Journal of Andrology, Vol. 24, No. 4, July/August 2003


# Osmotic Tolerance Limits and Properties of Rhesus Monkey (*Macaca mulatta*) Spermatozoa

JOSEP RUTLLANT, ANGELA C. POMMER, AND STUART A. MEYERS

*From the Sperm Biology Laboratory, Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, California.*

**ABSTRACT:** Fundamental cryobiological characteristics of rhesus spermatozoa must be determined for successful cryopreservation techniques to be established. The main objectives of the present study were to determine the osmotic behavior and osmotic tolerance limits of rhesus macaque spermatozoa. Cell volume changes over anisotonic conditions were assessed using an electronic particle counter and sperm motility was evaluated with a computer-assisted sperm analysis system. Analysis of membrane integrity and mitochondrial membrane potential was performed using flow cytometry. Rhesus monkey spermatozoa behave as linear osmometers in the osmotic range tested (75–900 mOsmol $kg^{-1}$), as shown by the Boyle van't Hoff plot ($r^2 = .99$). Rhesus spermatozoa have a mean cell volume of $36.8 \pm 0.5$ $\mu m^3$ at 22°C, with 77.2% of the intracellular volume being osmotically inactive. Results regarding sperm tolerance to osmotic stress showed that sperm motility was more sensitive than membrane integrity to deviations from isotonicity and, in addition, that rhesus sperm motility and membrane integrity were more sensitive to hypertonic than hypotonic conditions. Mitochondrial membrane potential did not explain the lack of sperm motility observed under anisosmolal conditions in our study. Although most spermatozoa were able to recover initial volume after osmotic stress, they were not able to recover initial motility.

Key words: Nonhuman primate, sperm, osmotic stress, motility, mitochondrial membrane potential.

**J Androl 2003;24:534–541**

Nonhuman primates have been a relevant model for the study of human disease because of the physiological and genetic similarities among members of the primate order. The preservation of nonhuman primates as important biomedical resources is critical, mainly for genetically unique or altered individuals. There is also an increasing need to preserve colonies of nonhuman primate species that are endangered because of human activity such as habitat destruction and hunting. The development of efficient nonhuman primate sperm cryopreservation would aid in successful propagation of germplasm through artificial insemination (AI) and in vitro fertilization (IVF) programs.

There have been attempts at freezing nonhuman primate sperm over the past 30 years, but reliable information on the most appropriate methods is limited (Morrell and Hodges, 1998). Cryopreserved sperm from some nonhuman primates have been used for AI and IVF, but macaque sperm, like human sperm, have highly variable cryoprotection requirements depending on the individual sperm donor. This biological variability has restricted progress in developing a cryopreservation protocol that preserves sperm motility in all semen samples.

Attempts to cryopreserve rhesus spermatozoa have been developed on the basis of empirical approaches (Leverage et al, 1972; Sanchez-Partida et al, 2000), and, as in other mammalian species, progress improving sperm survival could not be achieved simply by modifying established cryopreservation diluents (Hammerstedt et al, 1990). Cryopreservation requires the exposure of spermatozoa to extreme variations in temperature and osmolality. When a solution is cooled below the freezing point, pure water crystallizes out as ice, so that the solutes dissolved in the remaining liquid water fraction increases the osmotic strength of the solution (Watson, 2000). In the process of freezing cells in suspension, ice nucleation in the extracellular space creates osmotic pressure changes in the unfrozen fraction that affects the cells (Mazur, 1984; Watson and Duncan, 1988). It is generally recognized that the duration of exposure to such events should be minimized for optimal cell survival, implying that the cooling rate should be rapid. However, the cooling rate must be slow enough to allow water to leave the cells by osmosis, preventing intracellular ice formation, which is lethal (Mazur, 1984; Watson, 2000). To avoid these deleterious events and help cells to survive the process of cryopreservation, cryoprotective agents (CPAs) must be present during cooling and warming (Karow, 1969). During the addition of CPAs, cells gradually shrink as water

Correspondence to: Stuart A. Meyers, Sperm Biology Laboratory, Department of Anatomy, Physiology, and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA 95616 (phone: 530-752-9511; fax: 530-752-7690; e-mail: smeyers@ucdavis.edu).

Received for publication October 16, 2002; accepted for publication February 7, 2003.

flows out of the cell to a hypertonic environment, then cells return to normal volume as water and CPAs enter. The reverse process occurs during CPA removal. These osmotic events can severely damage the spermatozoa. A more fundamental understanding of the biophysical and biochemical characteristics that accompany the sperm freezing and thawing processes is an essential prerequisite to design successful cryopreservation protocols, as has been described for other mammalian spermatozoa (Holt and North, 1994). However, little information, if any, is known regarding the fundamental cryobiology and biophysics of nonhuman primate sperm.

There are primary biophysical properties with particular significance to cryopreservation, such as isosmotic cell volume ($V_{iso}$), which is the volume of the cell in osmotic equilibrium within an isosmotic solution; the osmotically inactive fraction of cell volume ($V_b$); the hydraulic conductivity ($L_p$), which reflects the membrane permeability to water; and the activation energy for $L_p$ ($E_a$), which is the temperature dependence of $L_p$. There are also crucial physiological properties that need to be preserved in order to be able to fertilize that can be affected as a result of osmotic stress, such as membrane integrity, motility, and mitochondrial membrane potential (MMP). Progressive motility is dependent on proper mitochondrial function, since these organelles must produce energy in the form of adenosine triphosphate (ATP) to power the flagellar motion that propels the sperm to the site of fertilization (Garner and Thomas, 1999).

