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Osmotic tolerance and membrane permeability characteristics of rhesus monkey (*Macaca mulatta*) spermatozoa ☆

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Abstract

Biophysical characteristics of the plasma membrane, such as osmotic sensitivity and water and cryoprotectant permeability are important determinants of the function of spermatozoa after cryopreservation. A series of experiments was conducted with rhesus macaque spermatozoa at 23 °C to determine their: (1) cell volume and osmotically inactive fraction of the cell volume; (2) permeability coefficients for water and the cryoprotectants dimethyl sulfoxide, glycerol, propylene glycol, and ethylene glycol; (3) tolerance to anisosmotic conditions; and (4) motility after a one step addition and removal of the four cryoprotectants. An electronic particle counter and computer aided semen analysis were used to determine the cell volume and permeability coefficients, and motility, respectively. Rhesus spermatozoa isosmotic cell volume was 27.7 ± 3.0 μm^3 (mean ± SEM) with an osmotically inactive cell fraction of 51%. Hydraulic conductivity in the presence of dimethyl sulfoxide, glycerol, propylene glycol, and ethylene glycol was 1.09 ± 0.30, 0.912 ± 0.27, 1.53 ± 0.53, and 1.94 ± 0.47 μm/min/atm, respectively. Cryoprotectant permeability was 1.39 ± 0.31, 2.21 ± 0.32, 3.38 ± 0.63, and 6.07 ± 1.1 ($\times 10^{-3}$ cm/min), respectively. Rhesus sperm tolerated all hyposmotic exposures. However, greater than 70% motility loss was observed after exposure to solutions of 600 mOsm and higher. A one step addition and removal of all four cryoprotectants did not cause significant motility loss. These data suggest that rhesus sperm are tolerant to hyposmotic conditions, and ethylene glycol may be the most appropriate cryoprotectant for rhesus sperm cryopreservation, as it has the highest permeability coefficient of the tested cryoprotectants.

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Due to their genetic similarity to humans, rhesus macaques are the most widely used non-human primate (NHP) by the international biomedical community [28]. Developing an effective method to cryopreserve rhesus sperm would contribute to maintaining the genetic diversity of the research population by improving breeding management through artificial insemination or other assisted reproductive technologies [55]. In addition, semen cryopreservation would allow the banking of important genetic lines which act as valuable models of human disease [38]. To date, there have only been a few live births reported using cryopreserved NHP sperm in combination with artificial insemination (chimpanzee [22], cynomolgus monkey [47], marmoset [39], and rhesus [44]). However, despite the potential importance of cryopreserved NHP sperm, to date there have been very few fundamental cryobiology studies focused on membrane permeability and structural characteristics of NHP sperm [40,43]. Captive-breeding programs perform repeated semen collection whenever a sample is needed. However, currently used semen collection procedures utilize penile- or rectal-stimulated electroejaculation, which can be discomforting as well as labor intensive [21,32]. It is also known that seasonal changes in semen collection and semen donor present variation in post-thaw survival [41]. Therefore, having the ability to cryopreserve semen from a certain male would allow a more uniform semen sample and obviate excessive collection.

Currently, in vitro fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) [25,26,55] as well as genetic modification techniques [5,6] are being developed for NHP. Appropriate cryopreservation protocols for NHP spermatozoa would facilitate successful implementation of many biomedical, assisted reproduction, and genetic engineering developments. There are several factors associated with cryopreservation which may lead to the loss of structural and functional integrity of spermatozoa [50,27,1]. During cryopreservation, cells are first exposed to hyperosmotic cryoprotective agent (CPA) solutions. This causes an initial efflux of intracellular water, followed by an influx of permeating CPA and water as the cells undergo equilibration.

Cells experience additional osmotic stress due to the change in solution osmolality as a result of extracellular ice formation during cooling and warming [48,56,34]. This series of osmotic changes in the cell environment during the cryopreservation procedure may adversely affect the integrity of the spermatozoa, resulting in the loss of the ability to participate in fertilization. To improve cryopreservation, it is necessary to perform systematic studies aimed at protecting the functional integrity of NHP spermatozoa. These include determination of methodologies for appropriate sperm handling, optimal CPAs, and optimal methods for their addition and removal. This study was conducted to develop a fundamental understanding of the osmotic characteristics of rhesus macaque spermatozoa to facilitate the improvement of rhesus spermatozoa cryopreservation.

Materials and methods

Animals

Rhesus macaques were housed and maintained at Yerkes National Primate Research Center. All animal procedures were conducted according to guidelines provided by institutional animal care and use committee protocols at Emory University. All research documented in this study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, National Research Council, 1996).

Semen collection

Semen used in this study was collected using penile electroejaculation from four adult animals (10–18 years old). The ejaculate was kept in an insulated thermos and transported to the laboratory within 30 min of collection. The sample tube was

then incubated at 37 °C in a humidified atmosphere containing 5% CO_2 in air allowing the semen to liquefy (10–15 min). The semen was extended 1:10 with a Hepes buffered Tyrodes lactate medium (TALP-Hepes [3]) containing 4 mg/ml bovine serum albumin to yield a concentration of about 100×10^6/ml. The medium and cell suspension were kept at room temperature. All experiments were conducted at room temperature (22–24 °C).

