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Chimpanzee (*Pan troglodytes*) Spermatozoa Osmotic Tolerance and Cryoprotectant Permeability Characteristics

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ABSTRACT: Osmotic properties of chimpanzee spermatozoa were studied at 22°C. An electronic particle counter was used to determine the isosmotic cell volume, and the volume response after exposure to four commonly used cryoprotectants: dimethyl sulfoxide, glycerol, propylene glycol, and ethylene glycol. The data were analyzed to determine the hydraulic conductivity and the permeability coefficients for the four cryoprotectants. The osmotically inactive volume fraction was determined using a Boyle van't Hoff plot of cells exposed to sodium chloride solutions. A computer-assisted semen analysis system was used to determine the osmotic tolerance of chimp spermatozoa, as well as the effects of a one-step addition and removal of 1 M permeating cryoprotectant on sperm motility. The isosmotic volume of chimpanzee sperm is 27.7 μm^3. The osmotically inactive cell fraction is 69%. Hydraulic conductivity was higher in the presence of ethylene glycol: 4.09 $\pm$ 0.76 (mean $\pm$ SEM) and propylene glycol: 3.91 $\pm$ 0.71 as compared to dimethyl sulfoxide: 3.49 $\pm$ 0.79 and glycerol: 2.83 $\pm$ 0.40 μm/min per atmosphere. The permeability of chimpanzee sperm in ethylene glycol (2.18 $\pm$ 0.40 $\times 10^{-3}$ cm/min) and propylene glycol (1.75 $\pm$ 0.17 $\times 10^{-3}$ cm/min) was higher than in glycerol (1.42 $\pm$ 0.12 $\times 10^{-3}$ cm/min) and dimethyl sulfoxide (0.82 $\pm$ 0.015 $\times 10^{-3}$ cm/min). Although chimpanzee sperm tolerated osmotic stress in the range of 169–400 mOsm very well, loss of motility was observed as the solution concentrations diverged from isosmotic condition. Exposure to the four cryoprotectants at 1 M did not cause a significant reduction in sperm motility. This information on membrane permeability characteristics and cryoprotectant tolerance will aid in designing more reliable cryopreservation protocols for chimpanzee sperm.

Key words: Cryopreservation; membrane permeability.

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Semen cryopreservation has been used in agriculture and human reproductive medicine since the introduction of glycerol as a cryoprotectant (Polge et al, 1949; Polge and Rowson, 1952). However, spermatozoa from many species fail to survive after cryopreservation. Substantial interspecies variation in post-thaw sperm quality exists and this is consistent with the hypothesis that the fundamental cryobiological properties of sperm from different species varies significantly. This must be considered when developing cryopreservation procedures for different species.

In the context of conservation biology, significant numbers of nonhuman primate (NHP) species are currently at risk of extinction in wild populations because of habitat destruction and hunting (Morrell and Hodges, 1998). In addition, many NHP in captive breeding programs have significant reproductive problems compared to NHP living in their natural habitats around the world (Gould and Johnson-Ward, 2000). The preservation of germplasm from these species could help captive breeding programs, which are aimed at conserving genetic diversity. Therefore, there is a need for the development of effective germplasm cryopreservation methods for many NHP species. Procedures for the retrieval of oocytes and embryos from mammalian species are relatively complicated, requiring exogenous gonadotropin stimulation, followed by invasive surgical collection procedure. Furthermore, oocyte cryopreservation has proven to be very challenging for many mammalian species, including NHP (Critser et al, 2002).

The ability to cryopreserve semen from NHP in combination with assisted reproductive technologies (ART) such as artificial insemination (AI) would provide flexibility in conservation efforts (Gould and Styperek, 1989; Gabriel Sanchez-Partida et al, 2000; Watson and Holt, 2001; VandeVoort, 2004). Although sperm cryopreservation technology in combination with more advanced ART such as in vitro fertilization and intracytoplasmic sperm injection are currently being developed for some NHP species (eg, rhesus macaques, cynomolgus) with poor motility (Wolf et al, 1989), the overall success of these ARTs would benefit from the development of improved sperm cryopreservation protocols.

