Osmotic Stress Induces Oxidative Cell Damage to Rhesus Macaque Spermatozoa¹

Megan J. McCarthy,³ Julie Baumber,³ Philip H. Kass,⁴ and Stuart A. Meyers^{2,3}

Departments of Anatomy, Physiology, and Cell Biology³ and Population Health and Reproduction,⁴
School of Veterinary Medicine, University of California, Davis, California

ABSTRACT

Cryopreservation introduces extreme temperature and osmolality changes that impart lethal and sublethal effects on spermatozoa survival. Additionally, evidence indicates that the osmotic stress induced by cryopreservation causes oxidative stress to spermatozoa as well. Our objective was to determine the effect of reactive oxygen species (ROS) on rhesus macaque (*Macaca mulatta*) sperm function and to determine whether osmotic stress elicits the production of ROS. In the first experiment, the xanthine-xanthine oxidase (X-XO) system was used to generate the ROS superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) in the presence or absence of the ROS scavengers superoxide dismutase and catalase, respectively. In the second experiment, osmotic stress was introduced by incubation of spermatozoa in a series of anisotonic media ranging from 100 to 1000 mOsm/kg in the presence or absence of the antioxidant alpha-tocopherol. Treatment with the X-XO system resulted in a significant increase in the generation of $O_2^{\cdot-}$ and H_2O_2 that was detectable using flow cytometry. The ROS generated by the X-XO system was dose dependent, and as the concentration of ROS increased, motility decreased and lipid peroxidation increased while no effect was observed on viability. Incubation of spermatozoa in anisotonic media also resulted in an increase in $O_2^{\cdot-}$ generation and lipid peroxidation that was significantly decreased in the presence of the powerful antioxidant alpha-tocopherol. These results clearly indicate that osmotic stress causes oxidative stress in rhesus macaque spermatozoa, which strongly supports the hypothesis that cryopreservation-induced osmotic stress may lead to oxidative cell damage.

cryopreservation, gamete biology, nonhuman primate, osmotic stress, oxidative stress, rhesus macaque, sperm, spermatozoa, superoxide anion

INTRODUCTION

Because of their physiological and genetic similarities to humans, rhesus macaques have become the most widely used nonhuman primate (NHP) for the study of human disease. The ability to efficiently cryopreserve semen from NHP not only would provide an important resource for biomedical research but also would contribute to preserving colonies of NHP species that are endangered as a result of habitat destruction and hunting [1, 2]. Although many advances have been made over the past 30 years to enhance the efficiency of NHP sperm

cryopreservation, a substantial amount of sublethal and lethal damage still results from exposure to the extreme temperature and osmolality effects that cryopreservation introduces [1, 3, 4]. As the cell is exposed to plunging temperatures, extracellular ice crystallization begins, which results in concentration of the surrounding solutes in the unfrozen aqueous channels between ice crystals [3, 5, 6]. In response to the developing hyperosmolar environment, cells lose water and shrink in volume until the intracellular and extracellular solute concentrations equilibrate [6]. During the thawing process, cells are subjected to a hypotonic environment that will cause an increase in cell volume in an effort to again equilibrate the intracellular and extracellular solute concentrations. If cell volume excursion during cryopreservation or thawing is driven beyond tolerance limits, damage will occur [2, 7, 8]. The tolerance of sperm to osmotic challenge varies among species, but the following factors remain consistent in determining the cellular response to volume changes: membrane phospholipid composition, water permeability, lipid phase transition temperature, Na^+/K^+ -ATPase activity, water channels, and ion channels [8, 9]. Understanding how these mechanisms work is extremely important in determining the most efficient sperm cryopreservation protocol.

