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The Effect of Reactive Oxygen Species on Equine Sperm Motility, Viability, Acrosomal Integrity, Mitochondrial Membrane Potential, and Membrane Lipid Peroxidation

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ABSTRACT: The objective of this study was to examine the influence of reactive oxygen species (ROS), generated through the use of the xanthine (X)–xanthine oxidase (XO) system, on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. Equine spermatozoa were separated from seminal plasma on a discontinuous Percoll gradient, and spermatozoa were incubated with 0.6 mM X and 0.05 U/mL XO for 30 minutes. Catalase (150 U/mL), superoxide dismutase (SOD, 150 U/mL), or glutathione (GSH, 1.5 mM) were evaluated for their ability to preserve sperm function in the presence of the induced oxidative stress. At the end of the 30-minute incubation, sperm motility was determined by computer-assisted semen analysis. Viability and acrosomal integrity were determined by Hoechst-*Pisum sativum* staining, and mitochondrial membrane potential was determined by staining with JC-1. Incubation with the X-XO system led to a significant ($P < .01$) increase in hydrogen peroxide production and an associated decrease ($P < .01$) in motility parameters. Total motility was significantly ($P < .01$) lower in the presence of X-XO compared with the case of the control (29% ± 9% vs 73% ± 1%, respectively). Catalase, but not SOD, prevented a decline in motility secondary to oxidative stress (71% ± 4% vs 30% ± 3%, respectively). The addition of glutathione had an intermediate effect in preserving sperm motility at the end of the 30-minute incubation (53% ± 3%). No influence of X-XO could be determined on viability, acrosomal integrity, or mitochondrial membrane potential. In order to promote lipid peroxidation, samples were incubated with ferrous sulfate (0.64 mM) and sodium ascorbate (20 mM) for 2 hours after the X-XO incubation. No increase in membrane lipid peroxidation was detected. This study indicates that hydrogen peroxide is the major ROS responsible for damage to equine spermatozoa. The decrease in sperm motility associated with ROS occurs in the absence of any detectable decrease in viability, acrosomal integrity, or mitochondrial membrane potential or of any detectable increase in lipid peroxidation.

Key words: hydrogen peroxide, oxidative stress, antioxidants.
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The spermatozoon, like all cells living under aerobic conditions, constantly faces the oxygen paradox; oxygen is required for life, but oxidative metabolism of biological molecules can be potentially toxic because of the formation of highly reactive oxygen species (ROS) that can modify cell functions or viability. In addition to the generation of ROS associated with oxidative metabolism, some cells also have an inherent capacity to generate ROS as part of normal cell function. For example, ROS generated by the *oxidative burst* in leukocytes are utilized for cell destruction in phagocytosis. Human spermatozoa posses the capacity to generate superoxide ($O_2^{-\cdot}$; Aitken and Clarkson, 1987), and the production of ROS is believed to involve a membrane-bound NADPH oxidase, similar to that present in leukocytes (Aitken et al, 1992, 1997). This production of low concentrations of hydrogen peroxide (H_2O_2) and $O_2^{-\cdot}$ by spermatozoa may have a functional role in the signaling events controlling capacitation and sperm–oocyte fusion (de Lamirande and Gagnon, 1993a,b; Aitken et al, 1995, 1998).

The capacity for ROS generation is significantly enhanced in abnormal spermatozoa (Iwasaki and Gagnon, 1992), particularly those cells with retention of residual cytoplasm (Gomez et al, 1996). Sperm with cytoplasmic droplets show a higher cellular content of cytoplasmic enzymes, including glucose-6-phosphate dehydrogenase. This enzyme is responsible for the flux of glucose through the hexose monophosphate shunt and the associated generation of NADPH. It is theorized that the NADPH generated via this system serves as the major source of electrons responsible for the production of $O_2^{-\cdot}$ by human spermatozoa. Therefore, the retention of residual cytoplasm creates a situation in which sufficient substrate would be available to support excessive NADPH-dependent ROS generation.

