Effect of glycerol and cholesterol-loaded cyclodextrin on freezing-induced water loss in bovine spermatozoa

G Li, J Saenz1, R A Godke1 and R V Devireddy

Department of Mechanical Engineering, Louisiana State University (LSU), Baton Rouge, LA 70803, USA and
1Embryo Biotechnology Laboratory, Department of Animal Sciences, LSU Agricultural Center, Baton Rouge, LA 70803, USA

Correspondence should be addressed to R V Devireddy; Email: devireddy@me.lsu.edu

Abstract

Recent experimental data show that incubating bovine sperm with cholesterol-loaded cyclodextrin (CLC) before cryopreservation increases the percentages of motile and viable cells recovered after freezing and thawing, compared with control sperm. In the present study, we report the effect of incubating bovine sperm with CLC on the subzero water transport response and the membrane permeability parameters (reference membrane permeability ($L_{pg}$) and activation energy ($E_{lp}$)). Water transport data during freezing of bovine sperm cell suspensions were obtained at a cooling rate of 20 °C/min under three different conditions: 1. in the absence of cryoprotective agents (CPAs); 2. in the presence of 0.7 M glycerol; and 3. in the presence of 1.5 mg/ml CLC and 0.7 M glycerol. With previously published values, the bovine sperm cell was modeled as a cylinder of length 39.8 μm and radius 0.4 μm, with osmotically inactive cell volume ($V_b$) of 0.61 $V_o$, where $V_o$ is the isotonic cell volume. By fitting a model of water transport to the experimentally obtained data, the best-fit water transport parameters ($L_{pg}$ and $E_{lp}$) were determined. The predicted best-fit permeability parameters ranged from $L_{pg}$ = 0.02 to 0.036 μm/min-atm and $E_{lp}$ = 26.4 to 42.1 kcal/mol. These subzero water transport parameters are significantly different from the suprazero membrane permeability values (obtained in the absence of extracellular ice) reported in the literature. Calculations made of the theoretical response of bovine spermatozoa at subzero temperatures suggest that the optimal cooling rate to cryopreserve bovine spermatozoa is 45–60 °C/min, agreeing quite closely with experimentally determined rates of freezing bovine spermatozoa.

Introduction

Soon after the serendipitous observation by Polge et al. (1949) that sperm cells survive freezing stress in the presence of glycerol, but not in its absence, the first calf was produced with cryopreserved spermatozoa in 1951 (Stewart 1951). Today, the worldwide cattle industry is based on artificial insemination and frozen semen, and cryopreservation has allowed exploitation of superior sires and achieved rapid, large-scale genetic improvement in cattle stocks coupled with a reduction in disease transmission (Foote 1999). This impact would not have been possible without successful freezing of bull spermatozoa (Parkinson & Whitfield 1987, Foote & Parks 1993, Woelders 1997, Vishwanath & Shannon 2000; also see Foote 2002 for a historical review on artificial insemination).

The process of cryopreservation represents an artificial interruption of the progress of the spermatozoon toward post-ejaculation capacitation and fertilization (Graham 1978, Hammerstedt et al. 1990). One of the initial steps in sperm capacitation is a loss of cholesterol from the plasma membrane (Langlais & Roberts 1985, Ehrenwald et al. 1988), and when sufficient cholesterol is removed, the membrane becomes unstable, enhancing its ability to fuse with the outer acrosomal membrane, and resulting in the acrosome reaction (Nolan et al. 1992). Adding cholesterol to the incubation medium inhibits the acrosome reaction (Visconti et al. 1999, Khorasani et al. 2000, De Jonge 2005). Altering the lipid composition of sperm plasma membranes affects not only the ability of sperm to capacitate and undergo acrosome reaction, but also the way sperm respond to cryopreservation (He et al. 2001, Awad & Graham 2002, Purdy & Graham 2004a, Moore et al. 2005). However, Purdy and Graham (2004a) found that when cyclodextrins, or cyclic oligosaccharides of glucose with a hydrophobic center capable of incorporating lipids (Gitler 1972, Klein et al. 1995), are preloaded with cholesterol (cholesterol-loaded cyclodextrin (CLC)) and then incubated with bull sperm before cryopreservation, higher
percentages of motile and viable cells are recovered after freezing and thawing than with control sperm. This added cholesterol probably benefits cells by eliminating or at least lowering the temperature at which the sperm plasma membranes undergo the lipid-phase transition from the fluid to the gel state as the cells are cooled (Blok et al. 1976, Watson 1981, Steponkus 1984, Holt & North 1986, Caffrey 1987, Drobnis et al. 1993). More importantly, Purdy and Graham (2004b) also showed that, after freezing and thawing, CLC-treated bovine sperm do undergo capacitation and the acrosome reaction at rates similar to control sperm, and fertilize oocytes in vitro and in vivo similarly well. Therefore, adding cholesterol to sperm membranes by CLC technology has been proven to be very useful in enhancing the cryosurvival of bovine sperm (Awad & Graham 2002, Purdy & Graham 2004a,b), as well as, as recently shown, equine sperm (Moore et al. 2005). However, there is little or no information available on the precise mechanism by which CLC treatment improves the post-freeze response of bovine sperm. One such mechanism could be the ability of CLC-treated bovine sperm to mediate beneficially the freezing-induced loss of intracellular water or water transport during freezing. Thus, the primary aim of this study was to determine the effect of CLC treatment on the subzero water transport response of bovine spermatozoa to measure any change in the bovine sperm membrane permeability to water during freezing with and without the CLC treatment.