Not only does cryopreservation introduce an osmotic stress to cells, substantial evidence indicates that cryopreservation causes oxidative damage to sperm as well [10–16]. From a physiological standpoint, the generation of low levels of reactive oxygen species (ROS) by spermatozoa plays an important role in the cascade of events controlling sperm capacitation [17–21], acrosome reaction [19, 22, 23], hyperactivation [17, 18], and sperm-oocyte fusion [23]. In contrast, excessive generation of ROS by defective spermatozoa [24–28], contaminating leukocytes [25, 29–32], or cryopreservation [13, 14, 27, 33] can have a detrimental effect on sperm function. Numerous free radicals are generated during normal cellular respiration, but the most common ROS generated by spermatozoa is the superoxide anion ($O_2^{\cdot-}$), which can then spontaneously or enzymatically (via superoxide dismutase) dismutate to hydrogen peroxide (H_2O_2) [34]. Although $O_2^{\cdot-}$ is capable of producing radicals of higher toxicity than H_2O_2 , the half-life is very short (1 msec), and membrane permeability is limited [27, 29, 35–37]. A limited ability to store antioxidant enzymes combined with a membrane rich in unsaturated fatty acids makes spermatozoa particularly susceptible to oxidative stress and peroxidative attack by these ROS [38, 39]. Specifically, high levels of ROS have been associated with extensive cell damage, including morphological defects [40], lipid peroxidation and DNA fragmentation [41], decreased ability to acrosome react or fuse with the oocyte [42], and compromised pregnancy after in vitro fertilization [43, 44].

The objective of the present study was to determine how rhesus macaque spermatozoa respond to high levels of ROS and whether osmotic stress alone is enough to elicit an oxidative response. First, we wanted to determine the effect of excessive ROS production on rhesus macaque sperm motility,

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²Correspondence: FAX: 530 752 7690; e-mail: smeyers@ucdavis.edu

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viability, and membrane lipid peroxidation. Second, using osmotic stress as an experimental model for cryopreservation, we wanted to determine whether osmotic stress alone is capable of introducing oxidative stress. Our overall goal is to investigate the role of oxidative stress during cryopreservation; however, in the present study, we focused on the interaction that oxidative stress may have with osmotic stress (separated from low-temperature stress).

MATERIALS AND METHODS

Superoxide dismutase (SOD), xanthine oxidase (XO), catalase (CAT), and α -tocopherol (type IV, from vegetable oil) were obtained from Sigma Chemical Co. Xanthine (X) was purchased from Calbiochem. Dihydroethidium (DHE), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), SYTOX Green, 4,4'-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY^{581/591}), and the LIVE/DEAD sperm viability kit (propidium iodide [PI] and SYBR-14) were obtained from Invitrogen. To minimize premature oxidation, DHE and H_2DCFDA were initially solubilized in dimethyl sulfoxide, held under N_2 , and stored at $-80^\circ C$ until needed.

Experiment 1

Experiment 1 studied use of the xanthine-xanthine oxidase (X-XO) ROS-producing system to evaluate use of DHE and H_2DCFDA for the detection of $O_2^{\cdot -}$ and H_2O_2 , respectively, in rhesus macaque sperm and to determine the effects of ROS on motility and membrane characteristics. Semen samples were obtained by electroejaculation from five male rhesus macaques (*Macaca mulatta*) as previously described [45]. Animals were housed at the California National Primate Research Center (UC Davis) and maintained according to Institutional Animal Care and Use Committee protocols at the University of California. Semen samples were collected directly into 50-ml centrifuge tubes containing 5 ml of Hepes-Biggers, Whitten, and Whittingham (BWW) media [46]. Following semen collection, samples were diluted in 5 ml of Hepes-BWW containing 2 mg/ml of polyvinyl alcohol (PVA) for a final concentration of 1 mg/ml of PVA. The samples were gently rocked for 5 min, after which the coagulum was removed and the sample was maintained at room temperature for 10 min to allow any ejaculate particulates to settle to the bottom. The upper 9.5 ml of semen were then transferred into a separate tube for determination of initial motility. A total of 2.5 ml of semen were layered over 3 ml of 80% buffered Percoll and centrifuged at $300 \times g$ for 25 min as described by Baumber and Meyers [47]. Following centrifugation, the supernatant was removed and the pellet washed twice in Hepes-BWW plus 1 mg/ml of PVA ($300 \times g$, 5 min). After two washes, the spermatozoa were resuspended in BWW plus 1 mg/ml of PVA to a final concentration of 25×10^6 sperm/ml in 500- μ l aliquots. Pretreatment motility was evaluated, and samples were then incubated at $37^\circ C$ (5% CO_2 in air) for 60 min according to the following treatments: 1) spermatozoa alone, 2) spermatozoa plus 1 mM xanthine, 3) spermatozoa plus 0.1 U/ml of XO, 4) spermatozoa plus 200 U/ml of SOD, 5) spermatozoa plus 200 U/ml of CAT, 6) spermatozoa + 0.1 mM X-0.01 U/ml of XO, 7) spermatozoa plus 0.3 mM X-0.025 U/ml of XO, 8) spermatozoa plus 0.6 mM X-0.05 U/ml of XO, and 9) spermatozoa plus 1 mM X-0.1 U/ml of XO [48]. A room-temperature control was also included. After the 60-min treatment, motility was evaluated, and the samples were processed for determination of viability, lipid peroxidation, $O_2^{\cdot -}$ production, and H_2O_2 production as further described below.