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Many studies have been conducted on human gamete cryobiology (Agca and Critser, 2002). Unfortunately, similar information for NHP, including the chimpanzee (Gopalakrishnan and Apkarian, 1990; Younis et al, 1998), vervet (Conradie et al, 1994), and rhesus monkey (Songsasen et al, 2002; Rutllant et al, 2003) is largely unknown. It is widely accepted that inappropriate semen handling before cooling is one of the prime reasons for low postthaw survival (Watson, 2000). Furthermore, factors such as individual differences, season, semen collection method (ie, penile electroejaculation, rectal probe), diluent components (eg, egg yolk, Tris buffer, sugars), and choice of permeating and nonpermeating cryoprotective agents (CPA) also have an impact on sperm survival (Holt, 2000).

During cryopreservation, cells experience osmotically driven volume changes as a result of CPA addition and removal before cooling, as well as during cooling and warming (Mazur, 1984). These transient volume excursions can be detrimental if CPA addition and removal is not performed in a manner that maintains the cell within the osmotic range that can be tolerated (Muldrew and McGann, 1994). Importantly, each CPA imposes different osmotic effects due to their membrane permeability coefficients (Gao et al, 1997; Gilmore et al, 1997). Therefore, using systematic quantitative approaches to cryopreservation that focus on the fundamental biophysical cell properties such as water and solute permeability coefficients and functional integrity is necessary to develop optimal cryopreservation protocols. In this study we performed a series of experiments that were aimed at providing a comprehensive understanding of the osmotic tolerance and permeability characteristics of chimpanzee spermatozoa to dimethyl sulfoxide (Me_2SO), glycerol (Gly), propylene glycol (PG), and ethylene glycol (EG).

Materials and Methods

Animals and Semen Collection

The chimpanzees (n = 4 males) were housed and maintained at Yerkes National Primate Research Center. All animal procedures were approved by the institutional animal care and use committee at Emory University. Semen was collected from sexually mature male chimpanzees (14–24 years old) by using a Yerkes-model artificial vagina (Gould, 1990). The ejaculate was kept in an insulated thermos at 37°C and transported to the laboratory within 30 minutes of collection. The sample tube was placed in an incubator at 37°C, which allowed the semen to liquefy (10–15 minutes). The semen was extended 1:10 with HEPES-buffered Tyrode's lactate medium (TL-HEPES) containing 4 mg/mL bovine serum albumin to yield a concentration of about 100 × 10^6/mL. All experiments were conducted at 22°C–24°C. The initial semen characteristics are given in Table 1.

Table 1. *Postcollection semen characteristics of the donor males*

Donor	Initial Motility (%)	Concentration (M/mL)
Donor chimp 1	91	123
Donor chimp 2	55	80
Donor chimp 3	80	240
Donor chimp 4	79	214

Media

A NaCl-free TL-HEPES solution was prepared and both hyposmotic as well as hyperosmotic TL-HEPES solutions were made by adding appropriate amounts of NaCl to yield solutions of 80, 160, 220, 260, 290, 350, 400, 600, and 880, mOsm. Phosphate-buffered saline (PBS) solutions were prepared by dilution of a 10× PBS stock to 160, 290, 600, and 860 mOsm. Solutions of Me_2SO, Gly, PG, and EG were prepared at a concentration of 1 M in isosmotic (290 mOsm) TL-HEPES. All materials were purchased from Sigma Chemical Co (St Louis, Mo) unless otherwise noted. Osmolalities were determined using a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, Utah).

Experiment 1: Isosmotic (V_{iso}) and Osmotically Inactive Cell Volume (V_b)

The goals of this experiment were: 1) to determine the isosmotic volume of chimpanzee spermatozoa; 2) to analyze the osmotic behavior of chimpanzee spermatozoa after exposure to anisosmotic solutions and to determine if the cells behave as linear osmometers; and 3) to determine the osmotically inactive volume of chimpanzee spermatozoa. Isosmotic cell volume was determined by adding 100 μL of a sperm cell suspension to 15 mL of isosmotic PBS (290 mOsm) and cell volumes were measured in triplicate at room temperature. Osmotically driven cell volume responses and the osmotically inactive cell volume (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). A Coulter counter ZM model (Coulter Electronics, Hialeah, Fla) with a 50-μm standard-resolution aperture tube was used to determine cell volume as described previously (Gilmore et al, 1995). The cell volume changes were recorded kinetically during the shrink or swell period and final cell volumes were determined by measuring the equilibrated states. Sperm cell volumes were calibrated for each anisosmotic solution using spherical styrene beads (Duke Scientific Corp, Palo Alto, Calif) 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 and the 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. Our hypothesis was that chimpanzee 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:

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

Table 2. *Reference chart used for performing osmotic treatments*

Osmolality (mOsm)	0	80	160	220	260	350	400	600	880
0	−150	−208	−342	−657	−1710	657	371	135	71
80	−107	−150	−246	−473	−1230	473	267	97	51
160	−65	−91	−150	−288	−750	288	163	59	31
220	−34	−47	−78	−150	−390	150	84	30	16
260	−13	−18	−30	−57	−150	57	32	11	6
350	34	47	78	150	390	−150	−84	−30	−16
400	60	84	138	265	690	−265	−150	−54	−28
600	165	230	378	726	1890	−726	−410	−150	−79
880	313	435	714	1373	3570	−1373	−776	−283	−150

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). An aliquot of the ejaculate from each of four animals was used for all combinations of factor levels (randomized complete block design; n = 4). These data were analyzed using the linear regression function in SigmaPlot® (SPSS; Chicago, Ill).

Experiment 2: Determination of Membrane Permeability Coefficients

A pair of coupled nonlinear differential equations that describe the cell volume and amount of solute in the cell as functions of time were derived by Kedem and Katchalsky (1958):

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

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

where noninteraction is assumed between water and CPAs where $0 < \sigma < 1 - P_s\bar{V}/(RTL_p)$.

An electronic particle counter (Coulter counter model ZM; Coulter Electronics) was used to measure the equilibrium volume of cells and changes in cell volume in response to anisosmotic conditions (McGann et al, 1982). In the kinetic experiments, a 100-μL aliquot of cells preloaded with 0.8 M CPA was abruptly diluted into 10 mL of an isotonic PBS solution and this extracellular concentration was assumed to be invariant with time. The electronic signals from cell debris and nondissociated cells (eg, cell doublets or triplets) accounted for less than 5% of total population and were separated from signals of single cells by using appropriate Coulter counter settings and by digital filtering. 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 Equations 2 and 3 using the Gear method (Bunow and Knott, 1995). The Marquard-Levenberg curve-fitting method (Bunow and Knott, 1995), as implemented in MLAB, was used to fit the experimental data and determine the values of the hydraulic conductivity (L_p) and cryoprotectant permeability (P_{CPA}, P_{Me_2SO}, P_{Gly}, P_{PG}, and P_{EG}) coefficients. A fixed value for V_b, determined independently from the Boyle van't Hoff plot, was used in the fitting calculation.

For this experiment we used fixed effects, randomized complete block design, with the main effect of 4 levels of CPA. We tested the hypothesis that the parameters of L_p, P_{CPA}, and σ are dependent on the type of CPA. An aliquot of an ejaculate from each of four animals was used for all factor levels. These data were analyzed using the SAS system with the α-level chosen to be 0.05, and the Tukey's least significant difference test was used to make pairwise comparisons (Westfall et al, 1999). Variance heterogeneity was not evident among the treatment levels.

Experiment 3: Effects of Anisosmotic Conditions on Chimpanzee Spermatozoa Motility

The goal of this experiment was to determine the effects of exposure to anisosmotic solutions on chimpanzee spermatozoa motility. The osmotic tolerance of chimpanzee sperm was determined using total motility as the endpoint. Two treatments were used: 1) exposure of cells to a series of anisosmotic solutions (80, 160, 220, 260, 350, 400, 600, and 880 mOsm); and 2) a return of the cells to isosmotic conditions after anisosmotic exposure. To determine the effects of exposure to anisosmotic solutions, a 10-μL aliquot of each sperm suspension (n = 4 males) was transferred into a 1.5-mL Eppendorf centrifuge tube containing 150 μL from one of the eight different osmotic solutions, yielding a final concentration of approximately 10×10^6 spermatozoa/mL. A 5-μL aliquot from each treatment was transferred to a preheated (37°C) microcell® (20 μL) stage and allowed to equilibrate for 30 seconds. Motility was determined after 5 minutes of incubation at room temperature using a computer-assisted semen analysis system (Hamilton Thorne, model HT M2030; Beverley, Mass). A minimum of 200 cells was analyzed in six to nine fields and three replicates were analyzed per male. Motility estimates were validated manually by the video playback option of the instrument.