Media

A NaCl free TALP-Hepes solution was prepared according to the standard recipe without adding NaCl. Isosmotic and anisosmotic TALP-Hepes solutions were prepared by adding an appropriate amount of NaCl to yield solutions of 80, 160, 220, 260, 285, 350, 400, 600, 880, and 1200 milliosmolal (mOsm). Phosphate buffer saline (PBS) solutions were prepared by dilution of a 10× PBS stock to 160, 285, 600, and 860 mOsm. Solutions of dimethyl sulfoxide (Me_2SO), glycerol (Gly), propylene glycol (PG), and ethylene glycol (EG) were prepared at a concentration of 1 M in isosmotic (285 mOsm) TALP-Hepes. All chemicals were purchased from Sigma Chemical (Saint Louis, MO) unless otherwise noted. Osmolalities were determined using a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, Utah).

Experiment 1

Determination of the isosmotic (V_{iso}) and osmotically inactive cell volume (V_b) of rhesus spermatozoa.

Experimental design. This experiment was designed to test the hypothesis that rhesus sperm behave as linear osmometers. To test this hypothesis, equilibrium cell volumes were fitted to the reciprocal of the extracellular osmolality of the solution, which is described by the Boyle van't Hoff relationship:

$$\frac{V}{V_{iso}} = \frac{M_{iso}}{M} \cdot \left(1 - \frac{V_b}{V_{iso}}\right) + \frac{V_b}{V_{iso}}, \quad (1)$$

where V is the cell volume at osmolality M, V_{iso} is the cell volume at isosmolality (M_{iso}), and V_b is the osmotically inactive cell volume (including both cell solids and osmotically inactive water).

For this experiment we used an incomplete randomized design. Isosmotic cell volume was determined by adding 100 µl of a sperm cell suspension to 15 ml of isosmotic PBS, and cell volumes were computed by averaging triplicate measurements from an aliquot of sperm from each animal. Osmotically driven cell volume responses and V_b were determined by adding 100 µl of a cell suspension in isosmotic PBS to 15 ml of anisosmotic solutions of PBS (160, 600, and 860 mOsm). Due to small sample volumes and the number of experiments for this project, the volume of sperm from only one animal was able to be measured at the 860 mOsm level ($n = 1$); volumes at the other levels were computed from independent measurements on aliquots of an ejaculate from three animals ($n = 3$). A Coulter counter ZM model (Coulter Electronics, Hialeah, FL) with a 50-µm standard-resolution aperture tube was used to determine cell volume as described previously [20]. The cell volume changes were recorded kinetically during the shrink or swell period and the final cell volumes were determined after equilibration. Sperm cell volumes were calibrated for each anisosmotic solution using spherical styrene beads (Duke Scientific Corporation, Palo Alto, CA) with a diameter of 3 µm (14.14 μm^3). To record and analyze the data, the Coulter counter was interfaced to a microcomputer using a CSA-1S interface.

Data analysis. The cell volume data were analyzed using "Cell Size Analyzer" software (The Great Canadian Computer Company, Edmonton, Canada). Cell volume estimates were based on the median value of the distribution of the osmotically active population. The relationship between cell volume and solution osmolality was analyzed using the linear regression function in SigmaPlot (SPSS, Chicago, Il). The osmotically inactive volume fraction (V_b) was determined by extrapolating the osmotic cell volume relationship to infinite osmolality.

Experiment 2

Determination of water (L_p) and CPA (P_{CPA}) membrane permeability coefficients for rhesus spermatozoa.

Experimental design. This experiment was designed to test the hypothesis that the parameters of L_p and P_{CPA} are dependent on the CPA. To test this hypothesis, we used a pair of coupled non-linear equations that describe the cell volume and intracellular solute concentration as functions of time (derived by Kedem and Katchalsky [30]) to model the changes in cell volume resulting from exposure to solutions containing CPAs and to determine the parameters of L_p and P_{CPA} [14]:

$$\frac{dV}{dt} = L_p ART[(M_n^i - M_n^e) + \sigma(m_s^i - m_s^e)] \quad (2)$$

and

$$\frac{dm_s^i}{dt} = \frac{(1 + \bar{V}_s m_s^i)^2}{(V - V_b)} \left[\left[\bar{m}_s(1 - \sigma) - m_s^i \frac{m_s^i}{(1 + \bar{V}_s m_s^i)} \right] \times \frac{dV}{dt} + AP_{CPA}(m_s^e - m_s^i) \right], \quad (3)$$

where

$$0 < \sigma < 1 - \frac{P_{CPA}\bar{V}}{RTL_p}. \quad (4)$$

Non-interaction of solvent and solute transport was assumed [31]; therefore, σ was set to the upper limit as shown in Eq. (4). The surface area for rhesus sperm was calculated as described in van Duijn [49]. Biometrical parameters for rhesus sperm were taken from Cummins and Woodall [8]. Midpiece width (1.5 ± 0.013 μm (mean ± SEM)) for rhesus sperm was estimated from a total of 10 spermatozoa from an ejaculate from each animal in the present study using light micrographs obtained with a 100× objective lens.