Electronic particle counters have been used to determine membrane permeability characteristics in the sperm of several species—humans (Laufer et al, 1977; Gilmore et al, 1995), pigs (Gilmore et al, 1996, 1998a), mice (Willoughby et al, 1996), bulls (Petrunkina et al, 2001), and horses (Pommer et al, 2002). The advantages of electronic particle counters are that they allow rapid and reproducible collection and analysis of data, a variety of cell shapes and sizes can be analyzed without any required assumption regarding shape, and multiple cell populations can be studied in a bulk sample (Acker et al, 1999).

The main objectives of the present study were to determine the osmotic behavior and the osmotic tolerance limits of rhesus monkey spermatozoa. For the first objective, an electronic particle counter able to detect cell volume changes over anisotonic conditions was used. For the second objective, computer-assisted sperm analysis (CASA) and flow cytometry were used to analyze motility, viability, and MMP.

## Materials and Methods

### Chemicals and Reagents

Propidium iodide and JC-1 were purchased from Molecular Probes (Eugene, Ore). All other chemicals were obtained from Sigma Chemical Company (St Louis, Mo).

### Animals and Semen Sample Collection

Semen samples were obtained by electroejaculation from male rhesus macaques (*Macaca mulatta*) (n = 7) as described elsewhere (Sarason et al, 1991). Animals were housed at California National Primate Research Center and maintained according to Institutional Animal Care and Use Committee protocols at the University of California. Semen samples were collected into 15-mL centrifuge tubes that containing 6 mL of HEPES-Biggers, Whitten, and Whittingham or Dulbeccos' phosphate-buffered saline (DPBS). After 15 to 30 minutes, the coagulum was removed, the semen was diluted with an additional 6 mL of the same medium, and the sperm suspensions were evaluated for motility and viability. After centrifugation, the sperm pellets were resuspended, and samples were subsequently divided into aliquots according to the experimental design.

### Media

The media used were isotonic (300 ± 5 mOsmol $kg^{-1}$) and anisotonic DPBS. Anisotonic solutions were prepared by diluting isotonic DPBS (hypotonic solutions; 75 and 150 mOsmol $kg^{-1}$) and by diluting 10-strength DPBS (hypertonic solutions; 450, 600, and 900 mOsmol $kg^{-1}$) with reagent-grade water for experiments in which the mean cell volume was determined. To achieve the final osmolalities of 75, 150, 450, 600, and 900 mOsmol $kg^{-1}$ in the experiments in which the sperm motility, MMP, and viability were assessed, an adjusted set of anisotonic solutions was prepared (20, 115, 490, 675, and 1050 mOsmol $kg^{-1}$, respectively), to avoid the dilution effect (1:4) of the sperm sample in the anisotonic media. Final osmolalities were always confirmed using a vapor pressure osmometer (model 5100 C; Wescor Inc, Logan, Utah) that was calibrated against 100, 285, and 900 mOsmol $kg^{-1}$ standards for accuracy within ±5 mOsmol $kg^{-1}$.

### Experimental Design

*Experiment 1. Osmotic Behavior of Rhesus Monkey Spermatozoa*—A Coulter Counter (Z2 model; Coulter Corp, Miami, Fla) with a standard 50-μm aperture tube was used. Sperm mean cell volume (MCV) was calibrated using spherical polystyrene latex beads of 3 different diameters (3, 5, and 10 μm, CC Size Standard 6602793, 6602794, and 6602796, respectively; Coulter Corp, Miami, Fla) measured in each of the iso- and anisotonic solutions used, as suggested by the manufacturer. The Coulter counter was interfaced to a microcomputer, and data were acquired using specific software (Accucomp; Coulter Corp, Miami, Fla). Twenty microliters of sperm suspension adjusted to $20 \times 10^6$ cells $mL^{-1}$ was placed in 20 mL of isotonic or anisotonic DPBS (final concentration 20 000 cells $mL^{-1}$). After 10 minutes, a histogram displaying particle count versus volume (cell volume distribution) was recorded, as well as different statistical parameters (mean, mode, median, and standard deviation of volume). To observe whether sperm cells subjected to anisotonic conditions could recover the isotonic volume, 20 μL of sperm suspension of a solution of $20 \times 10^6$ cells $mL^{-1}$ were placed in 2 mL of anisotonic DPBS. After 10 minutes, 18 mL of isotonic DPBS were added, and volume determinations were performed after 5 more minutes (Gilmore et al, 1998a). Sperm incubated in 300 mOsmol $kg^{-1}$ DPBS were used as control samples.

To determine the inactive cell volume and estimate whether monkey sperm membranes behave as linear osmometers, sperm volumes recorded in iso- and anisotonicity conditions were fitted to the Boyle van't Hoff equation:

$$\frac{V}{V_{iso}} = \frac{M_{iso}}{M}\left[1 - \frac{V_b}{V_{iso}}\right] + \frac{V_b}{V_{iso}}$$

where $V$ is the cell volume at the osmolality $M$, $V_{iso}$ is the cell volume at isotonicity ($M_{iso}$), and $V_b$ is the osmotically inactive cell volume.