For the second treatment, spermatozoa were returned to near isosmolality (approximately 290 mOsm) by transferring a calculated amount of an anisosmotic solution into a 1.5-mL Eppendorf tube. Various amounts of stock solutions were added to the tubes (see Table 2) to bring the cell suspensions back to isosmotic condition. For example, for the Eppendorf tube that contained solution at 350 mOsm, 47 μL of 80 mOsm stock solution was added to bring the cell suspension back to isosmotic condition. Motility was determined after 5 minutes of incubation at room temperature.

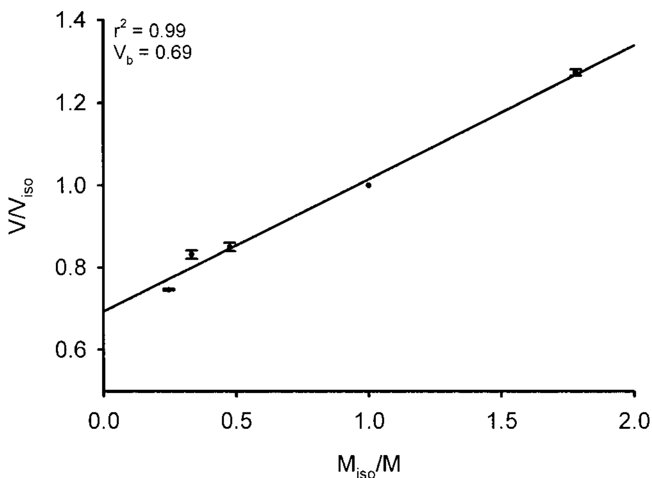

Figure 1. Boyle-van't Hoff plot showing relative volume (mean ± SEM) of chimpanzee sperm exposed to various concentrations (160, 290, 600, and 860 mOsm) of NaCl. The *y*-intercept indicates the osmotically inactive cell volume (V_b), which is 69% of the isosmotic volume.

For this experiment we used a two-way, mixed-effects model. Osmotic treatment was a repeated-measures factor, having two levels, and osmolality was a between-group factor having nine levels. These data were analyzed using the SAS system with the α-level chosen to be 0.05. Comparisons of the anisosmotic treatments to the isosmotic control were performed with the Dunnett's one-sided test (Westfall et al, 1999). Variance heterogeneity was not evident among the treatment levels.

Experiment 4: Effects of One-Step Addition and Removal of CPAs on Motility

The goal of this experiment was to determine the effects of an abrupt one-step addition and dilution of four CPAs on motility. In this experiment, a 10-μL aliquot of the sperm suspension from each donor was exposed to a 100-μL 1 M concentration of either Me_2SO, Gly, PG, or EG in Eppendorf centrifuge tubes. Motility was determined after 5 minutes of exposure to the CPAs. Then 400 μL of isosmotic TL-HEPES was added. The CPAs were removed by centrifuging the samples (400 × *g* for 5 minutes) and resuspending the sperm pellets in isosmotic TL-HEPES (190 μL) to a final volume of approximately 200 μL. Motility was determined after 5 minutes of incubation. For this experiment we used a mixed-effects, repeated-measures design, with exposure to CPA and TL-HEPES being repeated factors with two levels and CPA type having four levels. These data were analyzed using the SAS system, with the α-level chosen to be .05. Variance heterogeneity was not evident among the treatment levels.

Results

Experiment 1

The isosmotic cell volume of chimpanzee spermatozoa was calculated to be 21.7 ± 1.27 μm^3 (mean ± SEM). Analysis of cell volume in anisosmotic conditions indicated that chimpanzee sperm were linear osmometers in the range of 160 to 860 mOsm (r^2 = 0.99) and 69% of the total cell volume was osmotically inactive (total solids plus nonosmotically active water, V_b = 0.69). A Boyle van't Hoff plot of this relationship is shown in Figure 1.

Experiment 2

The changes in cell volume in the presence of Me_2SO, Gly, PG, and EG were measured over time, and these data were fitted to calculate L_p and P_{CPA} at 22°C. The values for these coefficients are listed in Table 3. None of the values for L_p were significantly different (P = .5855). However, the value of P_{CPA} for EG was significantly higher than that for Me_2SO (P = .0074).

Experiment 3

Figure 2 shows the percentage of motile spermatozoa, normalized to motility at 290 mOsm, as a function of osmolality for the two osmotic stress treatments. There was a significant interaction between the treatments (P = .0013). Loss of sperm motility increased as the solution concentration diverged from isosmotic. The osmolality levels of 80, 160, 600, and 880 caused a significant reduction in motility of sperm compared to those only exposed to an isosmotic solution. Few cells were motile after exposure to 80, 600, or 880 mOsm. Although the levels of motility increased upon return to isosmotic conditions, only the cells having been exposed to 160 mOsm regained a level of motility comparable to the untreated sperm.