For this experiment we used a fixed-effects, randomized complete block design, with CPA having four levels. An aliquot of an ejaculate from each of four animals was used ($n = 4$) for each treatment level. Sperm from each male was analyzed independently, and measurements on each aliquot were made in duplicate or triplicate, and averages of these were used in the data analysis. A Coulter counter ZM model (Coulter Electronics, Hialeah, FL) with a 50-μm standard-resolution aperture tube was used to determine changes in cell volume as described previously [37]. In the kinetic experiments, a 100 μl aliquot of cells, preloaded with 0.8 M CPA, was abruptly diluted into 10 ml of an isosmotic PBS solution, and this extracellular concentration was assumed to be invariant with time. The electronic signals from cell debris and non-dissociated cells (cell doublets or triplets) accounted for less than 5% of the total population. These were separated from signals of single cells by using appropriate Coulter counter settings and digital filtering (Fig. 1). The data were averaged over 100 ms intervals to reduce the size of the data set of the experiments. A commercial software package, MLAB (Civilized Software, Bethesda, MD), was used to solve Eqs. (2) and (3) using the Gear method [4]. The Marquard–Levenberg curve-fitting method [4], as implemented in MLAB, was used to fit the experimental data and determine the values of L_p and P_{CPA}. A fixed value for V_b, determined independently from the Boyle van't Hoff plot, was used in the fitting calculation.

Data analysis. These data were analyzed using the SAS system (The SAS institute, Cary NC), and the Tukey LSD test was used to make pairwise comparisons [53]. The α-level was chosen to be 0.05. There was no strong evidence for variance heterogeneity at the treatment levels.

Experiment 3

Effects of anisosmotic exposure on rhesus sperm motility.

Experimental design. This experiment was designed to test the hypotheses that increasing levels of anisosmotic exposure would decrease rhesus sperm motility, and this effect would be irreversible. The osmotic tolerance, using total motility as the endpoint, was determined using two treatments: (1) exposing cells to a series of anisosmotic TALP-Hepes solutions and (2) returning the cells to isosmotic conditions after anisosmotic exposure. For this experiment, we used a two-way, mixed-effects, repeated-measures model. Osmotic treatment was a within-group, repeated-measures factor, having two levels; osmolality was a between-group factor having 10 levels. An aliquot of an ejaculate from each of four animals was used for all combinations of factor levels (complete block design; $n = 4$).

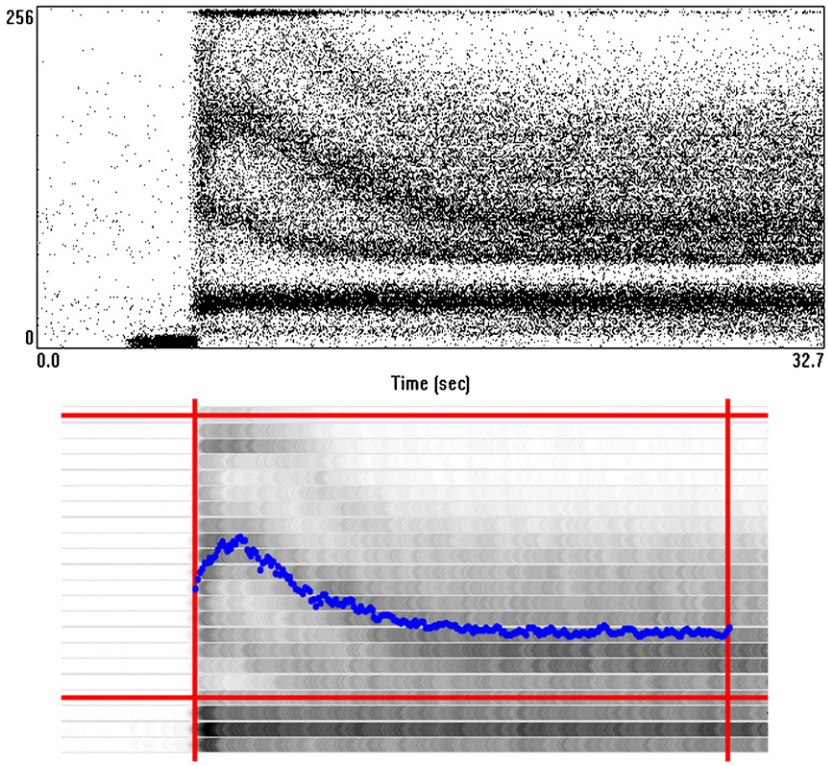

Fig. 1. This plot shows the cell volume change vs. time following an abrupt addition of a 0.8 M Me_2SO solution at room temperature. The upper panel shows the raw kinetic data points over time. The *x*-axis represents time and the *y*-axis represents the electronic signals which are proportional to cell volume. In the lower panel, the gray levels correspond to the cell count per unit time per unit volume. A filter window is defined by two vertical and two horizontal solid lines (top). Data points within the filter window are averaged to produce the final data set (blue dots) which is used for the curve fitting calculation. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

Sperm from each male was analyzed independently, and measurements on each aliquot were made in duplicate or triplicate, and averages of these were used in the data analysis. For the first treatment, a 10 μl aliquot of each sperm suspension was transferred into a 1.5 ml Eppendorf centrifuge tube containing 150 μl of the anisosmotic TALP-Hepes solutions, yielding a final concentration of approximately 10×10^6 spermatozoa/ml. A 5 μl aliquot from each anisosmotic treatment was transferred to a microcell (20 μl) stage, pre-heated to 37 °C, and allowed to equilibrate for 30 s. Motility was determined after a 5 min incubation at room temperature using a computer assisted semen analysis system (CASA; Hamilton Thorne, model HT M2030, Beverley, MA). A minimum of 200 cells was analyzed in 6–9 fields. Motility estimates were validated manually by the video playback option of the Hamilton Thorne instrument. For the second treatment, spermatozoa were returned to near isosmolality (270–325 mOsm) by transferring a calculated amount of an anisosmotic solution into the 1.5 ml Eppendorf centrifuge tube which contained the cell suspension [20]. Total motility was determined after a 5 min incubation period at room temperature.