*Experiment 2. Osmotic Tolerance Of Rhesus Monkey Spermatozoa*—Evaluation of sperm motility: For motility experiments, 50 μL of the sperm suspension ($150 \times 10^6$ ml$^{-1}$) was placed in 200 μL isotonic DPBS (300 mOsmol kg$^{-1}$) or a range of anisotonic DPBS solutions to get a final concentration of 75, 150, 450, 600, and 900 mOsmol kg$^{-1}$ and incubated at 22°C for 10 minutes ($30 \times 10^6$ mL$^{-1}$) prior to motility analysis. Sperm suspensions were then returned to near-isotonic conditions by adding 1.25 mL of isotonic DPBS ($5 \times 10^6$ mL$^{-1}$), as described by Gilmore et al (1998a). After 5 minutes of incubation, motility was analyzed again. At least 200 cells were evaluated using CASA for all experiments in which motility was determined (CEROS, version 10.9i; Hamilton Thorne Biosciences, Inc, Beverly, Mass). The settings used were: frame rate 60 Hz, frames acquired 30, minimum contrast 30, minimum cell size 4, threshold straightness 80, medium average path velocity (VAP) cutoff 25, low VAP cutoff 5, low straight line velocity cutoff 10, nonmotile head size 12, nonmotile head intensity 80, static size limits 0.5 to 2.5, static intensity limits 0.55 to 1.4, and static elongation limits 0 to 80.

Evaluation of mitochondrial function: For MMP experiments, 200 μL of the sperm suspension ($150 \times 10^6$ mL$^{-1}$) was added to 800 μL of isotonic or anisotonic DPBS, for a final sperm concentration of $30 \times 10^6$ mL$^{-1}$, and incubated at room temperature (22°C) for 10 minutes. Then, each 1-mL sample was divided into 2 500-μL samples. To the first set of tubes in each treatment, a final concentration of 2 μM JC-1 was added for 10 minutes, and the samples were evaluated using a flow cytometer, as described below. In the second set of tubes, sperm were diluted with 1.5 mL of isotonic DPBS ($7.5 \times 10^6$ mL$^{-1}$) for 10 minutes and then incubated for another 10 minutes with 2 μM JC-1 before being analyzed on the flow cytometer. Samples were also visually assessed by epifluorescence microscopy by a single observer and evaluated for the uptake of dye and fluorescence pattern.

Determination of viability: The same protocol as described in ''Evaluation of Mitochondrial Function'' was used for the viability studies. The only difference was that propidium iodide (5 μM) was used instead of JC-1.

Flow cytometry: Information on 10 000 spermatozoa from each sperm sample used in the evaluation of mitochondrial function and determination of viability studies were collected using a FACS analyzer flow cytometer (FACSCalibur; Becton-Dickinson Immunocytometry Systems, San Jose, Calif), and generated data were examined using CellQuest software (version 3.3; Becton-Dickinson Immunocytometry Systems, San Jose, Calif).

Statistical analysis: Data were analyzed using one-way analysis of variance (ANOVA) with Minitab statistical software (Minitab Inc, State College, Penn), with a level of significance set at 5%. ANOVA was used to evaluate treatment differences over a range of anisotonic treatments. End points included sperm total motility, viability, MMP, and MCV. Data were also blocked by individual male and pooled if no significant differences were observed among males. In some experiments, data were normalized to control values prior to analysis. Data are presented as means ± standard error (SE).

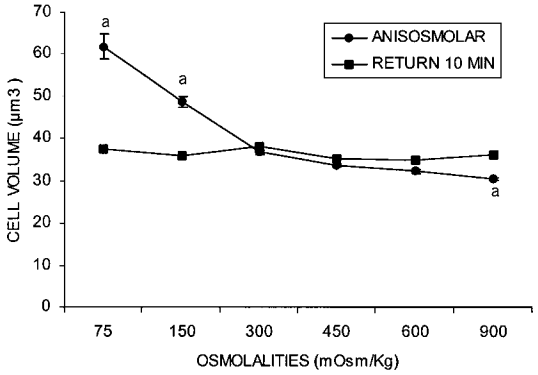


Figure 1. Sperm samples were incubated for 10 minutes at room temperature in isotonic or anisotonic buffer. MCV was then determined using a Beckman Coulter Counter (Z2). In separate tubes, after the initial 10-minute incubation, samples were diluted with isotonic buffer and incubated for 10 minutes before volume was determined. Superscript (a) denotes significance from control (300 mOsmol kg$^{-1}$) volume (n = 5 monkeys).

## Results

### Experiment 1

The isotonic MCV (mean ± SE) of rhesus monkey spermatozoa was determined to be 36.8 ± 0.5 μm$^3$ at 22°C measured with an electronic particle counter, with no significant difference among males ($P = .958$). The MCV was different from the control volume (300 mOsmol kg$^{-1}$) in 75, 150, and 900 mOsmol kg$^{-1}$ anisotonic solutions ($P < .05$) but not statistically different in 450 and 600 mOsmol kg$^{-1}$. Spermatozoa in all treatments were able to recover initial volume when returned to isotonic medium ($P > .05$) (Figure 1). MCV swelled approximately 1.68 times initial volume when exposed to 75 mOsmol kg$^{-1}$ DPBS and decreased to 0.83 times initial volume when incubated in 900 mOsmol kg$^{-1}$ DPBS (Table).

The osmotic response of monkey spermatozoa was determined over the range 75 to 900 mOsmol at 22°C. Data are presented as a Boyle van't Hoff plot (volume vs 1/osmolality) and depicts a linear response ($r^2 = 0.99$) and a $V_b$ of 77.2% (Figure 2).