Using the electronic particle sizing data from Experiment 1 and the motility curve data (Figure 2), as previously described, a volume change in the range of 91% to

Table 3. *Chimpanzee spermatozoa water and cryoprotectant permeability coefficient at 22°C*

	Mean ± SEM		
	L_p (μm/min/atm)	P_{CPA} (×10^{-3} cm/min)	σ
Dimethyl sulfoxide	3.49 ± 0.79	0.820 ± 0.015*	0.99 ± 0.0016
Glycerol	2.83 ± 0.40	1.42 ± 0.12	0.98 ± 0.0022
Propylene glycol	3.91 ± 0.71	1.75 ± 0.17	0.98 ± 0.0026
Ethylene glycol	4.09 ± 0.76	2.18 ± 0.40*	0.98 ± 0.0040

* These values are significantly different (P < .05).

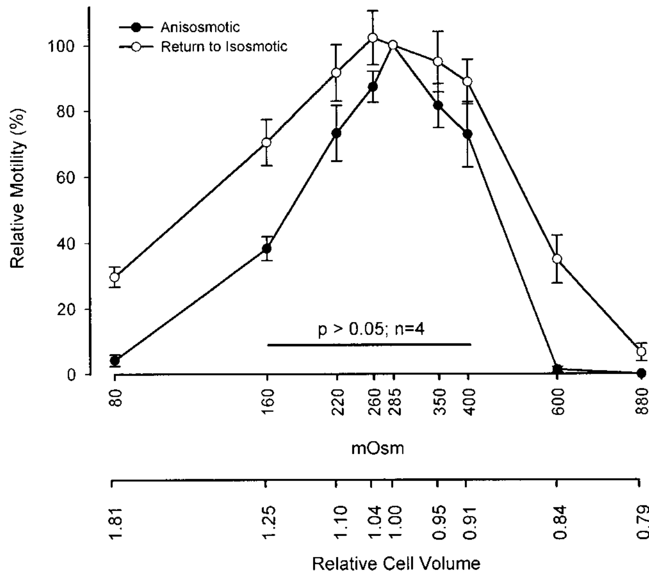

Figure 2. The percentage of motile chimpanzee spermatozoa (mean ± SEM), normalized to motility at 290 mOsm, for the two osmotic stress treatments. Treatments within the concentration range spanned by the horizontal bar did not cause a significant reduction in the motility of the spermatozoa after having been returned to isosmotic conditions ($P > .05$). The second *x*-axis shows the corresponding sperm cell volumes at the different treatment osmolalities.

125% (relative to isosmotic volume) was found to be compatible with retention of motility.

Experiment 4

Figure 3 shows calculated relative volume changes of chimpanzee spermatozoa during a one-step addition and fivefold dilution of 1 M Me_2SO, Gly, PG, and EG, on the basis of the values obtained from Experiments 1 and 2. Figure 4 shows chimpanzee sperm motility (normalized to the isotonic treatment) during exposure to solutions of 1 M Me_2SO, Gly, PG, and EG, and after their dilution. Although the average motility in each solution was lower than for the cells only exposed to an isotonic treatment, this difference was not significant ($P > .11$).

Discussion

Semen cryopreservation involves multiple steps from collection to long-term storage. Although it is generally assumed that most of the sperm cell injury is caused during the freezing, there is the potential for substantial damage during the course of sperm retrieval, handling (Alvarez et al, 1993; Gould and Young, 1996; Katkov and Mazur, 1999), and CPA addition and removal (Gilmore et al, 1997). These effects can be detrimental to the cellular and subcellular structures of the spermatozoa (Okada et al, 2001), potentially causing acrosomal vesiculation, fusion of the plasma membrane and acrosomal membranes, and