Data analysis. These data were analyzed using the SAS system, with the α-level chosen to be 0.05. Multiple comparisons of the anisosmotic treatments to the isosmotic control was performed with the Dunnetts one-sided test [53]. No evidence for strong variance heterogeneity was found at the treatment levels.

Experiment 4

Effects of a one-step addition and removal of CPAs on motility.

Experimental design. This experiment was designed to test the hypotheses that motility would be reduced by the exposure to CPAs, and this effect would be irreversible. Total motility was determined after a 5 min exposure to 1 M Me_2SO, Gly, PG, or EG. Motility was again determined after a 5-fold dilution of the CPA with TALP-Hepes. TALP-Hepes with no CPA was included as one of the treatments. For this experiment we used a two-way, mixed-effects, repeated-measures design, with CPA treatment being the within-group, repeated-measures factor, having two levels (in CPA solution, and after CPA dilution); and the CPA being the between-group factor having four levels. An aliquot of an ejaculate from each of four animals was used for all combinations of factor levels (complete block design; $n = 4$). Sperm from each male was analyzed independently, and measurements on each aliquot were made in duplicate or triplicate, and averages of these were used in the data analysis.

Data analysis. These data were analyzed using the SAS system with the α-level chosen to be 0.05. Multiple comparisons of the CPA treatments to the non-CPA control were performed with the Dunnetts one-sided test [53]. No evidence for strong variance heterogeneity was found at the treatment levels.

Results

Experiment 1

The isosmotic cell volume of rhesus sperm was calculated to be $27.7 \pm 3.0\ \mu m^3$ (mean ± SEM). Analysis of cell volume in the various osmotic conditions indicated that rhesus sperm were linear osmometers in the range of 160–860 mOsm ($r^2 = 0.99$), and that 51% of the total cell volume was osmotically inactive (total solids plus osmotically inactive water, $V_b = 0.51$). A plot of this relationship is shown in Fig. 1.

Experiment 2

The experimental system which included Coulter counter, data acquisition hardware and software enabled the recording of real-time signals generated by particles flowing through the orifice of coulter counter tube. The magnitudes of these signals were proportional to the size of the particles (cells and debris). When these data are plotted as time vs. magnitude, the relationship appears as in Fig. 1. The values for the coefficients of L_p and P_{CPA} determined from the curve-fitting are listed in Table 1. None of the values for L_p were significantly different from one another ($p = 0.32$). In contrast, P_{EG} was significantly greater than P_{Me_2SO} ($p < 0.05$).

Experiment 3

The effects of the anisosmotic treatments on spermatozoa motility, normalized to the isosmotic treatment, are shown in Fig. 2. A greater proportion of the sperm lost their motility as the solution concentration in which the cells were incubated diverged relative to isosmotic. Motility of the sperm exposed to the treatment levels of 220–400 mOsm was not significantly different. However, the hyposmotic treatments of 80 and 160, and the hyperosmotic levels of 600, 880, and 1200 mOsm caused a significant decrease in motility compared to the isosmotic treatment. Few cells maintained motility

Table 1
Rhesus sperm water (L_p) and CPA (P_{CPA}) permeability coefficients at 22 °C

	L_p(μm/min/atm) (mean ± SEM)	P_{CPA}($\times 10^{-3}$ cm/min) (mean ± SEM)	σ (mean ± SEM)
Me_2SO	1.09 ± 0.30	1.39 ± 0.31*	0.959 ± 0.007
Glycerol	0.91 ± 0.27	2.21 ± 0.32	0.913 ± 0.020
Propylene glycol	1.53 ± 0.53	3.38 ± 0.63	0.900 ± 0.027
Ethylene glycol	1.94 ± 0.47	6.07 ± 1.1*	0.905 ± 0.032

* These values are significantly different ($p < 0.05$).

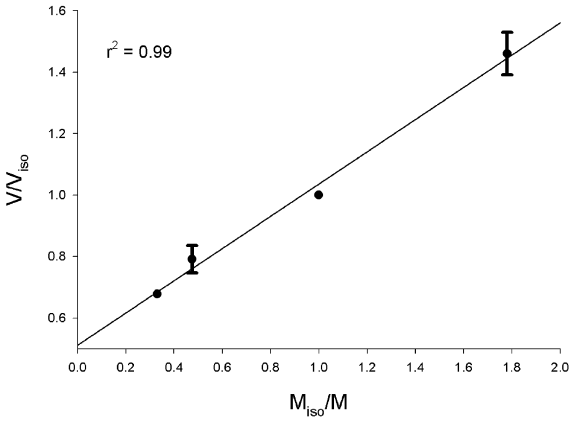

Fig. 2. Boyle-van't Hoff plot showing relative volume (mean ± SEM) of rhesus sperm exposed to various concentrations (160, 285, 600, and 860 mOsm) of NaCl. The *Y*-intercept indicates the osmotically inactive water volume (V_b), which is 51% of the isosmotic volume.