The unique morphology and small size of mammalian spermatozoa limit the applicability of standard cryomicroscopy techniques to measure the biophysical responses (water transport and intracellular ice formation) of spermatozoa during freezing. However, a novel method using a differential scanning calorimeter (DSC) has enabled the measurement of the water transport response during freezing of spermatozoa of several species, including mouse (Devireddy et al. 1999), man (Devireddy et al. 2000), horse (Devireddy et al. 2002a,b), dog (Thirumala et al. 2003), Pacific oyster (He et al. 2004), boar (Devireddy et al. 2004), green swordtail (Thirumala et al. 2005) and southern platyfish (Pinsetty et al. 2005). This DSC technique was used in this study to measure the membrane permeability parameters of bovine spermatozoa at a cooling rate of 20°C/min in three different media: 1. in the absence of any cryoprotective agents (CPAs); 2. in the presence of 0.7 M glycerol; 3. in the presence of 1.5 mg/ml CLC and 0.7 M glycerol. The experimentally determined membrane permeability parameters were then used to calculate the optimal rates of freezing bovine sperm cells in the presence and absence of glycerol and CLC.

**Materials and Methods**

**Semen collection**

In this study, semen was collected over a 2-month interval from mature, healthy, fertile Aberdeen Angus (n = 3) and Senepol (n = 5) bulls housed in temperature-controlled paddocks at the Genex Custom Collection Service near the Louisiana State University (LSU) campus. The bulls were 2–7 years in age (mean = 3.5 years for the Aberdeen Angus bulls and 3.4 years for the Senepol bulls), were in good body condition and had body weights of 603–1250 kg at the onset of semen collection. Semen was collected once or twice a week, with never fewer than 3 days between semen collections from one week to the next. Each bull was in semen production and thus, accustomed to the standard semen collection procedures at the bull stud facilities. Males were randomly selected from the bull group on the day of semen collection.

All bulls were collected by the same experienced technicians throughout the study. Briefly, two false mounts were made by the bulls before actual semen collection form a trained teaser steer. A total of nine separate semen ejaculates (collection days = 9) from eight bulls were randomly allotted as replicates across this experiment. One Aberdeen Angus bull (no. AN179) was collected twice during the collection interval. Semen was harvested with a 15 ml sterile glass tube, extended in a standard bovine egg yolk-based diluent and then placed into a 37°C water bath for equilibration before evaluation of progressive motility. Sperm concentration was calculated by a standard curve from a spectrophotometer, following standard commercial bull stud procedures. The semen sample was then transported in a 15 ml glass conical tube, held in a 50 ml water bath (37°C) in a standard Styrofoam container, to the LSU Bioengineering Laboratory (~10 min) for DSC experiments. Ejaculates from bulls were used randomly across the replicates of the DSC experiments, and all experiments were completed within 3–6 h of collection.

**Preparation of CLC**

Methyl-β-cyclodextrin (Sigma Aldrich, St Louis, MO, USA) was loaded with cholesterol, as described previously by Purdy and Graham (2004a,b). Briefly, 200 mg cholesterol were dissolved in 1 ml chloroform. In a second tube, 1 g methyl-β-cyclodextrin was dissolved in 2 ml methanol, and 0.45 ml cholesterol solution were added. The combined cyclodextrin and cholesterol solution was thoroughly mixed, and the solvents were then removed by a stream of nitrogen gas. The resulting crystals were stored at 22°C until use. To add cholesterol to sperm, a solution of 1.5 mg/ml CLC was made by adding 50 mg CLC to 1 ml TALP (Nolan et al. 1992) at 37°C and mixing vigorously in a vortex mixer, as described by Purdy and Graham (2004a).