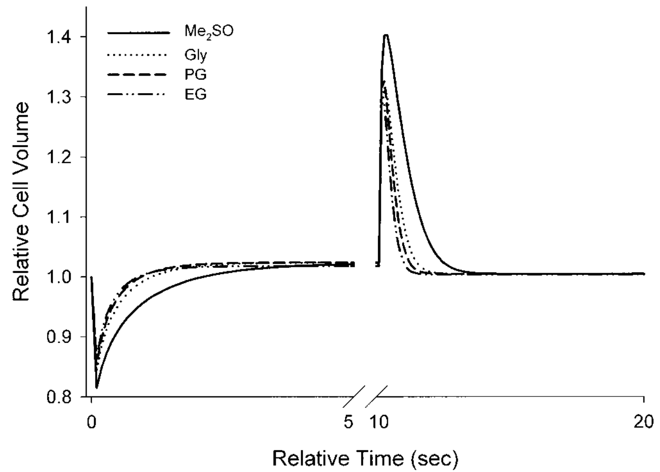

Figure 3. Theoretical simulation of the chimpanzee spermatozoa volume change for the one-step addition and dilution of 1 M dimethyl sulfoxide, glycerol, propylene glycol, and ethylene glycol at 22°C.

exocytosis of acrosomal content and sperm agglutination (VandeVoort, 2004). All of these potential alterations in sperm structure could cumulatively affect the function of sperm after cryopreservation, resulting in reduced ability to participate in fertilization (Watson, 2000).

To develop successful cell cryopreservation methods, it is imperative to understand cryobiologically relevant cell characteristics. The cell surface-to-volume ratio and the osmotically inactive cell volume are important factors related to formation of lethal intracellular ice during freezing. The isosomotic cell volume determined for chimpanzee spermatozoa in this study (21.7 μm^3) is smaller than that for human (28.2 μm^3; Gilmore et al, 1995), boar (26.3 μm^3; Gilmore et al, 1996), rhesus (36.8

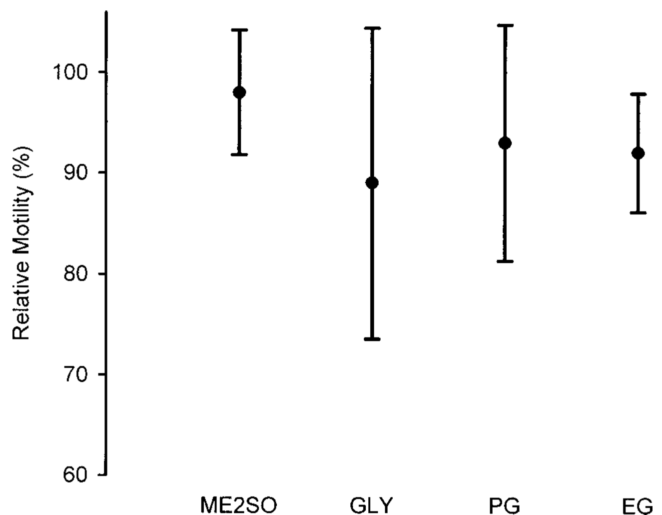

Figure 4. The motility of chimpanzee spermatozoa (normalized to the isosmotic treatment) exposed to solutions of 1 M dimethyl sulfoxide, glycerol, propylene glycol, or ethylene glycol after dilution (mean ± SEM). None of the cryoprotective agents had a significant effect on motility ($P > .05$).

μm^3; Rutllant et al, 2003), and bull spermatozoa (23.5 μm^3; Guthrie et al, 2002). The spermatozoa of most mammalian species studied to date behaved as ''ideal osmometers,'' in that their cell volume changes are linearly related to the reciprocal of the extracellular nonpermeating solute osmolality. The present study has demonstrated that chimpanzee sperm are also ideal osmometers. The osmotically inactive cell volume for chimpanzee spermatozoa (69%) determined in this study was higher than human (50%; Gilmore et al, 1995), bull (61%; Guthrie et al, 2002), mouse (60.7%; Willoughby et al, 1996), and boar spermatozoa (67.4%; Gilmore et al, 1996). This information is important in enabling mathematical modeling of the chimpanzee spermatozoa cell volume changes during series of anisosmotic exposures inherent in cryopreservation.

When cells are exposed to CPA solutions, they undergo volume excursion, first shrinking in response to an extracellular hyperosmotic solution, then returning to slightly greater volume than their initial isosmotic volume as the CPA enters the cell and water follows to maintain its chemical potential. The extent of the initial shrinkage and the subsequent time course of the cell to return to near-isosmotic volume are directly related to the cell's L_p and P_{CPA} coefficients. In general, higher L_p and P_{CPA} lead to smaller initial shrinkage and shorter time course of the cell to return to near-isosmotic volume. Knowledge of these parameters provides the means to predict optimal CPA addition and removal procedures (Gao et al, 1995).