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Oxidative stress is determined by the balance between the generation and degradation of ROS within a tissue. Spermatozoa and seminal plasma posses a number of enzymes and low–molecular weight antioxidants that scavenge ROS in order to prevent possible cellular damage. There are 3 major enzyme systems, the glutathione (GSH) peroxidase/reductase system (Li, 1975; Alvarez and Storey, 1989), superoxide dismutase (SOD; Nissen and Kreysel, 1983; Alvarez et al, 1987), and catalase (CAT; Jeulin et al, 1989), that have been described in seminal plasma. A number of other components of seminal plasma may act as antioxidants. Vitamin E (Chow, 1991), vitamin C (Niki, 1991), urate (Ronquist and Niklasson, 1984; Gavella et al, 1997), albumin (Alvarez and Storey, 1983), and taurine and hypotaurine (Alvarez and Storey, 1983) have all been characterized as antioxidants in seminal plasma. Together, the enzyme scavengers and low–molecular weight antioxidants make up the total antioxidant capacity of seminal plasma (Smith et al, 1996).

Oxidative stress denotes a condition associated with an increased rate of cellular damage induced by ROS (Sikka et al, 1995). The cause of the pro-oxidant–antioxidant shift may be an increase in ROS production, a decrease in antioxidant capacity, or possibly a combination of the two. For example, sperm preparation methods associated with assisted reproductive techniques involve the removal of seminal plasma and hence the antioxidant protection for spermatozoa, thereby increasing their susceptibility to oxidative stress. Oxidative stress in human spermatozoa has been associated with a reduction in sperm motility and viability and in sperm-oocyte fusion (de Lamirande and Gagnon, 1992a,b; Aitken et al, 1993a). Oxidative stress has also been shown to have a negative influence on enzyme function (de Lamirande and Gagnon, 1992a,b; Griveau et al, 1995), sperm DNA (Kodama et al, 1997), and proteins (Davies, 1987). An understanding of the role of oxidative stress in the pathophysiology of human sperm function has therefore become increasingly important in the study of human male fertility.

There is little published information on the influence of ROS on equine sperm function. In a study by Aurich et al (1997), the antioxidant ascorbic acid had protective effects on equine sperm membrane integrity after chilled semen storage, suggesting that ROS damage may be related to loss of sperm function during equine semen storage. The current study was conducted to evaluate the effects of ROS on equine spermatozoa in vitro.

The first aim of the current study was to determine the influence of ROS on equine sperm function, specifically, motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. The second aim was to compare the effect of CAT, SOD, and GSH on preserving these sperm parameters following ROS challenge. We hypothesize that incubation of equine spermatozoa in the presence of ROS generated by the X-XO system will have a detrimental effect on sperm function and that this can be minimized by the addition of antioxidants.

Materials and Methods

All reagents were obtained from Sigma Chemical (St. Louis, Mo) unless otherwise indicated. Glutathione (reduced free acid) and xanthine were obtained from Calbiochem (San Diego, Calif). Bovine serum albumin (BSA) Fraction V was obtained from Sigma (product A 9418).

Five experiments were conducted within this study. Experiment 1 was designed to measure the production of H_2O_2 by the xanthine–xanthine oxidase (X-XO) system. Experiments 2 through 5 investigated the effect of ROS generated by the X-XO system on equine sperm motility, acrosomal integrity/viability, membrane lipid peroxidation, and mitochondrial membrane potential, respectively. Experiments were replicated across 2 ejaculates from each of 3 stallions unless otherwise indicated.