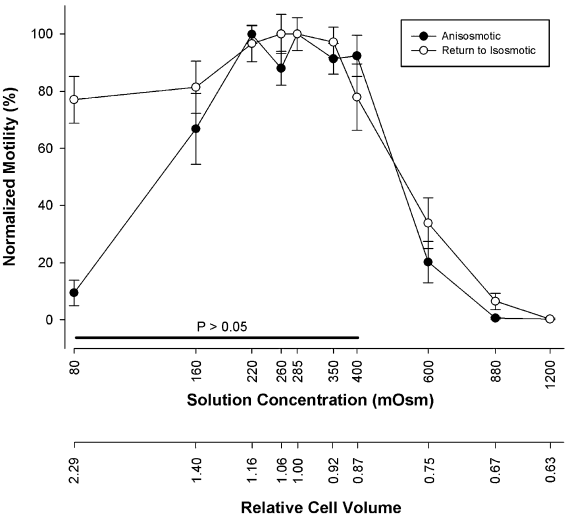

Fig. 3. The percentage of motile rhesus spermatozoa (mean ± SEM), normalized to motility at 285 mOsm, at each level of osmolality, for the two osmotic stress treatments. Treatments within the concentration range spanned by the horizontal bar did not cause a significant reduction in the motility of the spermatozoa after having been returned to isosmotic conditions. The second *x*-axis shows the equilibrium volumes that the sperm attain as a result of exposure to the treatment solutions.

during exposure to either 80 or 1200 mOsm. There was a significant interaction effect between osmolality and osmotic stress treatment. The cells exposed to the treatment levels of 80 and 160 mOsm recovered their motility to values similar to the untreated cells after being returned to isosmotic conditions. This was not true for the sperm exposed to 600, 880, or 1200 mOsm. Thus, the sperm having been returned to near-isosmotic conditions, after having been exposed to the treatment solutions ranging in concentration from 80 to 400 mOsm, were able to regain motility comparable to the sperm only exposed to isosmotic solutions.

Using electronic particle sizing data from Experiment 1 and the Boyle van't Hoff relationship, a volume change in the range of 84–130% normalized to isosmotic volume was found to be compatible with retention of the motility measured in an isosmotic environment (Fig. 2).

Experiment 4

The effect on the motility of rhesus sperm after exposure to a solution of 1 M Me_2SO, Gly, PG or EG, and subsequent dilution, normalized to the isosmotic treatment, is shown in Fig. 3. While the average motility in each solution was lower than the value for the cells only exposed to an isosmotic treatment, this difference was significant only for PG. After the 5-fold dilution, the motility of the sperm exposed to PG returned to a level similar to those only exposed to the isosmotic solution.

Discussion

Cryopreservation is a multi-step procedure in which cells are exposed to anisosmotic CPA solutions before cooling as well as during cooling and warming [35,56]. During each step, cells undergo volume excursions resulting from water and CPA transport across the plasma membrane, as a result of the concentration difference between the intracellular and extracellular compartments. These volume excursions may affect several cellular structures that are necessary for the sperm to fertilize an oocyte under either in vivo or in vitro conditions. It is desirable to achieve high post-thaw motility, but also to maintain complete functional integrity so that they may participate in fertilization without advanced assisted reproductive technologies such as IVF and ICSI. Current

protocols for cryopreserving primate semen use permeating (e.g., Gly, Me_2SO) and non-permeating media components (e.g., egg yolk, skim milk, raffinose, and lactose). While the protective action of egg yolk is due to preventing cold shock injury, the mode of action of permeating agents in protecting cells from damage has been attributed to their colligative action, preventing solutes from reaching harmful concentrations [36]. Addition of non-permeating mono- and disaccharides to the freezing medium will increase the likelihood of achieving intracellular vitrification by reducing the intracellular water concentration; these compounds can also act as an osmotic buffer during the dilution process [33]. It is our thesis that a better understanding of the effect of volume changes associated with the use of CPA will provide important information which may then be used for development of species-specific sperm cryopreservation procedures.

Regardless of its origin, each mammalian cell type has a unique size and morphology. It is well documented that there are significant morphologic differences between spermatozoa from different mammalian species [8]. The variations in sperm morphology among species becomes cryobiologically relevant due to different surface-to-volume ratios and osmotically inactive cell volumes, which are important factors governing water and CPA transport efficiency, as well as lethal intracellular ice formation temperatures at any given cooling rate [34]. Many mammalian cells, including spermatozoa, demonstrate an ideal osmotic response, where cell volume is a linear function of $(\text{osmolality})^{-1}$. The osmotically inactive cell volume fraction for rhesus spermatozoa (51%) determined in this study was very comparable with human (50%) [31], but lower than the values reported for bull (61%) [23], mouse (60.7%) [54], and boar spermatozoa (67.4%) [17]. This lower V_b determined for rhesus sperm suggests the possibility of relatively more water within the sperm from rhesus that would need removal by osmosis during freezing to minimize the formation of lethal intracellular ice compared to boar, bull and mouse spermatozoa under similar freezing regimes.