### Experiment 2

*Evaluation of Sperm Motility*—Normalized total sperm motility was significantly lower ($P < .05$) in 75, 600, and

*Normalized monkey sperm cell volume, motility, and viability after incubation in anisotonic solutions for 10 minutes and returning to isotonic conditions and MMP in anisotonic DPBS*

| Osmolality (mOsmol kg$^{-1}$) | Normalized | | | High |
|---|---|---|---|---|
| | Volume | Motility (%) | Viability (%) | MMP (%) |
| 75 | 1.68 | 63.76 | 84.92 | 81.77 |
| 150 | 1.32 | 89.88 | 95.67 | 79.49 |
| 300 | 1 | 100.00 | 100.00 | 76.93 |
| 450 | 0.92 | 79.76 | 98.78 | 62.48 |
| 600 | 0.88 | 30.59 | 86.62 | 41.78 |
| 900 | 0.83 | 8.94 | 80.36 | 41.15 |

900 mOsmol kg$^{-1}$ anisotonic conditions compared with controls (Figure 3). However, statistically significant differences in sperm motility were not observed ($P > .05$) when incubated in 150 and 450 mOsmol kg$^{-1}$ and compared with controls.

When sperm were returned to isosmolal conditions, only sperm previously incubated in 900 mOsmol kg$^{-1}$ DPBS were not able to recover control levels of motility ($P < .05$). A nonspecific decrease in motility was also observed after dilution into isosmolal DPBS; however, significant differences were not observed ($P = .27$).

*Evaluation of Mitochondrial Function*—Using the appropriate gating parameters for monkey sperm determined during preliminary experiments, the cell population (10 000 events) was divided into either high membrane potential (red/green fluorescence) or low membrane potential (green fluorescence), according to natural partitioning. The percentages of sperm with high and low MMPs subjected to different anisotonic conditions are shown in Figure 4A. Spermatozoa subjected to hypertonic conditions (600 and 900 mOsmol kg$^{-1}$) showed a significant decrease ($P = .004$) of the percentage of population with high MMP and, consequently, a significant increase ($P = .01$) of the percentage of population with low MMP compared with control samples. However, when the intensity of ''red'' and ''green'' (high and low MMP, respectively) was quantified, an unexpected increase ($P < .02$) of individual cell intensity was observed in spermatozoa exposed to hypotonic conditions (Figure 4B). Sperm suspensions that returned to isotonic conditions after being exposed for 10 minutes to anisotonic solutions showed no significant differences in percentage of population with high and low MMP (Figure 4C) or cell intensity (Figure 4D) compared with control samples.

*Sperm viability*—The percentage of membrane-intact spermatozoa after 10 minutes of incubation in anisotonic solutions is shown in Figure 5. Membrane integrity was apparently unaffected by exposure to anisotonic solutions, but, after returning to isotonic conditions, spermatozoa incubated previously in 900 mOsmol kg$^{-1}$ solution showed a significant decrease ($P < .05$) of viability demonstrated by the increased number of stained cells with propidium iodide.

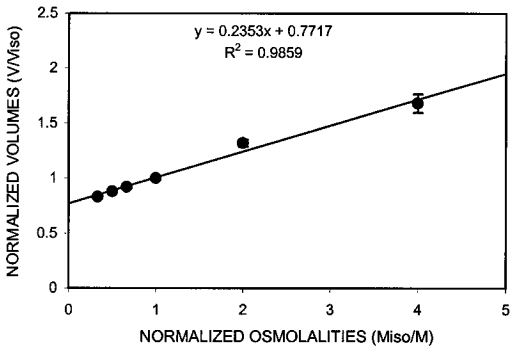


Figure 2. Boyle van't Hoff plot (volume vs osmolality$^{-1}$) of monkey spermatozoa at 22°C. Sperm samples were exposed to different osmolalities: 900, 600, 450, 300, 150, and 75 mOsmol kg$^{-1}$ DPBS. The y-intercept indicates that $V_b$, the osmotically inactive volume, is 77.2% of the isotonic cell volume (n = 5 monkeys).

## Discussion

Cells undergoing freezing are initially exposed to extracellular ice crystallization from extracellular water that results in hyperosmolal concentration of solutes (Watson, 1995, 2000). The cells respond to this insult by losing water and shrinking in volume so that the solute concentration between intra- and extracellular compartments can equilibrate. Conversely, as cells are exposed to a hypotonic extracellular environment, as in the case during thawing, cell volume is increased by passive diffusion of water. So the determination of the osmotic limits that sperm cells can tolerate could be applied to minimize the deleterious consequences associated with volume excursion and therefore improve cryopreservation techniques.

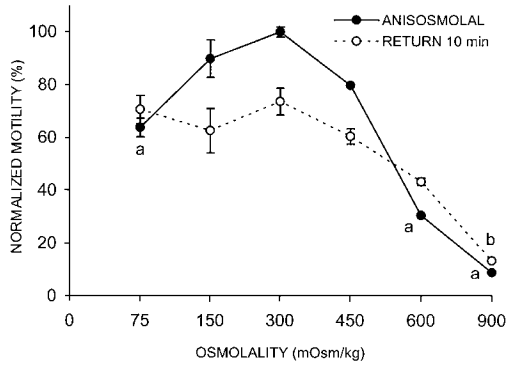


Figure 3. Monkey sperm samples were incubated for 10 minutes in isotonic or anisotonic DPBS, and total motility was analyzed using a CASA system. Samples were then diluted with adjusted DPBS, to bring solutions near to isotonicity, incubated for 5 minutes, and motility analyzed again. Superscript (a, b) denotes significant difference from control (300 mOsmol kg$^{-1}$) within each treatment group (n = 7 monkeys).