Preparation of Spermatozoa and Treatments

Semen was collected from 5 stallions by artificial vagina and was filtered and diluted 1:1 in a modified Tyrode albumin-lactate-pyruvate medium supplemented with 1% BSA (TALP-BSA; Padilla and Foote, 1991). A sperm-rich supernatant was obtained by centrifugation at 50 × *g* for 10 minutes (Meyers et al, 1995). Two milliliters of this supernatant was placed over an 80%/40% Percoll gradient (Drobnis et al, 1991) and centrifuged for 20 minutes at 300 × *g*. The sperm pellets were aspirated and resuspended in 2 mL of TALP-BSA and were washed at 300 × *g* for 10 minutes. Sperm were then resuspended in TALP-BSA to a final concentration of 25×10^6 cells/mL.

Reactive Oxygen Species Generation

Reactive oxygen species were generated by the X-XO system described by McCord and Fridovich (1968). Xanthine oxidase catalyzes the univalent and divalent reduction of ground-state oxygen to generate both $O_2^{-\cdot}$ and H_2O_2 with the oxidation of xanthine to uric acid. The presence of trace amounts of iron in the medium has a direct inactivating effect on xanthine oxidase through creation or exposure of reactive sulfur centers upon reduction of the enzyme by its substrates (Aitken et al, 1993a). This complication was avoided by incorporating the chelating agent diethylene triamine pentacetic acid (0.4 mg/mL; Britigan et al, 1990; Aitken et al, 1993a). Chelation of iron also minimizes the production of the hydroxyl radical by the transition metal–catalyzed Haber-Weiss reaction (Britigan et al, 1990; Aitken et al, 1993a).

Treatments in experiments 1, 2, 3, and 5 were done as follows: 1) sperm alone; 2) sperm + XO (0.05 U/mL); 3) sperm + xanthine (0.6 mM); 4) sperm + X-XO; 5) sperm + X-XO + CAT (from bovine liver; 150 U/mL); 6) sperm + X-XO + SOD (from bovine erythrocytes; 150 U/mL); and 7) sperm + X-XO + GSH (1.5 mM). Treatment samples were incubated for 30 minutes at room temperature under aerobic conditions.

Experiment 1: Production of Hydrogen Peroxide by the X-XO

System—The production of ROS by the X-XO system was confirmed by measuring the generation of hydrogen peroxide with the Amplex Red H_2O_2 assay kit (Molecular Probes, Eugene, Ore). This assay is based on a highly sensitive and stable probe for H_2O_2, 10-acetyl-3, 7-dihydroxyphenoxazine, the Amplex Red reagent. In the presence of horseradish peroxidase (1 U/mL), the Amplex Red reagent (200 μM) reacts with H_2O_2 in a 1:1 stoichiometry to produce a highly fluorescent product, resorufin. After incubation with the X-XO system, treatments were diluted 1:100 in phosphate buffer (50mM NaH_2PO_4; pH = 7.4), and 100 μL of the standards (final concentrations: 0.0, 0.05, 0.5, 5.0, and 50 μM), treatments, and blank (TALP alone) were pipetted into a 96-well microplate with duplicates for each sample. The Amplex Red assay was performed on each sample according to the manufacturers instructions. Fluorescence was measured using excitation at 560 nm and emission detection at 590 nm with a fluorescence microplate reader (HTS 7000; Perkin Elmer, Norwalk, Conn). Results are expressed as a single blank corrected concentration.

Experiment 2: The Effect of Reactive Oxygen Species on Equine Sperm Motility—Motility was recorded onto videotape at 37°C by phase–contrast videomicroscopy (Olympus BX60; Melville, NY; total magnification, 350×) at time zero minutes (T0) and time 30 minutes (T30) and was analyzed by the Cell Track computer automated sperm analysis (CASA) program (Motion Analysis Corporation, Santa Rosa, Calif).

Instrument settings for the CASA analysis were as follows: number of cells per sample, 200; minimum number of fields, 5 per sample; frame rate, 30 per second; duration of capture, 15 frames per second; minimum and maximum cell size, 2 and 8 pixels, respectively; minimum motile speed and maximum burst speed, 6 and 400 μm per second, respectively; distance scale factor, 2.4813 pixels/μm.

Straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), amplitude of lateral head displacement (ALH), average path velocity (VAP), and percentage total motility (% TM) were determined. This experiment was conducted on 2 ejaculates from each of 5 stallions.

Experiment 3: The Effect of Reactive Oxygen Species on Equine Sperm Viability and Acrosomal Integrity—After incubation with the X-XO system, sperm viability and acrosomal integrity were determined by staining with Hoechst 33258 and fluoroisothiocyanate-conjugated *Pisum sativum* agglutin (FITC-PSA; Vector Laboratories, Burlingame, Calif), respectively (modified from Casey et al, 1993). Hoechst 33258 (4 μL of a 50 μg/mL solution) was added to 200 μL of each sample and incubated (38°C) for 8 minutes. Two percent paraformaldehyde (200 μL) was then added, and samples were incubated for a further 10 minutes at room temperature. After fixation and staining, 8 μL of each sample was placed onto a polycarbonate filter (13 mm diameter, 3 μm pore size; Poretics Products, Livermore, Calif) and mounted in 1,4-diazabicyclo[2.2.2]octane (DABCO; 10% solution in ethanol). One hundred sperm from each treatment were examined with epifluorescence microscopy and scored to determine the percentage of live acrosome-intact, live acrosome-reacted, dead acrosome-intact, and dead acrosome-reacted spermatozoa by an examiner who was unaware of the treatment groups.

Experiment 4: The Effect of Reactive Oxygen Species on Lipid Peroxidation of Equine Spermatozoa—Treatments in experiment 4 were 1) sperm alone, 2) sperm + X (0.6 mM)-XO (0.05 U/mL), 3) sperm + X-XO + catalase (150 U/mL), 4) sperm + X-XO + SOD (150 U/mL), 5) sperm + X-XO + GSH (1.5 mM), 6) sperm + intermediate X (2 mM)-intermediate XO (0.1 U/mL), and 7) sperm + high X (10 mM)–high XO (0.5 U/mL). To maximize ROS production in treatments 6 and 7, XO was added in 2 equal allocations, at time 0 and time 15 minutes (Aitken et al, 1993a; Gomez et al, 1998). After incubation with the X-XO system, samples were centrifuged (300 × *g* for 10 minutes), the supernatant was removed, and the sperm pellet was resuspended in TALP (Ca^{2+}, Mg^{2+} and BSA free; Gomez et al, 1998) at the same cell concentration. Samples were then incubated with the lipid peroxidation promoters ferrous sulfate (0.64 mM) and sodium ascorbate (20 mM) for 2 hours at room temperature before determination of lipid peroxidation (Gomez et al, 1998). Lipid peroxidation was monitored by the end point generation of malondialdehyde detected by the LPO-586 kit (Biotech; Oxis International, Portland, Ore). Briefly, this assay detects malondialdehyde by the reaction of *N*-methyl-2-phenylindole with malondialdehyde to yield a stable chromophore with detection by spectrophotometry (586 nm; Lambda Bio, Perkin-Elmer, Norwalk, Conn). Results are expressed as a single concentration.

Experiment 5a: The Effect of Reactive Oxygen Species on Mitochondrial Membrane Potential of Equine Spermatozoa, as Determined by Fluorescence Multiwell Plate Reader—Treatments were the same as in experiment 1 with an additional treatment 8: sperm + X (1.8 mM)-XO (0.15 U/mL). After incubation with the X-XO system, samples were centrifuged at 300 × *g* for 10 minutes, the supernatant was removed, and the sperm pellet was resuspended in TALP without BSA at the same cell concentration. Samples were then incubated at room temperature for 40 minutes with 2 μM JC-1 (Molecular Probes; Gravance et al, unpublished data). The fluorescent dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′,-tetraethylbenzimidazoyl carbocyanine iodide) labels mitochondria with a high membrane potential orange (JC-1 aggregates) and labels mitochondria with a low membrane potential green (JC-1 monomers). After labeling with JC-1, a 200-μL aliquot of each sample was transferred into each of 2 duplicate wells of a 96-well microplate. Fluorescence was then assessed with excitation at 485 nm and with emission at 595 nm to detect total orange fluorescence or with emission at 535 nm to detect total green fluorescence in 2 separate measurements on each plate (HTS 7000; Perkin Elmer). Total fluorescence was blank corrected against TALP (no BSA) with 2-μM JC-1, and results are expressed as relative fluorescence units (RFU).

