cryo-induced capacitation, and will have discovered how to prevent this phenomenon. If some or none of these treatments prevent premature capacitation, then we will have shown that cryopreservation, by some mechanism, can override the normal requirements of capacitation and stimulate the phosphorylation events associated with the capacitation process by an undescribed pathway.

**Pitfalls.** The assays proposed in this experiment are the same as those of SA3.1 and subject to the same pitfalls. Crisp-1 protein purification is routinely performed in our laboratory.
E. HUMAN SUBJECTS
None

F. VERTEBRATE ANIMALS

F.1. Species and Numbers.
   Rats: 300/year

F.2. Rationale.
The experiments proposed require the use of epididymal sperm and many of the experiments call for the use of Crisp-1 protein which is obtained from homogenates of rat epididymes. We have proposed only the numbers of rats required to obtain the necessary sperm and Crisp-1 protein required for these experiments.

F.3. Description of the Proposed Use of Animals.
Rats will be housed under NIH standard conditions, will be killed by CO₂ asphyxiation before organs are collected for experimentation. No survival surgeries are proposed.

Animals are inspected daily by animal care personnel. Any problem with the animals is immediately referred to the Research Animal Resources veterinarian.

F.5. Euthanasia.
Euthanasia of rats will be either by CO₂ asphyxiation or anesthesia overdose.

G. LITERATURE CITED
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H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS.

None

I. CONSULTANTS/COLLABORATORS
None

**Time Line for Completion of Specific Aims.**

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