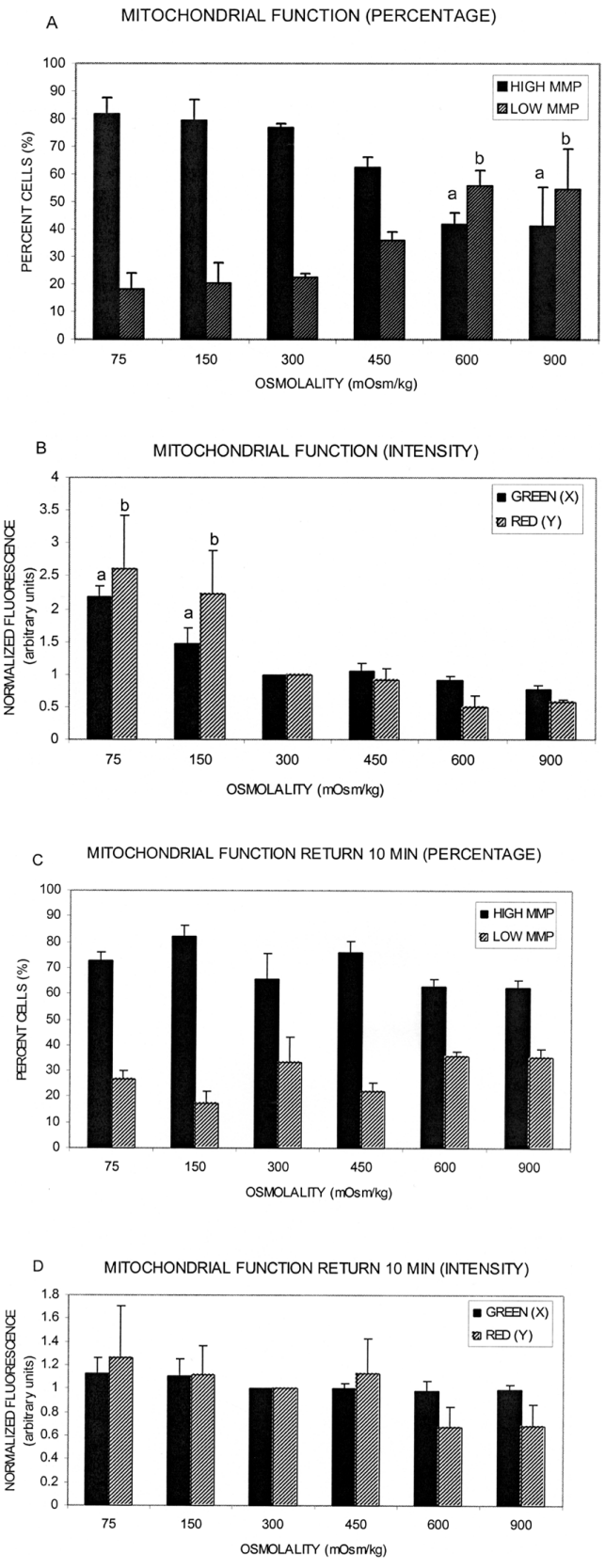


Figure 4. Sperm samples were incubated for 10 minutes at room temperature in isotonic or anisotonic DPBS. **(A)** and **(B)** A final concentration of 2 μM JC-1 was added to each treatment for 10 minutes, and the samples were read on the flow cytometer. **(C)** and **(D)** Samples previously subjected to anisotonicity conditions were diluted with 1.5 mL isotonic DPBS for 10 minutes and then incubated for another 10 minutes with 2 μM JC-1 before being analyzed on the flow cytometer. The graphs show percentage of cells with high and low MMP **(A, C)** and normalized fluorescence intensity for high and low MMP **(B, D)**. Red-labeled cells were considered to have high MMP, whereas those labeled green were considered to have low MMP. Superscripts (a, b) denote significance from control (300 mOsmol $kg^{-1}$) values (n = 3 monkeys).

### *Osmotic Behavior of Rhesus Monkey Spermatozoa*

The isotonic cell volume of rhesus monkey spermatozoa determined using an electronic particle counter was 36.8 ± 0.5 $\mu m^3$ at 22°C with 77.2% of the total cell volume being osmotically inactive ($V_b$) (both solids and bound water). The determination of the osmotic response and Boyle van't Hoff relationship of a cell type helps characterize the response of a cell to osmotic stress that occurs during the cryopreservation process. In the present study, monkey sperm behaved as linear osmometers in the range of 75 to 900 mOsmol $kg^{-1}$ ($r^2$ = .99). When a cell behaves as an ideal osmometer, the volume of the osmotically available water contained in the cell is inversely related to the osmolality of nonpermeable solutes in the external medium (Mazur, 1984). Then, the osmotically active cell volume ($V_{iso} - V_b$) indicates the amount of water in the cells that can be lost (unbound water) during the osmotic stress occurred during cryopreservation or in other physiological events. In monkey spermatozoa, only 22.8% of the total cell volume in isotonic conditions is osmotically active, which approximates 8.4 $\mu m^3$ of water. Compared with spermatozoa from various species studied thus far, 22.8% is the smallest active cell volume recorded (man 50%, Gilmore et al, 1995; murine 39.3%, Willoughby et al, 1996; boar 32.6%, Gilmore et al, 1996; and horse 29.3%, Pommer et al, 2002). During the freezing process, water must leave the cells by osmosis to prevent intracellular ice formation, which is lethal. The duration for water loss depends on the volume of water that has to be released from the cell, which is a small amount for macaque spermatozoa, and also on the membrane hydraulic conductivity ($L_p$) (Gao et al, 1995). Hydraulic conductivity values at different temperatures in the presence or absence of CPAs have not been determined for monkey spermatozoa but are the basis for ongoing investigations in our laboratory.