Experiment 5b: The Effect of Reactive Oxygen Species on Mitochondrial Membrane Potential of Equine Spermatozoa as Determined by Flow Cytometry—Treatments were as follows: 1) sperm alone, 2) sperm + X (0.6 mM)-XO (0.05 U/mL), 3) sperm + X-XO + CAT (150 U/mL), 4) sperm + X-XO + SOD (150 U/mL), and 5) sperm + X-XO + GSH (1.5 mM), and 6) sperm + X (1.2 mM)-X0 (0.1 U/mL). Treatment samples were incubated for 30 minutes at room temperature under aerobic conditions with 2-μM JC-1. After incubation, samples were diluted 1:5 in TALP (no BSA) containing 2μM JC-1 (Gravance et al,

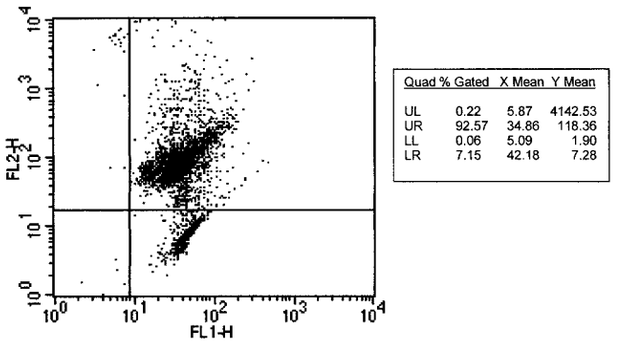

Figure 1. Cytogram of spermatozoa staining green (FL1-H) and orange (FL2-H) from a control (sperm alone) sample. JC-1 labels mitochondria with a high membrane potential orange (JC-1 aggregates) and mitochondria with a low membrane potential green (JC-1 monomers). Sperm staining orange appear in the upper right quadrant (UR); green-stained spermatozoa appear in the lower right quadrant (LR).

unpublished data), and diluted samples were immediately assessed for orange and green staining by flow cytometry. Forward (FS) and side scatter (SS) of the population of equine sperm to be analyzed were determined according to Gravance et al (unpublished data). A total of 10 000 gated events (based on FS and SS) were analyzed per sample; the sample running rates were approximately 100–300 events per second. A 488-nm filter was used for excitation of JC-1; emission filters of 535 nm and 595 nm were used, respectively, to quantify the population of sperm with green and orange fluorescence. Frequency plots were prepared for FL1 (green) and FL2 (orange) to determine the percent of the population stained green and orange (Figure 1). Results are expressed as percentage orange and percentage green populations. This experiment was conducted on 4 ejaculates from 3 stallions.

Statistical Analysis

Data were analyzed by analysis of variance (ANOVA); comparisons between individual means were performed with Duncan's multiple-range test. Differences with values of $P < .05$ were considered to be statistically significant. Data are presented as mean ± standard error of the mean (SEM).

Results

Experiment 1: Production of Hydrogen Peroxide by the X-XO System

The Amplex Red assay confirmed the production of hydrogen peroxide by the X-XO system (Figure 2). No significant differences were found in the production of H_2O_2 between the control (sperm alone) and sperm with either XO or X; however, the addition of X-XO together significantly ($P < .01$) increased H_2O_2 generation above the control. Catalase significantly ($P < .01$) reduced H_2O_2 generation in the presence of X-XO, whereas SOD and GSH did not reduce the generation of H_2O_2.

