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Brief Communication

Water transport in epididymal and ejaculated rhesus monkey (*Macaca mulatta*) sperm during freezing ☆

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ABSTRACT

In the present study, we report the effects of cooling ejaculated and epididymal rhesus monkey (*Macaca mulatta*) sperm with and without the presence of a cryoprotective agent, glycerol. Water transport data during freezing of ejaculated and epididymal sperm cell suspensions were obtained at a cooling rate of 20 °C/min in the absence of any cryoprotective agents and in the presence of 0.7 M of glycerol, as well. Using previously published values, the macaque sperm cell was modeled as a cylinder of length 73.83 μm with a radius of 0.40 μm and an osmotically inactive cell volume, V_b, of $0.772V_o$, where V_o is the isotonic cell volume. This translated to a surface area, SA to initial water volume, WV ratio of ~22 μm^{-1}. By fitting a model of water transport to the experimentally determined volumetric shrinkage data, the best-fit membrane permeability parameters (reference membrane permeability to water at 0 °C, L_{pg} or $L_{pg}[cpa]$ and the activation energy, E_{Lp} or $E_{Lp}[cpa]$) were found to range from: L_{pg} or $L_{pg}[cpa]$ = 0.0020–0.0029 μm/min-atm; E_{Lp} or $E_{Lp}[cpa]$) = 10.6–18.3 kcal/mole. By incorporating these membrane permeability parameters in a recently developed equation (optimal cooling rate, $B_{opt} = 1009.5 \cdot \exp^{(-0.0546 \cdot E_{Lp})} \cdot (L_{pg}) \cdot (\frac{SA}{WV})$; where the units of B_{opt} are °C/min, E_{Lp} or $E_{Lp}[cpa]$ are kcal/mole, L_{pg} or $L_{pg}[cpa]$ are μm/min-atm and SA/WV are μm^{-1}), we determined the optimal rates of freezing macaque sperm to be ~23 °C/min (ejaculated sperm in the absence of CPAs), ~29 °C/min (ejaculated sperm in the presence of glycerol), ~24 °C/min (epididymal sperm in the absence of CPAs) and ~24 °C/min (epididymal sperm in the presence of glycerol). In conclusion, the subzero water transport response and consequently the subzero water transport parameters are *not* significantly different between the ejaculated and epididymal macaque spermatozoa under corresponding cooling conditions.

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The ability to obtain and preserve viable semen from non-human primates post-mortem would be one approach to salvage a genetic resource when valuable males suddenly die. However, there is very little or no information available on the precise freezing characteristics and differences, if any, between ejaculated and epididymal non-human primate sperm. One such difference could be the relative ability of ejaculated and epididymal sperm to mediate freezing induced loss of intracellular water or water transport during freezing. Thus, the primary aim of this study is to measure the subzero water transport response of ejaculated and epididymal macaque spermatozoa at a cooling rate of 20 °C/min in two different media: (i) in the absence of any cryoprotective agents or CPAs and (ii) in the presence of 0.7 M glycerol. The experimental water transport response was analyzed to determine the differences, if any, between the cooling behavior of ejaculated and epididymal macaque sperm. The water transport response was also used to determine the macaque sperm membrane permeability parameters, and were subsequently used to calculate the optimal rates of freezing ejaculated and epididymal macaque sperm cells in the presence and absence of glycerol.

Materials and methods

Fresh semen was collected by penile electro-stimulation of two chair-trained males. To obtain epididymal sperm, testes were collected from animals at necropsy. Each epididymis was carefully removed from the testis and its contents squeezed into a standard petri dish. Fresh and epididymal sperm were then diluted with medium, counted, washed and extended in a standard egg yolk based diluent and placed into a 37 °C water bath for equilibration prior to evaluating progressive motility. The semen sample was then transported (Fed-Exed, overnight delivery) in a 50 ml conical tube, placed in a standard Styrofoam container to the LSU Bioengineering Laboratory for Differential scanning calorimetry (DSC)

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experiments. Ejaculates and epididymal semen samples were used randomly across the replicates of the DSC experiments and all experiments were completed within 3–6 h of delivery (or within 24–36 h after collection).

For DSC experiments in the absence of CPAs, macaque spermatozoa were concentrated by gentle centrifugation (300 g, ~25 °C) for 5 min and resuspended in residual supernatant. Similarly DSC experiments on macaque spermatozoa were also conducted in the presence of a permeating cryoprotective agent or CPA (0.7 M glycerol or 6% v/v glycerol). Stepwise addition of glycerol was performed at 25 °C to minimize the osmotic injury and to lessen the volumetric excursions of macaque sperm during the CPA loading process [1].