Various techniques including Coulter counter [20] electron microcopy [10], stopped-flow fluorometry [9], differential scanning calorimetry [12,13], and flow cytometry [57] have been employed to determine sperm characteristics. For example, while human sperm volume was calculated as 28.2 μm^3 with the use of Coulter counter [20], estimated volume for human sperm was relatively lower (22.2 μm^3) by utilizing electron microscopy [10]. The coulter counter method described in this report relies upon measurements of volume changes to determine permeability parameters. This can be challenging due to the morphologic characteristic of spermatozoon. Other techniques that have been utilized to determine membrane permeability values include flow cytometry, stopped-flow fluorometry, and differential scanning calorimetry. Many of these reports show that there is reasonable agreement among these techniques. Yeung et al, [57] compared the volumes measured by electronic sizing using a Coulter counter and flow cytometry in an effort to establish a reliable method for detecting changes in sperm volume in the presence of various osmolalities. In their study, flow cytometry analysis correlated well with volume measurement obtained by a Coulter counter ($R = 0.83$) suggesting that flow cytometry reflects Coulter counter findings for mouse sperm. Curry et al. [9] used stopped-flow fluorometry to measure the water permeability of boar, ram, and rabbit sperm, and they showed ''reasonably good agreement'' for boar sperm with previously published Coulter counter measurements 0.84 vs 1.03 μm/min/atm. The results of differential scanning calorimetry have been suggested to be more accurate in assessing sub-zero water permeability parameters compared to other techniques [11]. Unfortunately, this method is limited in that it cannot measure the permeability of cryoprotectants. The cell volume determined for rhesus spermatozoa in this study (27.7 μm^3) is comparable with human (28.2 μm^3; [20]) and boar (26.3 μm^3; [17]), but larger that of bull spermatozoa (23.5 μm^3; [23]). However, using the coulter counter technique Rutllant et al, [43] estimated rhesus sperm volume as 36.8 μm^3 which is relatively higher than the values reported here, and that for sperm from previous studies.

The volume changes cells undergo during the addition and removal of CPAs are determined by

the permeability of the cells to both water and the CPA. The degree of damage from osmotically induced volume changes depends on the magnitude of these volume changes and the osmotic tolerance. Therefore, controlling cell volume changes during this process is important for optimizing cryopreservation efficiency. These osmotically induced volume changes can be mathematically modeled once the permeability values have been determined, and, coupled with knowledge of the cells' osmotic tolerance, this modeling allows efficient determination of optimal CPA selection, as well as addition and removal procedures [16]. Optimization of spermatozoa cryopreservation becomes particularly important where frequent semen collection is not possible, or at least not desirable, such as with NHP. When the current rhesus spermatozoa membrane permeability values are compared with those from the human [20], mouse [42], and boar [19], there are some similarities, as well as marked differences (Table 2). The L_p values for rhesus and human sperm are similar in the presence of Me_2SO, Gly, and PG. However, the permeability of rhesus spermatozoa to water (1.94 μm/min/atm) in the presence of EG is about 2.5, 5, and 10 times higher to that for human (0.74 μm/min/atm), mouse (0.38 μm/min/atm), and boar (0.20 μm/min/atm) sperm, respectively. Given these lower L_p values of sperm from these species, poor cryosurvival outcome can be expected since slower water movement across the plasma membrane will cause greater osmotic stress to the cell during addition and removal of CPA. Therefore, optimal CPA addition and removal procedures, particularly for sperm from the boar and mouse, should be quite different. This information, in combination with the osmotic tolerance limits will make it possible to design stepwise methods to ensure that the cell's volume does not exceed those limits [16]. Another important observation from this comparison is that the average permeability of spermatozoa to EG from rhesus, human, mouse, and boar is the highest of all of the CPAs tested (Table 2). This suggests that EG may be a more appropriate CPA for these cells since the increased permeability will reduce the overall magnitude of osmotically driven volume changes during CPA addition and removal.

Tolerance to volume excursions is a biological property specific to a given cell type. Previous studies have shown that spermatozoa from different mammalian species have significantly different sensitivities to osmotically driven volume excursions [14,51,27]. Thus, osmotic tolerance limits need to be determined for spermatozoa from each species to predict optimal CPA addition and removal procedures to minimize detrimental volume excursions. The results of our experiments show that rhesus spermatozoa can tolerate exposure to extreme hyposmotic conditions. Although motility dropped nearly 90% while the cells were in the hypotonic media, approximately 80% of their initial motility was recovered when returned to isosmolality. It should be noted that the hyposmotic tolerance for rhesus sperm is greater than for sperm from other mammalian species studied to date, including mouse, human, boar, bull, and stallion [23,17,16,2,54,19]. Fig. 4 compares the osmo-

Table 2
A comparison of the permeability parameters of spermatozoa from rhesus, human, boar, and mouse

Species	L_p[1] (Me_2SO, Gly, PG, and EG)	P_{Me_2SO}[2]	P_{GLY}[2]	P_{PG}[2]	P_{EG}[2]
Rhesus	1.09, 0.91, 1.53, 1.94	1.39	2.21	3.38	6.07
Human[3]	0.84, 0.77, 1.23, 0.74	0.80	2.10	2.30	7.90
Boar[4]	0.12, 0.13, ND, 0.20	0.93	0.50	ND	2.0
Mouse[5]	ND, 0.38, 0.25, 0.38	ND	2.2	1.2	3.4

ND, not determined.
[1] μm/min/atm.
[2] $\times 10^{-3}$ cm/min.
[3] From [20].
[4] From [19].
[5] From [42].