Experiment 2

Experiment 2 studied the effect of osmotic stress on ROS production by rhesus macaque spermatozoa and the subsequent effect on motility and membrane characteristics. Semen was processed exactly as described for experiment 1 except that spermatozoa were incubated in the following anisomotic treatments: 100, 200, 300, 400, 500, 600, or 1000 mOsmol/kg of Dulbecco PBS (DPBS) plus 1 mg/ml of PVA for 30 min at room temperature [1]. After the hypotonic (100 and 200 mOsmol/kg), isotonic (300 mOsmol/kg), or hypertonic (400-1000 mOsmol/kg) incubations, motility was evaluated, and samples were centrifuged at $300 \times g$ for 5 min and returned to 300 mOsmol/kg of DPBS at a concentration of 25×10^6 sperm/ml. Five minutes after return to an isotonic environment, motility was evaluated, and cells were processed for determination of viability, lipid peroxidation, $O_2^{\cdot -}$ production, and H_2O_2 production.

Additionally, the effect of α -tocopherol during osmotic stress was evaluated. Spermatozoa were incubated in 100, 300, 600, and 1000 mOsmol/

kg of DPBS plus 1 mg/ml of PVA in the presence of 100 μ M α -tocopherol or vehicle (0.5% ethanol). Samples were processed as described above for determination of $O_2^{\cdot -}$ and lipid peroxidation.

Motility

Sperm motility was evaluated by means of computer-assisted sperm analysis with HTM Ceros, version 12.2 g (Hamilton Thorne Biosciences, Inc.). At least 200 cells in a minimum of four fields were evaluated for each treatment. The following instrument settings were used for computer-assisted sperm analysis: frame rate, 60 Hz; frames acquired, 30; minimum contrast, 80; minimum cell size, four pixels; static average velocity of the smoothed cell path (VAP) cutoff, 20 μ m/sec; static average velocity measured in a straight line from the beginning to the end of the track cutoff (VSL), 10 μ m/sec; progressive VAP threshold, 25 μ m/sec; progressive average value of the ratio VSL/VAP threshold (STR; measures the departure of the cell path from a straight line), 80%; static intensity limits, 0.6–1.4; static size limits, 0.6–2.31; and static elongation limits, 0–80. Percentage total motility and percentage progressive (forward) motility were determined at all time points.

Viability

SYBR-14 and PI were added to 500 μ l of each sample for a final concentration of 0.1 and 12 μ M, respectively. Spermatozoa were incubated for 10 min at room temperature, diluted to 1×10^6 sperm/ml, and analyzed by flow cytometry. SYBR-14 is a membrane-permeant nucleic acid stain and will stain all cells, whereas PI is membrane-impermeant dye that binds to DNA. Therefore, SYBR-14-labeled cells (green) were scored as "viable," and SYBR-14/PI-labeled cells (green and red) were scored as "nonviable." The excitation wavelength used for both fluorochromes was 488 nm, and the emission maxima of SYBR-14 and PI was 516 and 617 nm, respectively.

DHE Assay

The oxidation of DHE by the superoxide anion within a cell produces the DNA-sensitive fluorochromes, Et^+ and $2OHEt^+$, that together generate a red nuclear fluorescence in cells that excites at 488 nm, and it has an emission of 630 nm. DHE is an excellent probe for measuring intracellular $O_2^{\cdot -}$ generation [49–51] but does not distinguish between live and dead cells; therefore, a vitality probe must be used in combination with DHE to account for nonviable cells. SYTOX Green, which is a nuclear chromosome counterstain that is impermeant to live cells and, thus, only stains dead/nonviable cells, was used as the vitality probe. In preliminary experiments, we validated that SYTOX Green was an accurate viability probe for rhesus spermatozoa by using it in sperm cell populations that were 100% live, 75% live, 50% live, and 100% dead. To obtain 100% dead cells, an aliquot of live cells was submerged in LN_2 and thawed ($37^\circ C$ water bath) three times for 2 min each time. The dead cells were then mixed with the live cells in the above-described proportions and run on our FACScan flow cytometer to determine that the cell populations appeared in the correct quadrants.