**Loading of glycerol and CLC**

For DSC experiments in the absence of CPAs, bovine spermatozoa were concentrated by gentle centrifugation (300 g, ~25°C) for 5 min and resuspended in the residual supernatant. Similarly, DSC experiments on bovine spermatozoa were also conducted in the
presence of a permeating CPA (0.7 M glycerol or 6% v/v glycerol). We chose to study membrane transport in the presence of glycerol, since bovine sperm is routinely cryopreserved in the presence of 4–8% v/v glycerol (Rodriguez et al. 1975, Robbins et al. 1976; for a review of various bovine sperm-preservation media, see Vishwanath & Shannon 2000). Stepwise addition of CPAs was performed at 25°C to minimize the osmotic injury and to lessen the volumetric excursions of bovine spermatzoa during the CPA-loading process (Liu & Foote 1998, Devireddy et al. 1999, 2000). At room temperature (25°C), a stock of 1.4 M CPA was added to the sperm cells in five equal volume steps at 5-min intervals such that the final concentration of the CPA was 0.7 M. The equilibration time and the number of steps were chosen on the basis of equations developed by Kedem and Katchalsky (1958), and utilizing the suprazerol (obtained in the absence of extracellular ice) membrane permeability parameter values \( L_p \sim 0.7 \text{ mm/min atm} \) and \( E_b \sim 10 \text{ kcal/mol} \) reported in the literature for bovine sperm (Watson et al. 1992, Chaveiro et al. 2004). After the addition of glycerol, the samples were concentrated by gentle centrifugation (as described earlier). For DSC experiments in the presence of CLC, the cells after equilibration with glycerol (as described earlier). For DSC experiments in the presence of CLC, the cells after equilibration with glycerol were incubated at room temperature (25°C) for 10 min with 1.5 mg/ml CLC, a value shown to be optimal for bovine spermatozoa during the CPA-loading process (Purdy and Graham 2004). As stated earlier, the spermatozoa were concentrated by gentle centrifugation (300, g, ~25°C) for 5 min and resuspended in residual supernatant, in preparation for the DSC experiments.

**DSC experiments**

DSC dynamic cooling experiments were performed on concentrated bovine sperm samples in standard aluminum sample pans (Perkin Elmer, Norwalk, CT, USA) in the presence of *Pseudomonas syringae* (ATCC, Rockville, MD, USA), a natural ice nucleator. Briefly, 1 ml semen was concentrated by centrifugation (300 g) for 5 min, at either room temperature or 4°C, and resuspended in ~25 μl residual supernatant. Approximately 10 μl of this sperm suspension were loaded in a DSC sample pan with ~0.1 mg *P. syringae*. The DSC dynamic cooling protocol used to measure the water transport during freezing of bovine sperm is the same as reported in earlier studies on mammalian and aquatic sperm cells (Devireddy et al. 1998, 2000, 2004, Thirumala et al. 2003, 2005, He et al. 2004, Pinisetty et al. 2005). Briefly, in the DSC technique, the following two heat releases from the same cell suspension (or tissue system) are measured:

1. during freezing of osmotically active (live) cells in medium (at which the intracellular water is being transported across the cell membrane to freeze in the extracellular space)

2. during freezing of osmotically inactive (dead) cells in medium.

The temperature dependence of the difference in the measured heat release between the two cooling runs is correlated to water transport. To ensure the accuracy and repeatability of the experimental data, a set of calibration and control experiments was performed as detailed previously for a DSC-7 (Perkin Elmer) machine (Devireddy et al. 1998).

**Translation of heat release to cell volume data for dynamic cooling**

The heat-release measurements of interest are \( \Delta q_dsc \) and \( \Delta q(T)_dsc \) and are the total and fractional difference between the heat releases measured by integration of the heat flows during freezing of osmotically active (live) cells in medium and during freezing of osmotically inactive (dead) cells in medium. This difference in heat release has been shown to be related to cell-volume changes in several biologic systems (Devireddy et al. 1998, 1999, 2002a, 2004, Thirumala et al. 2003, Pinisetty et al. 2005) by the following equation:

\[
V(T) = V_o - \frac{\Delta q(T)_dsc}{\Delta q_dsc} (V_o - V_b) \quad (1)
\]