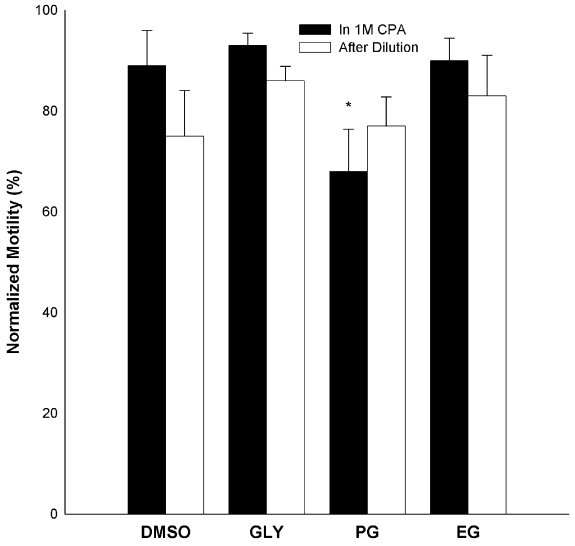

Fig. 4. The motility of rhesus spermatozoa exposed to a solution of 1 M dimethyl sulfoxide, glycerol, propylene glycol, or ethylene glycol, and after dilution (normalized to the isosmotic treatment; mean ± SEM). *Significantly different than the untreated sperm.

tic tolerance of spermatozoa from the rhesus, human, and boar. This comparison highlights the significantly higher degree of hyposmotic tolerance of rhesus spermatozoa relative to these other mammals. This extreme degree of tolerance suggests that after thawing, CPAs can be diluted from rhesus sperm in a single step (which was supported by the results from our final experiment). This will allow a rapid return of these cells to a medium more appropriate to their physiological requirements. This extreme tolerance to hyposmotic conditions was not mirrored when the cells were exposed to hyperosmotic solutions, however. While it was found that rhesus sperm were able to regain motility after exposures up to 400 mOsm, when the concentration increased above that level the spermatozoa were not able to recover their motility to pre-treatment levels. For hyperosmotic exposures, rhesus sperm motility responded intermediate to that of sperm from the boar and the human (Fig. 4).

Despite extensive efforts, sperm from the porcine, murine, equine, and canine remain difficult to cryopreserve. One of the common characteristics of sperm from these species is their high sensitivity to osmotic stress [19,54,2,46]. In addition, among those species studied, mouse and boar sperm showed significantly lower L_p values compared to rhesus and human spermatozoa [20]. According to fundamental cryobiology theory, the low osmotic tolerance and L_p may be largely responsible for previous failure of sperm cryopreservation attempts from these species. Therefore, more careful optimization is needed to cryopreserve sperm from these particular species using the known permeability values coupled with knowledge of the osmotic tolerance limits as was done for human spermatozoa [16].

To date, sperm from numerous other mammalian species have also been investigated for their hyperosmotic tolerance. Rhesus spermatozoa sensitivity to intermediate (350–600 mOsm) hyperosmotic conditions in this study is similar to the sensitivity of mouse, canine, and bull spermatozoa from previous studies [54,46,23]; although rhesus spermatozoa were slightly more tolerant to 600 mOsm than were sperm from bulls and mice. On the contrary, abrupt exposure of stallion spermatozoa to a 600 mOsm NaCl solution and return to an isosmotic condition was extremely detrimental to their motility [2]. It is important to note that among mammalian species studied to date, boar sperm motility has shown the highest osmotic sensitivity, being significantly reduced after exposure to only slightly hyperosmotic solutions [19].

Rutllant et al. [43] recently conducted a series of experiments to determine the effects of anisosmotic stress (75–900 mOsm) on rhesus sperm plasma membrane integrity, progressive motility, and mitochondrial membrane potential (MMP). They found that both plasma membrane integrity and motility are more sensitive to hyperosmotic than hyposmotic stress. In addition, they found that motility of spermatozoa was more sensitive to osmotic stress than was plasma membrane integrity. Our results agree in that high recovery of motility was observed following extreme hyposmotic treatment. However, one of the interesting observations from their study was that although a high percentage of spermatozoa subjected to a 600 or 900 mOsm solution had lower MMP compared to the untreated sperm, this difference was diminished when the sperm sample was returned to isos-

motic conditions. Their study also did not detect any correlation between MMP and motility after exposing rhesus spermatozoa to anisosmotic conditions, and the authors concluded that sperm motility is affected by factors other than mitochondrial integrity.

Determination of an optimal CPA addition and dilution protocol is an important aspect of developing a sperm cryopreservation procedure, since it is necessary to avoid osmotic damage as well as minimize the number of steps needed for effective cryopreservation. It should be noted that there may also be chemical effects (true chemical toxicity) of the CPA on functional integrity of spermatozoa, and this may be reduced by lowering the CPA equilibration time or temperature [24,29,7]. Although this aspect of CPA effects is beyond the scope of the current study, one could use the procedure described here to determine temperature dependent osmotic characteristics in an attempt to minimize cell injury. As with other cells, spermatozoa are relatively more resistant to volume changes if they are induced by hyperosmotic permeating CPA solutions. This is because isosmotic cell shape and volume is regained when equilibrium is reached; thus less volume change is experienced in terms of both magnitude and duration. Although the exact mechanism of hyperosmotic-induced cell death is not fully understood, the extended shrinkage of the cell may cause membrane folding and subsequent irreversible membrane fusion [52,15,54,1]. It is also possible that some damage to the sperm is caused by the high ionic strength of the solutions [46].