Dihydroethidium (2 μ M) and SYTOX Green (0.05 μ M) were added together for 15 min before the end of treatment incubation [51]. Then, cells were diluted to 1×10^6 cells/ml and analyzed by flow cytometry. DHE was effectively used for the evaluation of $O_2^{\cdot -}$ by rhesus spermatozoa using the ROS-generating system (X-XO with or without varying levels of the $O_2^{\cdot -}$ scavenger SOD). Comparison of DHE fluorescence in control samples to that in samples treated with X-XO allowed the establishment of a threshold for identification of positively DHE-labeled cells and, thus, cells producing $O_2^{\cdot -}$.

H_2DCFDA Assay

The production of H_2O_2 was evaluated using the cell-permeant probe H_2DCFDA . This reduced fluorescein is freely permeable across cell membranes, but once inside the cell, cellular esterases remove the acetate groups, resulting in the impermeant product H_2DCF . Oxidation of H_2DCF by H_2O_2 yields dichlorofluorescein (DCF), which has an excitation/emission spectra of 488/530 nm. Preliminary experiments were performed to determine the optimal concentration, temperature, and time for loading spermatozoa with H_2DCFDA for maximum assay sensitivity. The efficiency of this probe was tested by incubating spermatozoa in 1 mM to 25 μ M H_2O_2 with and without CAT, testing both incubation time and temperature.

Fifteen minutes before the end of the treatment incubation, 50 μ M H_2DCFDA and 5 μ M PI were added. Spermatozoa were then diluted to 1×10^6 sperm/ml and analyzed by flow cytometry. Inclusion of the viability stain PI allowed the differentiation between live and dead cells in the production of H_2O_2 . Comparison of DCF fluorescence in control samples to those treated

FIG. 1. Treatment with xanthine-xanthine oxidase (X-XO) resulted in a decrease in total motility ($P < 0.01$) and progressive motility ($P < 0.01$), whereas the addition of catalase (CAT, 200 U/ml) improved both total motility ($P < 0.05$) and progressive motility ($P < 0.05$) to near control levels. Superoxide dismutase (SOD, 200 U/ml) improved motility, but this improvement was not significant. Data were analyzed using ANOVA with repeated measures, and values represent the mean $\pm$ SEM ($n = 5$).

with H_2O_2 allowed the establishment of a threshold for identification of positively DCF-labeled cells and, thus, cells producing H_2O_2 .

BODIPY^{581/591}C₁₁ Assay

Lipid peroxidation was evaluated using the fluorescence lipid probe BODIPY^{581/591}C₁₁ (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid). When this probe becomes incorporated into the sperm membrane, it fluoresces red until lipid radicals peroxidize the membrane, at which time the probe undergoes an emission shift to green (red, nonperoxidized; green, peroxidized). Before experimental treatment, spermatozoa were incubated in 1 μ M BODIPY^{581/591}C₁₁ for 30 min at room temperature to load the probe into the cell membranes. Spermatozoa were then washed two times ($300 \times g$, 5 min) to remove excess probe and resuspended to 25×10^6 sperm/ml in their respective treatments in the presence or absence of the lipid peroxidation promoters ferrous sulfate (1 μ M) and sodium ascorbate (5 μ M). Because it is possible for dead cells to undergo lipid peroxidation, the vitality probe PI (final concentration, 12 μ M) was added during the last 5 min of treatment incubation so that dead lipid-peroxidized cells could be distinguished from live lipid-peroxidized cells. Spermatozoa were then diluted to 1×10^6 sperm/ml and analyzed by flow cytometry. During preliminary experiments, the ideal BODIPY^{581/591}C₁₁ and ferrous sulfate/sodium ascorbate concentrations were determined. To verify that lipid peroxidation

was working through ferrous ion promotion, 100 μ M ethylenediaminetetraacetic acid (EDTA) was added to determine if lipid peroxidation could be decreased through the chelation of iron. Comparison of the fluorescence in control samples to those treated with X-XO in the presence of ferrous sulfate and sodium ascorbate allowed the establishment of a threshold for identification of positively BODIPY^{581/591}C₁₁-labeled cells and, thus, cells that were undergoing lipid peroxidation.