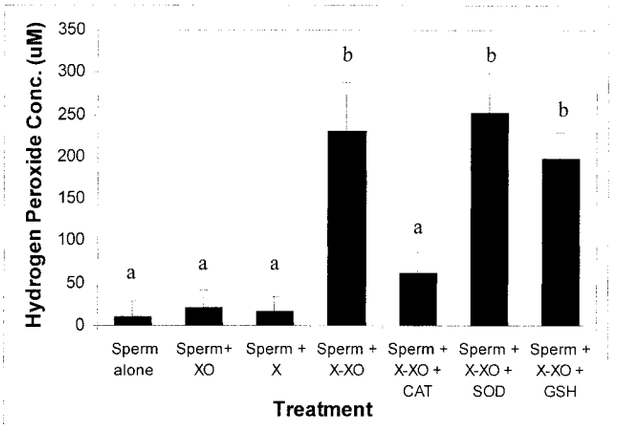

Figure 2. Mean (±SEM) concentrations of hydrogen peroxide (in micromoles) in equine sperm suspensions after a 30-minute incubation with and without xanthine (X, 0.6mM), xanthine oxidase (XO, 0.05 U/mL), or the antioxidants catalase (CAT, 150 U/mL), superoxide dismutase (SOD, 150 U/mL), and glutathione (GSH, 1.5 mM). Hydrogen peroxide concentrations were determined by the Amplex Red assay kit. Means with different superscripts (a,b) differ ($P < .05$; n = 6 ejaculates).

Experiment 2: The Effect of Reactive Oxygen Species on Equine Sperm Motility

At T0, there were no significant differences between treatments in any motility parameter. At T30, incubation with the X-XO system was associated with a significant ($P < .01$) decline in % TM and VSL compared with the case of the control (Figure 3). Similar changes were also demonstrated with VCL, ALH, LIN, and VAP (results not shown). Catalase prevented the decrease in all motility parameters observed upon incubation with the X-XO system, whereas SOD did not. The addition of GSH had an intermediate effect in preventing the decline in % TM, VSL, VCL, and VAP; however, GSH prevented the de-

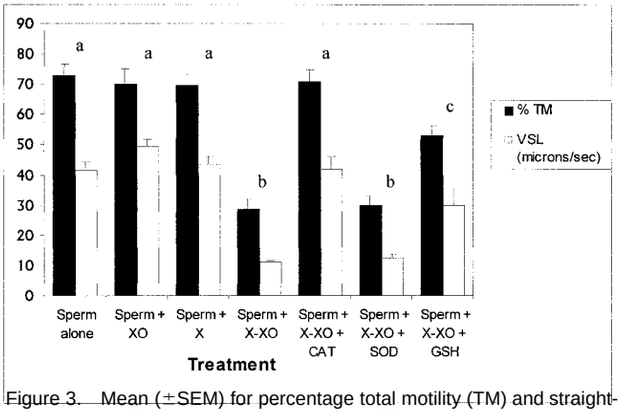

Figure 3. Mean (±SEM) for percentage total motility (TM) and straight-line velocity (VSL) for spermatozoa after a 30-minute incubation with or without the presence of xanthine (X, 0.6 mM); xanthine oxidase (XO, 0.05 U/mL); or the antioxidants catalase (CAT, 150 U/mL), superoxide dismutase (SOD, 150 U/mL), and glutathione (GSH, 1.5 mM). Means with different superscripts (a,b,c) differ ($P < .05$; n = 10 ejaculates).

cline in LIN when spermatozoa were incubated with X-XO.

Experiment 3: The Effect of Reactive Oxygen Species on Equine Sperm Viability and Acrosomal Integrity

At T30, there were no significant treatment effects on either viability or acrosomal integrity. The generation of ROS by the X-XO system had no effect on the percentage of live intact sperm (90.2% ± 1.7%) compared with that of control (89.0% ± 2.6%).