Rhesus monkey spermatozoa respond similarly to sperm from other mammalian species exhibiting a linear response to a given osmotic range (human, Gilmore et al, 1995; boar, Du et al, 1994; Gilmore et al, 1996; mouse, Willoughby et al, 1996; impala, wart hog, elephant, and lion, Gilmore et al, 1998b; horse, Pommer et al, 2002). The observed linear osmotic behavior included hypo- and

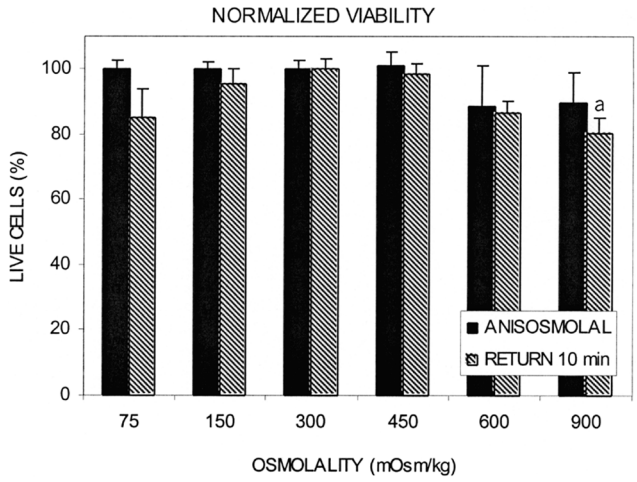


Figure 5. Monkey sperm samples were incubated for 10 minutes in isotonic or anisotonic DPBS, and duplicated samples were returned to isotonic conditions. A final concentration of 5 μM propidium iodide was added to each treatment for 10 minutes, and the samples were analyzed using a flow cytometer. Superscript (a) denotes significant difference from control (300 mOsmol $kg^{-1}$) within each treatment group (n = 5 monkeys)

hypertonic conditions (75–900 mOsmol $kg^{-1}$), which suggests that the values for exosmotic and endosmotic hydraulic flows were similar in the tested osmolality range.

Rhesus monkey sperm cell volume has not been previously reported; therefore, we cannot compare our results to other studies. However, using measures obtained by automated sperm morphometric analysis (Gago et al, 1999) and scanning electron microscopy (Martin et al, 1975), the estimated macaque sperm volume is approximately 40 to 45 $\mu m^3$. As has been described in mouse (Willoughby et al, 1996), human (Laufer et al, 1977; Gilmore et al, 1995), and horse (Pommer et al, 2002) sperm, the estimates obtained from electronic cell sizers are slightly smaller than estimates obtained from microscopic measurements.

### *Osmotic Tolerance of Rhesus Monkey Spermatozoa*

The present study demonstrates that a high percentage of macaque sperm (>80%) maintain viability or membrane integrity in the osmotic range tested (75–900 mOsmol $kg^{-1}$). Only sperm subjected for 10 minutes to 900 mOsmol $kg^{-1}$ solution and returned to isotonicity showed a significant decrease in viability ($P < .05$) compared with control spermatozoa (isotonic conditions), but even then the viability was greater than 80%. The deleterious effect on membrane integrity of the returning process to isotonicity after being incubated in hypertonic conditions has been described before in human (Gao et al, 1993), mouse (Willoughby et al, 1996; Songansen and Leibo, 1997), feline (Pukazhenthi et al, 2000) and, recently, in equine (Pommer et al, 2002) spermatozoa. Several hypotheses have been suggested for this finding (Gao et al, 1993; Willoughby et al, 1996; Pukazhenthi et al, 2000). The first hypothesis is that spermatozoa respond as red blood cells do, which undergo a process called posthypertonic hemolysis (spermolysis in this case), which consists of an overload of normally impermeable solutes due to membrane leakage on hypertonic stress. Then, when the cell returns to isotonic conditions, it swells to above its normal isotonic volume and lyses (Lovelock, 1953; Zade-Oppen, 1968). Another possible explanation is that cell shrinkage induces membrane loss, so that, when they return to isotonic conditions, the cells lyse in attempting to return to their normal volume (Steponkus and Wiest, 1979). It has been observed by scanning electron microscopy that sperm subjected to hypertonic conditions display exvaginations of the head membrane, resulting in a wrinkled surface (Hammerstedt et al, 1990; Gao et al, 1993). If this wrinkled surface results in a fusion of contiguous membranes under the influence of hypertonic stress, as has been described in other cell types (Homann, 1998), an abrupt return to isosmolal conditions would possibly result in cell lysis.

Although the percentage of cells showing adequate membrane integrity was high through the osmotic ranges tested here, sperm motility markedly declined in response to deviations from isotonicity. Samples exposed to 75 mOsmol $kg^{-1}$ resulted in an almost 40% decrease in motility, and samples exposed to the extreme hypertonicity tested in the present study (900 mOsmol $kg^{-1}$) resulted in a decrease of more than 90% from the isotonic motility (control). Similar to human (Gao et al, 1993; Curry and Watson, 1994), mouse (Willoughby et al, 1996; Songansen and Leibo, 1997), ram (Curry and Watson, 1994), boar (Gilmore et al, 1998a), bovine (Liu and Foote, 1998), and equine (Pommer et al, 2002) sperm, macaque sperm motility was more sensitive to anisotonic conditions than membrane integrity. However, it appeared that, in macaque sperm, in contrast to other species, the shrinking process was more detrimental than the swelling process in terms of motility in the osmotic range tested. Sperm progressive motility is of primary importance, because it is required for sperm to reach the site of fertilization and penetrate into the oocyte. Therefore, viable but nonmotile sperm are not useful for AI and IVF purposes. These observations suggest that, during the cryopreservation of macaque sperm, the freezing process might be more detrimental than the thawing process. A correlation between posthypertonic injury of human sperm and the time of cell exposure to the hypertonic environment before returning to isosmotic conditions has been observed (Gao et al, 1993). The shorter the time, the less cell injury, which indicates that cells can tolerate severe shrinkage for a short time.