Note that the heat release readings \( \Delta q(T)_dsc \) and \( \Delta q_dsc \) are obtained separately at a cooling rate of 20°C/min in the three freezing media studied; that is, with no CPAs, with glycerol, and with glycerol and CLC. The unknowns needed in Eqn (1), apart from the DSC heat-release readings, are \( V_o \) (the initial or the isotonic cell volume) and \( V_b \) (the osmotically inactive cell volume) and were taken from the literature (van Duijn 1960, van Duijn & van Voorst 1971, Drevius 1972, Hammerstedt et al. 1978, Cummins & Woodall 1985, Révay et al. 2004). As stated earlier, the concentration of cells used in the DSC experiments was significantly larger than the values recommended or used in conventional cryopreservation protocols. This increased cell concentration in the DSC experiments is essential to generate a measurable difference in the heat release (\( \Delta q_dsc \)) between the two cooling runs. However, according to our earlier measurements with lymphocytes, the increased cell concentration does not affect the ratio of \( \Delta q(T)_dsc \) and \( \Delta q_dsc \) or the DSC-measured water transport data obtained with Eqn (1) is not affected by cell concentration (Devireddy et al. 1998). Thus, the DSC-measured water transport obtained with Eqn (1) corresponds quite closely to the freezing behavior of bovine sperm cells under conventional cryopreservation protocols.

**Water transport model**

The reduction in cellular volume that occurs during freezing has been modeled thermodynamically (Mazur 1963, Levin et al. 1976) and is described by the
Following equation:
\[
d\frac{V}{dT} = -\frac{L_p A_r RT}{B} (C_f - C_o)
\] (2)
in which \(L_p\) the plasma membrane permeability to water, is defined as
\[
L_p = -\frac{L_{pg}[cpa]}{R} \exp\left(-\frac{E_{lp}[cpa]}{R} \left(\frac{1}{T} - \frac{1}{T_R}\right)\right)
\] (3)

where \(L_{pg}\) or \(L_{pg}[cpa]\) is the reference membrane permeability (\(\mu\)m/min-atm) at a reference temperature, \(T_R\) (= 273.15 K) in the absence and presence of CPA; \(E_{lp}\) or \(E_{lp}[cpa]\) is the apparent activation energy (kJ/mol) or the temperature dependence of the cell membrane permeability in the absence and presence of CPA; \(V\) is the sperm volume at temperature, \(T\) (K); \(A_r\) is the effective membrane surface area for water transport, assumed to be constant during the freezing process; \(R\) is the universal gas constant; \(B\) is the constant cooling rate (K/min); and \(C_f\) and \(C_o\) represent the concentrations of the intracellular and extracellular (unfrozen) solutions.

In the present study, we modeled the bovine sperm cell as a long cylinder with length \((L)\) of 39.8 \(\mu\)m and radius \((r_c)\) of 0.4 \(\mu\)m, which translates to an initial (or isotonic) cell volume \(V_o = 20 \mu\text{m}^3\) and \(A_r = 100 \mu\text{m}^2\) (Cummins & Woodall 1985). The osmotically inactive cell volume, \(V_b\), was taken to be 0.61 \(V_o\), a value reported earlier for bovine spermatozoa by Guthrie et al. (2002). The various assumptions made in the development of Mazur’s model of water transport are discussed in detail elsewhere (Mazur 1963, Levin et al. 1976, Toner 1993). The two unknown water transport 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, were determined by curve-fitting the water transport model to experimentially determined volumetric shrinkage data during freezing.

**Numerical methods**

A nonlinear, least-squares curve-fitting technique was implemented by a computer program to calculate the water transport parameters that best fit the volumetric shrinkage data, as previously described by Bevington and Robinson (1992). The optimal fit of Eqn (3) to the experimental data was obtained by selecting a set of parameters that minimized the residual variance (\(\chi^2\)) and maximized a goodness-of-fit parameter \((R^2)\) (Smith et al. 1998). All the curve fitting results presented have an \(R^2\) value greater than or equal to 0.98 indicating that there was a good agreement between the experimental data points and the fit calculated using the estimated water transport parameters.

**Theoretical prediction of optimal rates of cooling**

Thirumala and Devireddy (2005) reported that for a variety of biologic systems a comparison of the published experimentally determined values of \(B_{opt}\) (in °C/min) agreed quite closely with the value obtained with the following generic optimal cooling rate equation (GOCRE):

\[
B_{opt} = 1009.5 \cdot \exp\left(-0.054 E_{lp}\right) \cdot \left(\frac{SA}{WV}\right)
\] (4)

In Eqn 4, \(L_{pg}\) and \(E_{lp}\) represent the membrane permeability parameters (in \(\mu\)m/min-atm and kcal/mol respectively), while \(SA/WV\) (in \(\mu\)m) represents the ratio of the available surface area for water transport \((SA = A_r)\) to the initial volume of intracellular water \((WV = V_o - V_b)\). From the assumed values of \(V_o\) and cell dimensions, the ratio of \(SA\) to \(WV\) for bovine sperm is 12.5/\(\mu\)m. The use of Eqn (4) greatly simplifies the prediction of optimal freezing rates and is based on the assumption that the optimal rate of cryopreservation of any cellular system can be defined as the freezing rate at which 5% of the initial water volume is trapped inside the cells at −15°C (Thirumala & Devireddy 2005). Once \(L_{pg}\) and \(E_{lp}\) are determined by the fitting procedure described above, we propose to utilize Eqn (4) to predict the optimal rates of freezing bovine spermatozoa.