These values allow the calculation of the volume history of the cell after exposure to a given CPA during its addition and removal, as well as during cooling and warming if activation energies of L_p and P_{CPA} are known (Mazur, 1970, 1984; Gao et al, 1995). This current finding is very consistent with human sperm permeability characteristics in the presence of the same CPAs, although the previously reported P_{EG} value is 3.6 times higher for human sperm than chimpanzee sperm (Gilmore et al, 1995). Furthermore, the L_p values for chimpanzee sperm in the presence of these CPAs is much higher than human spermatozoa (0.84, 0.77, 1.23, and 0.74 μm/min per atmosphere, respectively). Previously determined boar spermatozoa L_p values in the presence of Me_2SO, Gly, and EG (0.12, 0.13, and 0.20 μm/min per atmosphere, respectively) are also much lower than those for chimpanzee sperm, although P_{Me2SO} and P_{EG} values (0.93×10^{-3} cm/min and 2.0×10^{-3} cm/min) are comparable (Gilmore et al, 1998). Studies on sperm permeability characteristics of various mammalian species including mouse, boar, bull, and human show that spermatozoa from these species have different characteristics for different CPAs. However, it should be noted that one common characteristic of the sperm from all aforementioned species is the highest permeability to EG. During CPA dilution, cells may experience significant swelling due to rapid influx of water, which can be very damaging to sperm functional integrity. Rapid efflux of the CPA during its removal will reduce this swelling. The current results suggest that EG may be a superior CPA for cryopreserving chimpanzee sperm since the L_p and P_{CPA} values are the largest among those tested.

In this study, we determined that chimpanzee sperm motility loss was moderate (20%–25%) after exposure to intermediate hyposmotic (160 mOsm) or hyperosmotic (400 mOsm) solutions. Additional 65% to 70% motility loss occurred when the hyposmotic (80 mOsm) and hyperosmotic (600 mOsm) range was broadened. These data suggest that chimpanzee sperm are less sensitive to hyposomotic exposure than human sperm (Gao et al, 1995). Although human sperm lose about 70% of their motility after exposure to 160 mOsm and return to isosmotic conditions, chimpanzee sperm experienced only 30% motility loss under the same osmotic conditions (Gao et al, 1995). In contrast, when human and chimpanzee sperm were exposed to a 600 mOsm solution and subsequently returned to an isosmotic condition, human sperm maintained about 90% motility, whereas chimpanzee sperm retained only about 35% of their motility. This suggests that human spermatozoa are more robust to hyperosmotic conditions than chimpanzee sperm. To date, among the species studied, boar spermatozoa demonstrated the highest sensitivity to deviations from an isosmotic condition compared to human, bull, and rhesus on the basis of motility assessment (Gao et al, 1995; Guthrie et al, 2002; Rutllant et al, 2003). According to Gilmore et al (1998), boar spermatozoa can only swell to within 1.02 times and shrink to within 0.97 times their isosmotic volume and maintain at least 70% motility. Another study conducted on mouse sperm showed that their motility is substantially more sensitive to osmotic stress than their mitochondrial or plasma membrane integrity and thus mouse sperm should be maintained within 76%–124% of their isosmotic volume to maintain about 80% of pretreatment motility (Willoughby et al, 1996).

Ozasa and Gould (1982) determined that harmful effects of osmotic stress on chimpanzee sperm could be reduced by supplementing the freezing media with as little as 2 mM taurine. Similar results were also reported for cynomolgus monkey sperm (Li et al, 2003). It was determined that the addition of 5 mM proline, 10 mM glutamine, and 10 mM glycine to Tes-Tris–egg yolk freezing medium significantly increased post-thaw survival of cynomolgus monkey sperm assessed by motility and acrosome integrity. Although the true nature of the protection provided by amino acids is not known, the authors attributed improved survival to a possible osmoregulatory role during cryopreservation. Others also suggested that the beneficial effects of amino acids may be due to re-

duction of the formation of reactive oxygen species during the cryopreservation procedure (Alvarez and Storey, 1983a,b). In the same context, a similar effect was also observed when mouse sperm was exposed to anisosmotic challenges in the presence of two commonly used semen extenders, skim milk and egg yolk (Agca et al, 2002). For sperm cryopreservation, a one-step addition of a CPA at a concentration of 0.5–1 M in combination with whole milk or egg yolk–Tris (10%–20%) semen extenders is commonly used for many species. On the basis of our general knowledge of fundamental cryobiology of spermatozoa, the optimal CPA will be the one that most rapidly permeates the cell, causing the least amount of volume excursion during its addition and removal. The current study has demonstrated that chimpanzee sperm are most permeable to EG and least permeable to Me_2SO.