Flow Cytometric Analysis

Flow cytometry was performed using a FACScan flow cytometer (Becton, Dickinson and Company) equipped with a 488-nm excitation laser, and data were analyzed using CellQuest software (Becton, Dickinson and Company). SYBR-14, SYTOX Green, and DCF were measured with a 530/30 bandpass filter, whereas PI was measured with a 585/42 bandpass filter and DHE with a 650LP bandpass filter. Adjustments were made to address and eliminate fluorochrome spectral overlap so that each cell population was seen as distinct. To limit the evaluation of DHE, DCF, and BODIPY^{581/591}C₁₁ fluorescence to viable spermatozoa, only the subpopulation outside of SYTOX Green- or PI-positive cells, respectively, were included in the evaluation. A total of 10 000 gated events were analyzed per sample.

Statistical Analysis

Repeated-measures ANOVA was used to determine the significance of treatment and whether treatment elicited a dose response. The same statistical method was used to look at the effect treatment had on sperm motility, viability, and lipid peroxidation. When the latter repeated-measures ANOVA was significant, post-hoc comparisons were performed to determine which treatments were significantly different from each other. A P value of less than 0.05 was considered to be statistically significant.

All X-XO treatments were compared to control (37°C), and 0.6 mM X-0.05 U/ml of XO plus SOD or CAT was compared to 0.6 mM X-0.05 U/ml of XO alone. All osmotic treatments were compared to control (300 mOsmol/kg).

RESULTS

Experiment 1

Experiment 1 studied use of the X-XO ROS-producing system to evaluate use of DHE and H_2 DCFDA for the detection of $O_2^{\cdot -}$ and H_2O_2 , respectively, in rhesus macaque sperm and to determine the effects of ROS on motility and membrane characteristics. The addition of increasing levels of ROS over a time period of 60 min had no effect on viability but negatively affected motility (Fig. 1). A decrease was found in total ($P < 0.005$) and progressive ($P < 0.01$) motility with increasing concentrations of X-XO. The addition of CAT was able to increase both total (X-XO, 39%; X-XO + CAT, 78%; control, 86%; $P < 0.05$) and progressive (X-XO, 18%; X-XO + CAT, 62%; control, 76%; $P < 0.05$) motility to near control levels. However, SOD was not as effective and increased total (X-XO, 39%; X-XO + SOD, 53%; control, 86%; $P = 0.38$) and progressive (X-XO, 18%; X-XO + SOD, 38%; control, 76%; $P = 0.26$) motility by a much smaller margin.

Incubation in the presence of the X-XO system exhibited a significant and dose-dependent increase in $O_2^{\cdot -}$ ($P < 0.01$)

FIG. 2. Flow cytometric histograms showing the distribution of dihydroethidium (DHE; A) and 2',7'-dichlorodihydrofluorescein diacetate (DCF; B) fluorescence in rhesus spermatozoa incubated with and without xanthine-xanthine oxidase (X-XO). DHE measures superoxide ($O_2^{\cdot -}$) production, whereas DCF measures hydrogen peroxide (H_2O_2) production. Data were analyzed using ANOVA with repeated measures, and values represent the mean $\pm$ SEM ($n = 5$).

FIG. 3. Treatment with varying levels of xanthine-xanthine oxidase (X-XO) significantly increased superoxide ($O_2^{\cdot-}$; DHE positive) and hydrogen peroxide (H_2O_2 ; DCF positive) production, whereas the addition of superoxide dismutase (SOD; 200 U/ml of SOD + 0.6 mM xanthine and 0.05 U/ml of XO) and catalase (CAT; 200 U/ml of CAT + 0.6 mM xanthine and 0.05 U/ml of XO) were able to decrease $O_2^{\cdot-}$ and H_2O_2 , respectively. Data were analyzed using ANOVA with repeated measures, and values represent the mean $\pm$ SEM ($n = 5$). ** $P < 0.01$ vs. control.