**Results**

**Semen collection**

The mean semen volumes per ejaculate and sperm concentration per ml were 7.9 ml (range = 2.1–11.5 ml) and 0.563 × 10^9 (range = 1.2–0.127 × 10^9) for the Aberdeen Angus bulls and 6.9 ml (range = 2.5–10 ml) and 0.473 × 10^9 (range = 0.302–0.689 × 10^9) for the Senepol bulls respectively. No bull had more than the accepted range for percent abnormal sperm for all bulls in the commercial stud (abnormal sperm under 30%). During the semen collection interval in this study, there was no statistical difference in semen volume per ejaculate and in sperm concentration per ml between the two bull breed types (P < 0.01). The overall mean semen volume (± S.E.) was 7.3 ± 1.1 ml per ejaculate with a concentration of 0.530 × 10^9 (± 0.105) sperm per ml for all bulls used in the experiment. The mean sperm concentration after extending was 0.301 × 10^9 (± 0.043) sperm per ml.

The mean percent progressive motility and the percent live sperm per ejaculate were 55% (range = 50–65%) and 67% (range = 60–80%) for the Aberdeen Angus bulls and 58% (range = 50–65%) and 76% (range = 65–85%) for the Senepol bulls. Moreover, no statistical difference was detected for percent progressive motility and percent live sperm between bull breed types. The overall mean percent progressive motility was 57%, and the percent live sperm per ejaculate was 73% for all bulls used in the study.

**Dynamic cooling response and water transport parameters**

Figure 1 shows a comparison of the water transport data at a cooling rate of 20°C/min for samples cooled without...
CPAs (Fig. 1A), with glycerol (Fig. 1B) and with CLC (Fig. 1C). The best-fit parameters for $L_{pg}$ and $E_{LP}$ are shown in Table 1. The volumetric response generated by these parameters in Eqn (2) are shown in Fig. 1 as solid lines. The model simulated equilibrium cooling response is also shown in Fig. 1A–C and is generated by setting the left-hand side (LHS) of Eqn (2) = 0 and balancing the intracellular and extracellular unfrozen chemical activity of water on the right-hand side (RHS) at a particular subzero temperature. Equilibrium is achieved at each temperature when the internal and external osmotic pressures are equal (i.e. $\pi_i = \pi_o$).

The experimentally measured water transport data obtained during freezing of bovine sperm that were cooled in the absence of CPAs (Fig. 1A) and in the presence of glycerol (Fig. 1B) were statistically significantly different from each other ($P < 0.01$), by Student’s $t$-test, over the entire temperature range of interest ($-0.53^\circ$C to $-25^\circ$C). Similarly, the water transport data obtained in the absence of CPAs (Fig. 1A) and in the presence of CLC (Fig. 1C) were also statistically different from each other ($P < 0.01$). Additionally, the differences in the water transport parameters between sperm that were cooled in the presence of glycerol (Fig. 1B) and in the presence of CLC (Fig. 1C) were also statistically significant, albeit at a lower confidence level ($P < 0.05$). For further analysis of the statistical significance of the differences in the measured water transport data between the three treatments (without CPAs, with glycerol, and with glycerol and CLC), we performed one-way analysis of variance.

**Figure 1** Volumetric response of bovine sperm as a function of subzero temperatures obtained by the DSC technique at 20 $^\circ$C/min. (A–C) Water transport data for bovine sperm with no CPAs, for bovine sperm loaded with glycerol and for bovine sperm treated with CLC respectively. The model-simulated equilibrium cooling response obtained is shown in all the panels, i.e. the line without any data points. The model simulated dynamic cooling response is shown as a solid line and was obtained by using the best-fit membrane permeability parameters ($L_{pg}$ and $E_{LP}$) shown in Table 1, in the water transport equation (Eqns (2) and (3)). The nondimensional volume is plotted along the y-axis, and the subzero temperatures are shown along the x-axis. The error bars represent the standard deviations in the data.
Table 1 Water transport parameters for bovine sperm in the absence of CPAs, with glycerol and with cholesterol-loaded cyclodextrin (CLC) at a cooling rate of 20 °C/min.