Using the permeability coefficients determined from the current study, theoretical volume excursions resulting from the addition and dilution of 1 M concentrations of Me_2SO, Gly, PG, and EG were calculated (Figure 3). This estimation revealed that the predicted relative volume expansion was as high as 1.6-fold (Me_2SO) and 1.4–1.5-fold for Gly, PG, and EG. It was previously shown that an abrupt addition of 1 M Gly caused less motility loss (20%) to human sperm compared to a one-step removal (60%) (Gao et al, 1995). Later studies conducted on other mammalian spermatozoa strongly supported this early observation, indicating that spermatozoa become dysfunctional when they experience high volume expansion. For example, it was shown for bull sperm that one-step addition and removal of 1 M concentration of Me_2SO, Gly, and EG caused 90%, 31%, and 6% motility loss, respectively (Guthrie et al, 2002). The motility loss reported for stallion sperm was greater (55%, 92%, 51%, and 24%) for Me_2SO, Gly, PG, and EG, respectively (Ball and Vo, 2001). It is notable that one of the common osmotic characteristics of the sperm from these species is their greater tolerance to one-step addition and removal of 1 M EG than one-step addition and removal of 1 M Gly, Me_2SO, or PG. The current study has shown that chimpanzee sperm exposed to 160 mOsm NaCl have the ability to recover 70% of their pretreatment motility, which is much higher than human, boar, bull, and stallion sperm (37%, 10%, 25%, and 41%, respectively) (Gilmore et al, 1995, 1996; Guthrie et al, 2002; Ball and Vo, 2001).

On the basis of these observations, we hypothesized that chimpanzee sperm will tolerate a one-step addition and removal of 1 M Gly, Me_2SO, PG, or EG without significant motility loss. The results demonstrated that this treatment resulted in a statistically insignificant reduction (approximately 20%) in motility compared to the control, supporting the hypothesis. In an early study by Sadleir (1966), chimpanzee sperm were frozen in either 7% Gly or Me_2SO in extender containing 20% egg yolk and 1.5% fructose (approximately 1100–1150 mOsm). It was concluded that Gly was relatively better CPA than Me_2SO for freezing chimpanzee sperm on the basis of post-thaw motility. Their observation is consistent with the current study in that the highest level of volume excursion is predicted for Me_2SO. Gould and Styperek (1989) froze chimpanzee sperm in Ham F10 medium supplemented with 15% human cord serum and either 7.8% Me_2SO or 7.8% Gly. They further demonstrated that despite the high post-thaw motility (70%), chimpanzee sperm had difficulty penetrating hamster oocytes under in vitro conditions (24.4%). After AI using frozen-thawed semen in their study, 2 of 11 (18%) recipient females conceived successfully. Younis et al (1998) investigated the effects of supplementing freezing media (7.8% Gly and test yolk buffer) with antifreeze peptide II (AFP) or insulin transferrin selenium (ITS) on chimpanzee sperm motility and acrosomal integrity. Although they found beneficial effects of supplementing freezing media with AFP and ITS on post-thaw sperm motility, acrosomal integrity was not protected at a similar level. Okada et al (2001) equilibrated cynomolgus monkey sperm in Tris buffer egg yolk containing 10% Gly (approximately 1470 mOsm). Post-thaw live fraction of the spermatozoa after Percoll separation had significantly higher acrosomal abnormalities (swollen, vesiculated, and ruptured) than control (79% vs 8.1%). The current finding is also in agreement with those previous studies in that high post-thaw motility recovery was achieved. Thus, the reduced ability to penetrate hamster oocytes of frozen-thawed chimpanzee sperm may be related to acrosomal integrity after freezing and thawing.

In conclusion, it appears from the present data that chimpanzee sperm have a high tolerance to anisosmotic conditions as assessed by maintenance of motility. Therefore, osmotic stress may not be the most likely reason for the poor fertility of frozen-thawed chimpanzee sperm observed in the previous studies. It may be that, although motility is protected to a high level, acrosomal integrity may be damaged by the same osmotic conditions. This raises an important aspect of chimpanzee sperm cryobiology that should be addressed in future studies.

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