After the determination of rhesus spermatozoa membrane permeability coefficients to water and the four different permeating CPA, it was possible to calculate the volume excursion history of rhesus spermatozoa following one-step addition of a 1 M concentration of these CPAs, and a subsequent five-fold dilution (Fig. 5) [16]. The volume excursion was calculated to be greatest for Me_2SO, due to the larger P_{CPA} value. The range of cell volume excursion resulting from this treatment was between 0.8 and 1.27 times isosmotic. The calculated osmotic swelling from this treatment is not expected to cause a substantial loss in motility due to the rather extreme tolerance of rhesus spermatozoa to swelling as determined by Experiment 3 (Fig. 2). The highest degree of osmotic shrinkage (equivalent to an exposure to approximately 490 mOsm) lies just outside the range of anisosmotic values shown in our study to maintain a normal level of motility. Thus, it was anticipated that only a modest reduction in motility would occur from this treatment. While the average values for the motility after the Me_2SO addition and dilution were reduced, they were not significantly different from the motility of spermatozoa only exposed to an isosmotic solution. The motility reduction after CPA dilution for the other 3 CPAs was also not significant, as anticipated Fig. 6.

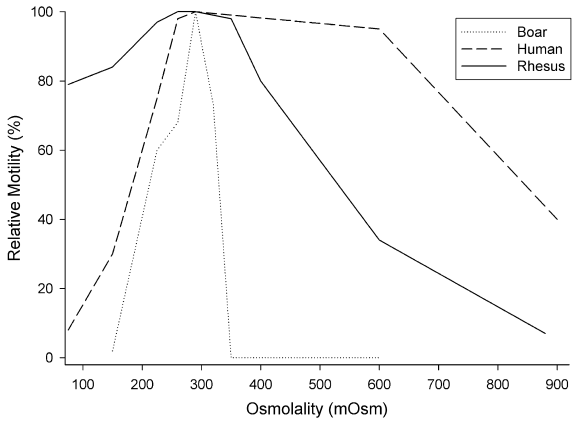

Fig. 5. A comparison of the osmotic tolerance of spermatozoa from rhesus, human, and boar.

Comparison of the present results with previous osmotic and CPA sensitivity studies conducted on spermatozoa from other mammalian species using the same CPAs (Me_2SO, Gly, PG, and EG) reveals insights into the osmotic nature of the effect of the CPA addition and removal. For example, one-step addition and removal of 1 M Gly resulted in a significant reduction in human sperm motility, and step-wise dilution was predicted, and subsequently shown, to increase the motility recovery rate [16]. Later, Gilmore et al. [18] found that EG is a superior CPA to maintain motility of human sperm cryopreservation than Gly, which can be explained by human spermatozoa's significantly higher P_{EG} (7.94×10^{-3} cm/min) values as compared to P_{Gly} (2.07×10^{-3} cm/min) [20]. In the stallion, abrupt removal of Gly in isosmotic media resulted in a

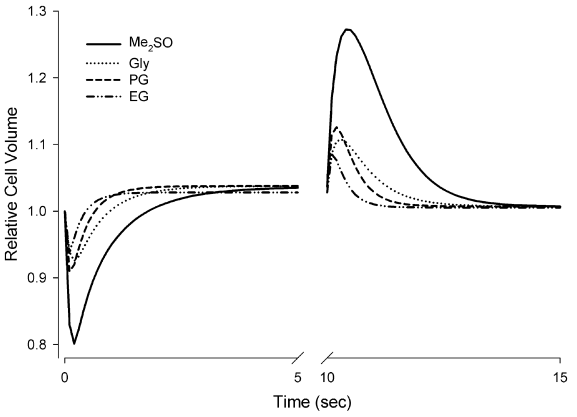

Fig. 6. Theoretical simulation of the rhesus spermatozoa volume change for the one-step addition and dilution of 1 M dimethyl sulfoxide, glycerol, propylene glycol, and ethylene glycol at 23 °C.

marked decline in sperm motility, membrane damage, and reduction of the mitochondrial membrane potential compared to the other CPAs [2]. In contrast, the abrupt removal of EG from stallion sperm resulted in the lowest decline in motility [2]. Songsasen et al. [46] showed that canine sperm are also sensitive to anisosmotic conditions, losing significant motility after having been exposed to a 0.6 M Me_2SO solution (900 mOsm). To our knowledge, the present report is the first to show that abruptly exposing spermatozoa to a 1 M CPA solution, and an abrupt dilution, had no significant effect on motility, which is consistent with the high level of osmotic tolerance of rhesus sperm compared to sperm from other species. The requirement for stepwise dilution of Gly from human sperm suggests that the hyposmotic swelling of the sperm during dilution is the cause of the motility loss seen. The significant difference in human sperm hyposmotic tolerance compared to rhesus sperm is consistent with the conclusion that the osmotic swelling during abrupt dilution of Gly from sperm from these two species is responsible for the differential sensitivity.

Recently, there have been two reports of successful cryopreservation of rhesus semen using a Tris–egg yolk based media containing 5% Gly. Sanchez-Partida et al. [44] obtained 87% post-thaw motility and a 63% fertilization rate after IVF, with no significant difference compared to fresh sperm (58%). However, they did show that acrosome integrity was lower for cryopreserved (74%) than fresh sperm (92%). Following AI, the pregnancy rate (62%) was comparable with fresh sperm (57%). Despite the lower post-thaw motility recovery (65%), Si et al. [45] reported a high fertilization rate after IVF (82%). These reports are very encouraging for the cryopreservation efforts of rhesus spermatozoa. Future studies should to be aimed at developing a further understanding basic sperm cryobiology for this and other NHP, including the determination of permeability values at cold temperatures. The knowledge would provide a foundation for the development of more reliable protocols, which could be used by national repositories to address global needs of assisted reproduction for NHP.

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