<table>
<thead>
<tr>
<th>Freezing media</th>
<th>$L_{pg}$ or $L_{pg}[cpa]$ (µm/min-atm)</th>
<th>$E_{lp}$ or $E_{lp}[cpa]$ (kcal/mol)</th>
<th>Optimal cooling ratea (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CPAs</td>
<td>0.036</td>
<td>42.1</td>
<td>45</td>
</tr>
<tr>
<td>Glycerol</td>
<td>0.025</td>
<td>30.9</td>
<td>58</td>
</tr>
<tr>
<td>CLC-treated</td>
<td>0.02</td>
<td>26.4</td>
<td>60</td>
</tr>
</tbody>
</table>

*Obtained with Eqn (4); see Materials and Methods for a more detailed description.*

(ANOVA) with SigmaStat from SPSS (Chicago, IL, USA; www.spss.com/software/science) at the usual probability level of $P = 0.05$. The ANOVA analysis confirmed the statistical significance of the differences in the measured water transport for bovine sperm frozen in the three media. Thus, the addition of either glycerol or CLC alters the bovine sperm membrane permeability to water during freezing. Finally, the differences in the measured water transport response among bulls were found to be either statistically not significant ($P < 0.99$) or statistically significant at $P < 0.01$.

Figure 2 shows the contour plots of the goodness-of-fit parameter ($R^2 = 0.98$) in the $L_{pg}$ and $E_{lp}$ (or $L_{pg}[cpa]$ and $E_{lp}[cpa]$) space that ‘fit’ the volumetric shrinkage data at 20°C/min without CPAs, with glycerol and with CLC. Any combination of $L_{pg}$ and $E_{lp}$ (or $L_{pg}[cpa]$ and $E_{lp}[cpa]$) shown to be within the contour will ‘fit’ the volumetric shrinkage data in that media with an $R^2$ value over 0.98. The common region within all the three contours represent the combination of $L_{pg}$ and $E_{lp}$ (or $L_{pg}[cpa]$ and $E_{lp}[cpa]$) that will fit the measured water transport data concurrently in the three media investigated. An examination of the contours suggests that the parametric space corresponding to the CLC-treated bovine sperm samples is almost completely enclosed within the corresponding contour obtained for bovine spermatozoa in the presence of glycerol. This suggests that the membrane transport properties obtained for CLC-treated samples can predict the water transport response of bovine spermatozoa in the presence of glycerol, while the converse is not necessarily true. Moreover, the contour space corresponding to bovine spermatozoa in the absence of CPAs is significantly larger than that obtained in the presence of glycerol or of CLC.

Optimal rates of cooling bovine spermatozoa

By incorporating the best-fit parameters of water transport (Table 1) into Eqn (4), the theoretically predicted optimal rates of freezing bovine sperm cell suspensions were obtained and are also listed in Table 1. For independent verification of the predicted rates of optimal freezing bovine spermatozoa, additional numerical simulations were also performed at various cooling rates (5–100°C/min) with Eqns 2 and 3 and the best-fit parameters. The results from the numerical simulations for cells that were frozen in the absence of CPAs, in the presence of glycerol and in the presence of CLC, are shown in Fig. 3A–C respectively. For example, an examination of the model simulations in Fig. 3C shows that bovine sperm cells cooled in the presence of CLC are essentially dehydrated at cooling rates under 60°C/min. At cooling rates over 60°C/min, the amount of water trapped inside the tissue cells increases rapidly with increasing cooling rate. A more detailed analysis of the water transport simulations was also performed, as described in earlier studies (Devireddy et al. 1999, 2000, 2004, Thirumala et al. 2003), to determine the optimal rate of freezing bovine sperm in the three freezing media investigated. Briefly, we found that the predicted optimal rates of freezing obtained by analyzing the water transport simulations are 43°C/min, 57°C/min and 62°C/min for bovine sperm frozen in the absence of CPAs, in the presence of glycerol and in the presence of CLC respectively. These values are within 5% of the values obtained with Eqn (4) and shown in Table 1. Thus, the predicted rates of optimally freezing bovine sperm obtained with Eqn (4) or by a detailed analysis of the water transport simulations (shown in Fig. 3) essentially agree and show that the theoretically predicted optimal rate of freezing bovine sperm is 45–60°C/min.