The DSC dynamic cooling protocol used to measure the water transport during freezing of macaque sperm is the same as reported in earlier studies on mammalian and aquatic sperm cells [2–5]. Briefly, in the DSC technique, two heat releases from the same cell suspension (or tissue system) are measured: (i) 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) and; (ii) 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 as:

$$V(T) = V_o - \frac{\Delta q(T)_{dsc}}{\Delta q_{dsc}} \cdot (V_o - V_b) \tag{1}$$

Note that the heat release readings $\Delta q(T)_{dsc}$ and Δq_{dsc} are obtained separately at a cooling rate of 20 °C/min in the two freezing media studied, i.e., with no CPAs and with glycerol for both the ejaculated and the epididymal macaque sperm. A cooling rate of 20 °C/min was chosen because prior studies have demonstrated that the water transport data obtained at a higher cooling rate (e.g. 20 °C/min) could predict the freezing behavior at lower cooling rates, while the converse is not true [2,4]. Presumably, because the data at 20 °C/min being farther away from equilibrium contains more dynamic information that cooling rates that are closer to equilibrium [3,5]. Thus, we chose to perform our water transport measurements at a cooling rate of 20 °C/min. The unknowns needed in Eq. (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 [6,7].

Mathematical model of water transport

The reduction in cellular volume that occurs during freezing has been modeled thermodynamically [8,9] and is described by the following relationship,

$$\frac{dV}{dT} = -\frac{L_p A_c RT}{B}[C_i - C_o] \tag{2}$$

with L_p, the plasma membrane permeability to water defined as,

$$L_p = L_{pg}[cpa]\exp\left(-\frac{E_{Lp}[cpa]}{R}\left(\frac{1}{T} - \frac{1}{T_R}\right)\right) \tag{3}$$

where L_{pg} or $L_{pg}[cpa]$ is the reference membrane permeability (μm/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_c 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); finally C_i and C_o represent the concentrations of the intracellular and extracellular (unfrozen) solutions.

In this study, we modeled the macaque sperm cell as a long cylinder with length (L) 73.83 μm and a radius (r_o) of 0.40 μm which translates to an initial (or isotonic) cell volume $V_o \sim 37\ \mu m^3$ and $A_c \sim 186\ \mu m^2$ [6]. The osmotically inactive cell volume, V_b, was taken to be $0.772V_o$, a value reported earlier for macaque spermatozoa [7]. The various assumptions made in the development of Mazur's model of water transport are discussed in detail elsewhere [8,9]. 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, are determined by curve-fitting the water transport model to experimentally obtained volumetric shrinkage data during freezing.

A nonlinear least squares curve-fitting technique was implemented using a computer program to calculate the water transport parameters that best-fit the volumetric shrinkage data as previously described [10]. 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.

Prediction of optimal cooling rates

Thirumala and Devireddy [11] reported that for a variety of biological systems a comparison of the published experimentally determined values of B_{opt} (in °C/min) agreed quite closely with the value obtained using a Generic Optimal Cooling Rate Equation (GOCRE) that defines:

$$B_{opt} = 1009.5 \cdot \exp^{(-0.0546 \cdot E_{Lp})} \cdot (L_{pg}) \cdot \left(\frac{SA}{WV}\right) \tag{4}$$

In this equation, L_{pg} and E_{Lp} represent the membrane permeability parameters (in μm/min-atm and kcal/mol, respectively), while the term SA/WV (in μm^{-1}) represents the ratio of the available surface area for water transport ($SA = A_c$) to the initial volume of intracellular water ($WV = V_o - V_b$). Based on the assumed values of V_b and cell dimensions, the ratio of SA to WV for macaque sperm is $\sim 22\ \mu m^{-1}$. The use of Eq. (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 [11]. Once L_{pg} and E_{Lp} are determined using the fitting procedure described above, we propose to utilize Eq. (4) to predict the optimal rates of freezing macaque spermatozoa.

Results and discussion

Semen was successfully retrieved from 17 males that were older than 4 years, while none of the males younger than 4 years yielded any sperm cells. The 17 remaining samples contained semen with an average sperm cell concentration of 144.0 million/mL, an average motility of 72.8% and a mean grade of 2.6. Age had no influence on initial motility or grade.

Fig. 1 shows a comparison of the water transport data from the ejaculated and epididymal macaque sperm at a cooling rate of 20 °C/min, without CPAs (Fig. 1A) and with glycerol (Fig. 1B). The best-fit parameters for L_{pg} and E_{Lp} are shown in Table 1. The volumetric response generated by using the best-fit parameters (taken from Table 1) in Eq. (2) are shown in Fig. 1 as solid lines (______). The model simulated equilibrium cooling response is also shown in Fig. 1A and B and is generated by setting the left hand side (LHS) of Eq. (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., $\pi_i = \pi_o$).

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