Progressive motility is dependent on proper mitochon-

drial function, because mitochondria produce energy in the form of ATP to power the flagellar motion that propels the sperm (Garner and Thomas, 1999). Results from the present study indicate that anisosmotic conditions, hyposmotic and hyperosmotic, affect MMP differently. Hyperosmotic conditions induced a decrease in the percentage of spermatozoa with high MMP, but there are 2 observations that indicate that sperm motility is affected by factors other than MMP. The first is the lack of correlation between the percentage of cells exhibiting high MMP and the percentatge of motile cells. Spermatozoa subjected to 600 and 900 mOsmol $kg^{-1}$ had a similar percentage of cells with high MMP (approximately 41%); however, the percentage of motile cells was significantly and disproportionately lower (30% and 9%, respectively). The second observation is that sperm suspensions that returned to isotonic conditions after being exposed for 10 minutes to anisotonic solutions showed no significant differences in the percentage of the population with high and low MMP compared with control samples, so a recovery of MMP was observed. However, sperm previously incubated in 900 mOsmol $kg^{-1}$ DPBS were not able to recover control levels of motility. In the process of ATP synthesis, a high MMP is needed to constitute the electrochemical proton gradient that the enzyme ATP synthase will use to drive the energetically unfavorable reaction between ADP and $P_i$ that makes ATP. However, a high MMP does not indicate concentration of ATP produced, which, ultimately, is the energy used by the flagellum. Perhaps JC-1 is a good indicator of MMP, but it does not assess either the final level of ATP or the lack of motility observed in our study.

Hypotonic solutions did not affect cell proportion with high and low MMP; however, individual cell fluorescence intensity increased unexpectedly in hypotonic conditions (Figure 5B). As in many other species and other cell types, macaque sperm experience a volume expansion that can reach 1.68 times the isotonic volume under hypotonic conditions (75 mOsmol $kg^{-1}$) and still maintain more than 84% viability (membrane integrity). Folded membrane structures or storage of excess surface membrane in isotonicity has not been reported for sperm cells (Hammerstedt et al, 1990); therefore, an increase in cell volume could be accompanied with membrane stretching processes, because sperm have a fixed membrane surface. As was discussed elsewhere (Mazur, 1990), cell membranes are incapable of stretching more than a minute amount. Membrane stretch due to volume expansion could likely provoke sublethal membrane leakiness, as has been discussed elsewhere (Noiles et al, 1993), that could increase the uploading of JC-1 in the mitochondria, inducing a higher individual cell fluorescence intensity.

In summary, the present study has determined several important biophysical characteristics of macaque spermatozoa that can be applied to improve current cryopreservation protocols. The results showed that 1) macaque spermatozoa exhibited a linear osmotic response in the range of 75–900 mOsmol $kg^{-1}$; 2) macaque spermatozoa in isotonic media had a mean cell volume of 36.8 ± 0.5 $\mu m^3$ at 22°C as measured with an electronic particle counter, with 77.2% being osmotically inactive; 3) motility was more sensitive than membrane integrity to changes in osmolality; 4) macaque sperm motility and membrane integrity were more sensitive to hypertonic than hypotonic conditions; 5) MMP did not explain the lack of sperm motility observed in our study; and 6) although most spermatozoa were able to recover initial volume after osmotic stress, they were not able to recover initial motility. This information, combined with current investigations of macaque sperm membrane hydraulic conductivity ($L_p$) at various temperatures, in the presence or absence of CPA, will be used to limit osmotic stress during cryopreservation.

## Acknowledgments

We gratefully acknowledge Dr Mary Delaney, Department of Animal Science, College of Agriculture and Environmental Sciences, for technical assistance with the Z2 Coulter counter and Abigail Spinner, California National Primate Research Center, for technical assistance with flow cytometry.

## References

Acker JP, Pasch J, Heschel I, Rau G, McGann E. Comparison of optical measurement and electrical measurement techniques for the study of the osmotic responses of cell suspensions. *Cryo-Lett.* 1999;20:315–324.

Curry MR, Watson PF. Osmotic effects on ram and human sperm membranes in relation to thawing injury. *Criobiology.* 1994;31:39–46.

Du J, Tao J, Kleinhans FW, Peter AT, Critser JK. Determination of boar spermatozoa water volume and osmotic response. *Theriogenology.* 1994;42:1183–1191.

Gago C, Pérez-Sánchez F, Yeung CH, Tablado L, Cooper TG, Soler C. Morphological characterization of ejaculated cynomolgus monkey (*Macaca fascicularis*) sperm. *Am J Primatol.* 1999;47:105–115.

Gao DY, Ashworth E, Watson PF, Kleinhans FW, Mazur P, Critser JK. Hyperosmotic tolerance of human spermatozoa: separate effects of glycerol, sodium chloride and sucrose on spermolysis. *Biol Reprod.* 1993;49:112–123.