Discussion

Water transport parameters in the absence of extracellular ice

As mentioned earlier, there are currently no experimental techniques, with the exception of the DSC technique, that yield data on how spermatozoa dehydrate during freezing in the presence of extracellular ice. However, there are a few techniques, such as the time to lysis method (Drevius 1971, Watson et al. 1992), the Coulter Counter technique (Gilmore et al. 1995) and the stopped-flow method based on the self-quenching of an entrapped fluorophore (Curry et al. 2000, Chaveiro et al. 2004), to measure the volumetric response of spermatozoa to external changes in osmolality, at suprazero temperatures. The parameters obtained by these techniques are essentially in agreement: suprazero water transport permeability parameter of $L_p \sim 0.3–10\,\mu m/min$-atm; activation energy at suprazero temperatures of $E_a \sim 3–14\,kcal/mol$ (Curry 2000). More specifically, Drevius (1971) and Watson et al. (1992), using the time to lysis method, have reported a $L_p$ value for bovine spermatozoa of $\sim 10\,\mu m/min$-atm and an activation energy of $\sim 3\,kcal/mol$. Recently, Chaveiro et al. (2004), using the stopped-flow method, have reported a $L_p$ value for bovine spermatozoa of $\sim 0.68\,\mu m/min$-atm in the absence of CPAs and values of $L_p$ of $\sim 0.91–0.29\,\mu m/min$-atm in the presence of various CPAs, including glycerol. These earlier studies provide a good understanding of suprazero water (and CPA) transport response...
Figure 2 Contour plots of the goodness-of-fit parameter $R^2 (=0.98)$ for the parametric space of bovine sperm cooled in the absence of CPAs (A), with glycerol (B) and with CLC (C). A combined plot of all three contours is shown in panel D. The predicted best-fit parameters for the three media are shown as (#) for media with no CPA (A and D), as (+) for media with glycerol (B and D), and as (*) for media with CLC and glycerol (C and D) – see Table 1 for a listing of the best-fit permeability parameter values. The membrane permeability at 0°C, $L_{pg}$ (µm/min-atm) is plotted on the y-axis, while the apparent activation energy of the membrane, $E_{lp}$ (kcal/mol), is plotted on the x-axis.
for spermatozoa of various mammals, including man, ram, bull, rabbit and mouse (Watson 1995, Holt 2000).

**Convergence of theoretical and experimental optimal rates of freezing bovine sperm**

Unfortunately, the suprazero permeability parameters obtained in the absence of extracellular ice by the techniques described above cannot predict the experimentally determined optimal rate of freezing bovine sperm or other mammalian sperm (Ravie & Lake 1982, Duncan & Watson 1992). For example, an $L_p$ value of 0.5 µm/min-atm and an activation energy of 3 kcal/mol results in a theoretically predicted optimal rate of freezing bovine sperm of $\sim 5000^\circ$C/min, when, in fact, experiments show that the ‘optimal cooling rate’ for bovine spermatozoa is 30–100°C/min, with a major decrease in viability after cooling at 300°C/min (Rodriguez et al. 1975, Robbins et al. 1976, Foote & Parks 1993, Woelders 1997, Woelders et al. 1997, Woelders & Malva 1998, Kumar et al. 2003). One explanation of this discrepancy is that the values of water transport parameters at subzero temperatures in the presence of extracellular ice are markedly different than those reported in the literature at suprazero temperatures. In particular, if $L_{pg}$ at subzero temperatures is lower by two orders of magnitude than $L_p$ at suprazero temperatures, and $E_L$ at subzero temperatures is higher by a factor of five than the corresponding $E_a$ at suprazero temperatures, then the discrepancy between numerical simulations and

\[ \text{Figure 3} \text{ Volumetric response of bovine spermatozoa at various cooling rates as a function of subzero temperatures, using the best-fit water transport parameters (shown in Table 1). The changes in the normalized cell volume ($V/V_o$) as a function of temperature for different cooling rates are shown in the absence of CPAs (A), with glycerol (B) and with CLC (C) respectively. The water transport curves represent the model simulated response for different cooling rates (from left to right: 5, 20, 40, 60, 80 and 100°C/min), using the best-fit parameters shown in Table 1. The subzero temperatures are shown along the x-axis, while the nondimensional volume is plotted along the y-axis.} \]
Effect of extracellular ice on bovine sperm membrane transport properties

The water transport parameters obtained in this study by the DSC technique during freezing of bovine spermatozoa are significantly lower than the reported suprazero permeability values for other mammalian species (Curry 2000). A dissimilarity between the suprazero and subzero water transport parameters was also found for mouse (Devireddy et al. 1999, human (Devireddy et al. 2000), horse (Devireddy et al. 2002a,b), dog (Thirumala et al. 2003) and boar (Devireddy et al. 2004) sperm cells. This discrepancy between the membrane permeabilities may be associated with possible changes in the sperm cell plasma membrane during suprazero cooling, including a lipid-phase transition of 0–4°C (Noiles et al. 1995) and/or cold-shock damage or ‘chilling’ injury during cooling (Blok et al. 1976, Watson 1981, Steponkus 1984). These changes in membrane transport properties might also be associated with solidification in the extracellular medium (phase change) phenomena, including changes in the membrane fluidity (Drobnis et al. 1993) and lyotropic (i.e. independent of cooling rate) membrane phase changes and corresponding alterations of water transport (Caffrey 1987, Noiles et al. 1995). The presence of extracellular ice has also been shown to alter the cell membrane transport properties (McGrath 1988).