(Figs. 2A and 3) and H_2O_2 production ($P < 0.0001$) (Figs. 2B and 3) that was sensitive to the presence of SOD (Fig. 3) and CAT (Fig. 3), respectively.

In accordance with increased ROS production, high levels of lipid peroxidation were observed during incubation with the X-XO system. As the level of X-XO increased, an increase in the level of lipid peroxidation was observed ($P < 0.005$) (Fig. 4). Although the response was not affected by the addition of SOD, it was significantly decreased by the addition of CAT ($P < 0.01$), which supports the fact that H_2O_2 , as opposed to $O_2^{\cdot-}$, is a major contributor to the lipid peroxidation of sperm cell membranes.

Experiment 2

Experiment 2 studied the effect of osmotic stress on ROS production by rhesus macaque spermatozoa and the subsequent effect on motility and membrane characteristics. In agreement with earlier work from our laboratory [1], both total and progressive motility significantly decreased when exposed to anisotonic conditions ($P < 0.0001$ and $P < 0.0001$, respectively), with 0% total motility observed at 600-1000 mOsmol/kg (data not shown). Viability was also significantly decreased by osmotic stress, with the larger effect observed in hypertonic conditions (data not shown; $P < 0.0005$).

When spermatozoa were subjected to osmotic stress, extremely hypotonic (100 mOsmol/kg) or extremely hypertonic (1000 mOsmol/kg) conditions, a significant increase in $O_2^{\cdot-}$ generation was observed ($P < 0.01$ and $P < 0.05$, respectively) (Fig. 5). Although we were unable to detect significant H_2O_2 production, lipid peroxidation was significantly high in both hypotonic (100 mOsmol/kg; $P < 0.05$) and hypertonic (500, 600, and 1000 mOsmol/kg; $P < 0.0005$, $P < 0.0001$, and $P < 0.005$, respectively) treatments (Fig. 5). The addition of ferrous sulfate and sodium ascorbate were required to produce a measurable increase in lipid peroxidation, and this effect was

counteracted by the addition of the iron chelator EDTA (Fig. 6). Taken together, these results indicate that low but significant levels of H_2O_2 were present and that the observed peroxidative damage was caused by ferrous ion-promoted H_2O_2 generation of the hydroxyl radical.

Because of the high levels of $O_2^{\cdot-}$ and lipid peroxidation observed under osmotic stress, a brief experiment was conducted in which rhesus sperm were subjected to 100, 300, 600, and 1000 mOsmol/kg conditions in the presence or absence of the powerful antioxidant α -tocopherol. The addition of 100 μ M α -tocopherol or vehicle (0.5% ethanol) had no significant affect on viability or motility (total/progressive; data not shown), but α -tocopherol had a profound effect on $O_2^{\cdot-}$ production and lipid peroxidation. A significant decrease in $O_2^{\cdot-}$ production was observed under all conditions (100 mOsmol/kg, $P < 0.01$; 300 mOsmol/kg, $P < 0.05$; 600 mOsmol/kg, $P < 0.005$; 1000 mOsmol/kg, $P < 0.05$) (Fig. 7A), and lipid peroxidation was significantly reduced in both hypotonic (100 mOsmol/kg, $P < 0.01$) and hypertonic (600 mOsmol/kg, $P < 0.01$; 1000 mOsmol/kg, $P < 0.01$) (Fig. 7B) conditions.

DISCUSSION

In the present study, we determined that DHE and H_2DCFDA , used in conjunction with either SYTOX Green or PI to exclude dead cells, are reliable probes for the detection of intracellular ROS in rhesus spermatozoa. Incubation with X-XO elicited a dose-dependent production of $O_2^{\cdot-}$ and H_2O_2 that appeared to level out at our highest concentrations, indicating a saturation effect in this enzyme system. Interestingly, the observed increase in $O_2^{\cdot-}$ production by rhesus spermatozoa is similar to observations in equine spermatozoa [36] but different than that reported for porcine spermatozoa [52], suggesting species differences in ROS production induced by X-XO. Porcine spermatozoa exhibited a mild increase in $O_2^{\cdot-}$, but the

FIG. 4. The lipid peroxidation response of rhesus spermatozoa to increasing concentration of xanthine-xanthine oxidase (X-XO) and the sensitivity to the antioxidants superoxide dismutase (SOD; 200 U/ml) and catalase (CAT; 200 U/ml). Treatment with X-XO significantly increased lipid peroxidation (BODIPY^{581/591} C₁₁ positive), whereas only CAT was able to significantly decrease the observed increase in lipid peroxidation. All lipid peroxidation assays were conducted in the presence of 1 μ M ferrous sulfate and 5 μ M sodium ascorbate. Data were analyzed using ANOVA with repeated measures, and values represent the mean $\pm$ SEM ($n = 5$). ** $P < 0.01$ vs. control.