Gao DY, Liu J, Liu C, McGann LE, et al. Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. *Hum Reprod.* 1995;10:1109–1122.

Garner DL, Thomas CA. Organelle-specific probe JC-1 identifies membrane potential differences in the mitochondrial function of bovine sperm. *Mol Reprod Dev.* 1999;53:222–229.

Gilmore JA, McGann LE, Liu J, Gao DY, Peter AT, Kleinhans FW, Critser JK. Effect of cryoprotectant solutes on water permeability on human spermatozoa. *Biol Reprod.* 1995;53:985–995.

Gilmore JA, Du J, Tao J, Peter AT, Critser JK. Osmotic properties of boar spermatozoa and their relevance to cryopreservation. *J Reprod Fert.* 1996;107:87–95.

Gilmore JA, Liu J, Peter AT, Critser JK. Determination of plasma mem-

brane characteristics of boar spermatozoa and their relevance to cryopreservation. *Biol Reprod.* 1998a;58:28–36.

Gilmore JA, McGann LE, Ashworth E, Acker JP, Raath JP, Bush M, Critser JK. Fundamental cryobiology of selected African mammalian spermatozoa and its role in biodiversity preservation through the development of genome resource banking. *Anim Reprod Sci*. 1998b;53: 277–297.

Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive? *J Androl.* 1990;11:73–88.

Holt WV, North RD. Effects on temperature and restoration of osmotic equilibrium during thawing on the induction of the plasma membrane damage in cryopreserved ram spermatozoa. *Biol Reprod.* 1994;51: 414–424.

Homann U. Fusion and fission of plasma-membrane material accommodates for osmotically induced changes in the surface area of guard-cell protoplasts. *Planta.* 1998;206:329–333.

Karow A. Cryoprotectant: A new class of drugs. *J Pharm Pharmacol.* 1969;21:209–223.

Laufer N, Segal S, Yaffe H, Svartz H, Gover NB. Volume and shape of normal human spermatozoa. *Fertil Steril.* 1977;28:456–458.

Leverage WE, Valerio DA, Schultz AP, Kingsbury E, Dorey C. Comparative study on the freeze preservation of spermatozoa: Primate, bovine and human. *Lab Anim Sci.* 1972;22:882–889.

Liu Z, Foote RH. Bull sperm motility and membrane integrity in media varying in osmolality. *J Dairy Sci.* 1998;81:1868–1873.

Lovelock JE. The hemolysis of human red blood cells by freezing and thawing. *Biochim Biophys Acta.* 1953;10:414–426.

Martin DE, Gould KG, Warner H. Comparative morphology of primate spermatozoa using scanning electron microscopy. I. Families Hominidae, Pongidae, Cercopithecidae and Cebidae. *J Hum Evol.* 1975;4: 287–292.

Mazur P. Freezing of living cells: mechanisms and implications. *Am J Physiol.* 1984;247:C125–C142.

Mazur P. Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. *Cell Biophys.* 1990;17:53–92.

Morrell JM, Hodges JK. Cryopreservation of nonhuman primate sperm: Priorities for future research. *Anim Reprod Sci.* 1998;53:43–63.

Noiles EE, Mazur P, Watson PF, Kleinhans FW, Critser JK. Determination of water permeability coefficient for human spermatozoa and its activation energy. *Biol Reprod.* 1993;48:99–109.

Petrunkina AM, Harrison RAP, Hebel M, Weitze KF, Töpfer-Petersen E. Role of quinine-sensitive ion channels in volume regulation in boar and bull spermatozoa. *Reproduction* 2001;122:327–336.

Pommer AC, Rutllant J, Meyers SA. The role of osmotic resistance on equine spermatozoal function. *Theriogenology* 2002;58:1373–1384.

Pukazhenthi B, Noiles E, Pelican K, Donoghue A, Wildt D, Howard J. Osmotic effects on feline spermatozoa from normospermic versus teratospermic donors. *Cryobiology.* 2000;40:139–150.

Sanchez-Partida LG, Maginnis G, Dominko T, Martinovich C, McVay B, Fanton J, Schatten, G. Live rhesus offspring by artificial insemination using fresh and cryopreserved sperm. *Biol Reprod.* 2000;63:1092–1097.

Sarason R L, Vandevoort CA, Mader DR, Overstreet JW. The use of nonmetal electrodes in electroejaculation of restrained but unanesthetized macaques. *J Med Primatol.* 1991;20:122–125.

Songansen N, Leibo SP. Cryopreservation of mouse spermatozoa. II. Relationship between survival after cryopreservation and osmotic tolerance of spermatozoa from three strains of mice. *Cryobiology.* 1997; 35:255–269.

Steponkus PA, Wiest SC, Freeze-thaw induced lesions in the plasma membrane. In: Lyons JM, Graham D, Raison JK, eds. *Low temperature stress in crop. The role of the membrane.* New York: Academic Press; 1979:231–254.

Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. *Reprod Fert Dev.* 1995;7:871–891.

Watson PF, Duncan AE. Effect of salt concentration and unfrozen water fraction on the viability of slowly frozen ram spermatozoa. *Cryobiology.* 1988;25:131–142.

Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* 2000;60–61:481–492.

Willoughby CE, Mazur P, Peter AT, Critser JK. Osmotic tolerance limits and properties of murine spermatozoa. *Biol Reprod.* 1996;55:715–727.

Zade-Oppen AM. The effect of mannitol, sucrose, raffinose and dextran on posthypertonic hemolysis. *Acta Physiol Scand.* 1968;74:195–206.