Effect of glycerol and CLC on bovine sperm membrane transport properties

As stated earlier, we have measured the effect of adding glycerol and CLC to the subzero water transport response of bovine spermatozoa. As shown in Table 1, addition of glycerol leads to a reduction in both the measured value of reference membrane permeability and the activation energy of bovine spermatozoa. This trend is consistent with results reported in previous studies on membrane permeability parameters of other mammalian sperm cells (Thirumala et al. 2003, Devireddy et al. 2004) and in isolated rat hepatocytes (Smith et al. 1998). Moreover, the addition of CLC further reduces the bovine sperm membrane water transport parameters, although the predicted rates of optimal freezing with CLC are within 10% of those obtained in the presence of glycerol (Table 1). As shown in Eqn (4), a decrease in the value of $L_{pg}$ lowers the predicted optimal cooling rate. However, decreasing $E_{lp}$ has the opposite effect. Thus, the decrease in the value of $E_{lp}$ coupled with an increase in the value of $L_{pg}$ in the presence of CLC does not significantly alter the predicted optimal rate of freezing CLC-treated bull sperm when compared with the optimal rate of freezing in the presence of glycerol. This result is intriguing and suggests that the beneficial effects of incubating bovine sperm with CLC prior to freezing partly depend on its ability to mediate membrane transport across the cell membrane, so far as this relates to the rate of intracellular water loss during an imposed freezing stress.

Although several studies (e.g. Graham & Foote 1987, Parks & Lynch 1992, De Leeuw et al. 1993, White 1993, Zeron et al. 2002, Amirat et al. 2005) have detailed the beneficial effects of adding lipids, cholesterol or fatty acids to biomembranes before cooling, we are unaware of any previous studies on the effect of adding CLC or other fatty acids to subzero water transport properties of cells. Although it seems reasonable to expect ‘alterations’ in the bovine sperm membrane transport properties in the presence of CLC, a recent study by Purdy et al. (2005) found that CLC treatment did not significantly alter membrane fluidity of bovine sperm after suprazero temperature changes. However, as we report in the present study, the addition of CLC does alter the water transport response (and hence the membrane permeability) of bovine sperm.
during freezing. It is possible that these measured alterations in the water transport response of bovine sperm are correlated to the beneficial effects of CLC during cryopreservation. Clearly, further studies are needed. It is also unclear whether varying the concentration of CLC from the 1.5 mg/ml used in the present study might result in corresponding changes to the measured subzero water transport response of bovine sperm. We propose to conduct, in future, similar experiments with bovine, equine and porcine spermatozoa for further delineation of the effect of CLC treatment on the subzero water transport response of mammalian spermatozoa.

Summary and Conclusion

In conclusion, water transport (volumetric shrinkage) data for bovine spermatozoa in the presence of extracellular ice, CPA (glycerol), and CLC during freezing were obtained by the DSC technique at 20 °C/min. The DSC volumetric data were curve fitted to a model of water transport (Eqns 2 and 3) to predict the water transport parameters (L_pe, E_pe and E_pe[CPA]) (or L_pe[CPA] and E_pe[CPA]). We modeled the bovine spermatozoa as a cylinder of length 39.8 μm and radius 0.4 μm with an osmotically inactive cell volume of 0.61 V_o. The ‘best-fit’ parameters of water transport for bovine spermatozoa in the absence of CPAs are L_pe = 0.0036 μm/min-atm and E_pe = 42.1 kcal/mol (goodness of fit, R_2 = 0.99); the corresponding parameters in the presence of 0.7 M glycerol are L_pe = 0.025 μm/min-atm and E_pe = 30.9 kcal/mol (R_2 = 0.99), and in the presence of 1.5 mg/ml CLC and 0.7 M glycerol, they are L_pe = 0.02 μm/min-atm and E_pe = 26.4 kcal/mol (R_2 = 0.99). The parameters obtained in this study are significantly different from the water transport parameters reported in the literature for bovine and other mammalian spermatozoa obtained in the absence of extracellular ice at suprazero (and subzero) temperatures. The new parameters obtained in this study help to explain the discrepancy between the predicted optimal cooling rates based on suprazero permeability parameters (on the order of thousands of degrees C per min) and the experimentally determined optimal cooling rates (on the order of tens of degrees C per min). We also report that the addition of CLC alters the bovine sperm subzero water transport properties. The experimentally determined water transport data and modeling presented here may lead to a rational way of optimizing freezing procedures for bovine spermatozoa.

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