FIG. 5. The effect of osmotic stress on rhesus sperm superoxide ($O_2^{\cdot-}$) production and lipid peroxidation. Exposure to anisotonic conditions resulted in a significant increase in superoxide ($O_2^{\cdot-}$) production (DHE positive) as well as a significant increase in lipid peroxidation (BODIPY^{581/591}C₁₁ positive). All lipid peroxidation assays were conducted in the presence of ferrous sulfate (1 μ M) and sodium ascorbate (5 μ M). Data were analyzed using ANOVA with repeated measures with orthogonal contrasts, and values represent the mean $\pm$ SEM ($n = 5$). * $P < 0.05$, ** $P < 0.01$ vs. control.

majority of the response was observed in H_2O_2 generation [52]. The high levels of ROS introduced by X-XO had no effect on rhesus sperm viability but did negatively affect both total and progressive motility. In the presence of CAT, however, motility was returned to near control levels, suggesting that H_2O_2 is the main ROS affecting rhesus sperm motility and supporting previous reports for other species [53–57].

In addition to negatively affecting motility, ROS have long been implicated in the lipid peroxidation of sperm membranes from both fresh and cryopreserved semen because of the relatively high polyunsaturated fatty acid content of sperm membranes [24, 37, 38, 58]. In the present study, rhesus spermatozoa incubated in the presence of X-XO exhibited significant membrane lipid peroxidation, which was localized primarily in the midpiece. This is consistent with previous reports [35, 39, 59, 60], but low levels were observed in the posterior region of the head as well. The exact mechanism for ROS-induced lipid peroxidation is unknown, but it has been shown that ROS generated by treatment with X-XO affects a key sperm metabolism enzyme, glucose-6-phosphate-dehydrogenase (G_6PD) [61]. It is believed that diffusion of H_2O_2 across the cell membrane may cause the inhibition of G_6PD that subsequently leads to a decrease in the availability of NADPH. A decrease in NADPH may then lead to an accumulation of glutathione and reduced glutathione, leaving spermatozoa vulnerable to ROS attack because of a compromised antioxidant defense system [60, 62].

It is clear from the present study that rhesus spermatozoa are susceptible to oxidative stress, and it also is clear that osmotic stress alone can illicit oxidative stress as well, a direct link that to our knowledge has not been shown previously in rhesus spermatozoa. Incubation of rhesus spermatozoa under anisotonic conditions resulted in a significant increase in $O_2^{\cdot-}$ production, which interestingly has just been observed with

equine spermatozoa as well [63]. The exact mechanism to explain the increased generation of this ROS in response to osmotic stress remains unclear; however, work with other cell types suggests that hyperosmotic-induced cell swelling may activate the membrane-associated phospholipase A_2 . Activation of phospholipase A_2 may then lead to an increase in arachidonic acid, which subsequently activates the NADPH oxidase complex, resulting in increased production of $O_2^{\cdot-}$ [64, 65]. Even in the absence of a clear explanation for osmotic-induced ROS production, it is clear from the present study and from previous work that an interaction occurs between the two stresses [64, 66, 67].

The high levels of lipid peroxidation observed in rhesus spermatozoa under osmotic stress were not surprising. A significant level of membrane stretching occurs under osmotic stress, resulting in a decrease in lateral packing of lipids [68] and reorganization of the actin cytoskeleton [4], leaving the cell membrane vulnerable to ROS attack. Surprisingly, H_2O_2 was not detected during anisotonic conditions, which was unexpected, especially in the presence of ferrous ion promotion. Consequently, it is quite possible that low levels of H_2O_2 were present and that our assay unfortunately lacked the sensitivity to measure this ROS. The probe used to detect lipid peroxidation, BODIPY^{581/591}C₁₁, only responds to changes in chain-propagating species (alkoxyl-RO $\cdot$ and peroxy radicals ROO $\cdot$) and does not allow detection of the specific ROS (i.e., $O_2^{\cdot-}$ or H_2O_2) that participate in the first chain initiation of the lipid peroxidation cascade [69]. Ferrous sulfate was added in these experiments to accelerate the lipid peroxidation cascade and allow a more direct study of the effects on sperm function [70, 71]. When ferrous sulfate is added in the presence of ROS, the initiation of a lipid peroxidation cascade, termed the Fenton reaction, begins [72]. In the presence of $O_2^{\cdot-}$ (or sodium ascorbate), the ferric ion (Fe^{3+}) is reduced to form the ferrous

FIG. 6. The effect of osmotic stress on rhesus sperm lipid peroxidation in the presence or absence of ferrous sulfate (1 μ M) and sodium ascorbate (5 μ M). In the absence of ferrous ion promoters, very low levels of lipid peroxidation (BODIPY^{581/591}C₁₁ positive) were observed under hypertonic conditions, which were significantly different from what was seen in the presence of ferrous ion promotion. Exposing cells to hypertonic conditions in the presence of ferrous ion promoters as well as 100 μ M EDTA resulted in lipid peroxidation levels comparable to those in the absence of ion promoters. Values represent the mean $\pm$ SEM (paired t -test, $n = 4$). ** $P < 0.01$ vs. control.

FIG. 7. **A** The antioxidant α -tocopherol (100 μ M) significantly decreased superoxide ($O_2^{\cdot-}$) production (DHE positive) in anisotonic as well as isotonic conditions. **B** Lipid peroxidation (BODIPY^{581/591}C₁₁ positive) was also significantly reduced in the presence of α -tocopherol under anisotonic conditions, with no significant decrease observed under isotonic conditions. Data were analyzed using ANOVA with repeated measures with orthogonal contrasts, and values represent the mean $\pm$ SEM (n = 4). * $P < 0.05$, ** $P < 0.01$ vs. control.

ion (Fe^{2+}) and oxygen (O_2); this step is termed the Haber-Weiss reaction [73]. The second step (i.e., the Fenton reaction) occurs when Fe^{2+} is oxidized by H_2O_2 to form Fe^{3+} , the hydroxyl anion (OH^-), and the hydroxyl radical ($OH^{\cdot}$). It is now the chain-propagating species, $OH^{\cdot}$, that will cause lipid peroxidation of the sperm membrane and, thus, a shift in BODIPY^{581/591}C₁₁ fluorescence from red to green. Although undetectable, it is possible that low levels of H_2O_2 were present, and when combined with high levels of $O_2^{\cdot-}$, a significant level of cell membrane lipid peroxidation resulted.

In an effort to protect rhesus spermatozoa from the deleterious effects of osmotic-induced oxidative stress, the enzymatic antioxidants CAT and SOD were added but, unfortunately, proved to be unsuccessful. This may have been a result of the method in which rhesus spermatozoa were exposed to osmotic stress, because the method used did not allow pretreatment incubation with either antioxidant. Alternatively, the nonenzymatic antioxidant α -tocopherol was used with much better success. α -Tocopherol is a powerful lipophilic antioxidant that can intercept lipid peroxyl radicals and break the chain of lipid peroxidation [37], which was clearly observed when this antioxidant was added to rhesus spermatozoa in the presence of ROS. Under both hypotonic and hypertonic conditions, addition of 100 μ M α -tocopherol significantly reduced both $O_2^{\cdot-}$ production and lipid peroxidation. It is not known if the addition of α -tocopherol during rhesus sperm cryopreservation could improve postthaw quality.

No advantage was observed by adding this antioxidant during equine sperm cryopreservation [74], although one of the hypothesized reasons for this failure was a sensitivity to the ethanol vehicle used in that study. Our present study had no indication of vehicle effect with α -tocopherol treatment.

These results clearly indicate, to our knowledge for the first time, that osmotic stress directly causes oxidative stress in rhesus macaque spermatozoa and strongly suggests that osmotic changes associated with cryopreservation lead to oxidative cell damage. Because osmotic and oxidative changes are major components of the cryopreservation process, these data support a very strong interaction between the two components and suggest that this interaction likely is a significant mechanism for cryoinjury to a number of cellular compartments during cryopreservation.

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