Experiment 4: The Effect of Reactive Oxygen Species on Lipid Peroxidation of Equine Spermatozoa

Malondialdehyde was increased (0.82 ± 0.51 μM) significantly ($P < .05$) above the control (0.43 ± 0.19 μM) with the addition of X-XO at the highest concentration. No significant increase in lipid peroxidation was observed with the intermediate (0.5 ± 0.27 μM) or low (0.42 ± 0.18 μM) concentration of X-XO.

Experiment 5: The Effect of Reactive Oxygen Species on Mitochondrial Membrane Potential of Equine Spermatozoa

There were no significant treatment effects on mitochondrial membrane potential after incubation with the X-XO system. The generation of ROS by 0.6 mM X − 0.05 U/mL XO had no effect on orange (9133 RFU) or green (15 798 RFU) fluorescence measured on the multiwell plate reader compared with the case of the control (8631 and 14 222 RFU, respectively). The generation of ROS by 0.6 mM X − 0.05 U/mL XO had no effect on the percentages orange (88%) or green (11%) populations determined by flow cytometry, compared with the case of control (88% and 11%, respectively).

Discussion

The results presented in this study are similar to those reported with human spermatozoa, showing an increase in the production of H_2O_2 (Griveau et al, 1995) and the superoxide anion (de Lamirande and Gagnon, 1992a; Aitken et al, 1993a) after incubation with the X-XO system. An inhibition of motility has also been observed following incubation of the X-XO system with human (de Lamirande and Gagnon, 1992a,b; Aitken et al, 1993a) and murine (Baiardi et al, 1997) spermatozoa. In the study of de Lamirande and Gagnon (1992a), human spermatozoa completely lost motility 1.5 hours after incubation with the same concentration of X-XO used in our study (0.6 mM X per 0.05 U/mL XO).

Although increased generation of ROS resulted in a marked decline in sperm motility, there was no detectable decrease in either sperm viability or acrosomal integrity. This finding contrasts that of Baiardi et al (1997), who demonstrated a 13% decline in the viability of mouse spermatozoa incubated with X-XO at a lower concentration than was used in our study. de Lamirande and Gagnon (1992a) also noted a 10%–20% decline in viability of human spermatozoa incubated with the same concentration of X-XO used in our study. Differences in experimental methodology may account for the different observations regarding the effect of ROS generated by the X-XO system on sperm viability. Likewise, there may be species differences in the susceptibility of spermatozoa to oxidative stress or perhaps may be species differences in the antioxidant capacity associated with spermatozoa.

Because the X-XO system generates both H_2O_2 and $O_2^{-\cdot}$, the ability of CAT, a H_2O_2 scavenger, to prevent a decline in sperm motility in the presence of the X-XO system indicates that H_2O_2 is the primary cytotoxic ROS. In contrast, SOD did not prevent the decline in motility parameters with X-XO, therefore $O_2^{-\cdot}$ was not the cytotoxic ROS responsible for the observed motility loss. These observations are consistent with previous research on human spermatozoa that also demonstrated H_2O_2 to be the ROS responsible for the loss of motility following incubation with the X-XO system (Aitken et al, 1993a).

Glutathione at the concentration used had an effect intermediate to CAT and SOD in preserving sperm motility. It would be of interest to determine whether or not a higher concentration of GSH would prevent the observed decline in motility parameters. This appears likely because other investigators have reported beneficial effects of GSH in blocking cytotoxic effects of ROS on human spermatozoa at concentrations of GSH that were higher than those used in our study (Griveau and Le Lannou, 1994; Baker et al, 1996). Because GSH is a single component of the glutathione peroxidase (GPX)/reductase (GRD) system, glutathione reductase is required for the regeneration of GSH from oxidized glutathione (GSSG). The activity of GPX/GRD has not been reported for equine spermatozoa, and the ability of equine spermatozoa to regenerate GSH remains to be determined.

There are several proposed mechanisms to account for the decline in sperm motility associated with oxidative stress. Peroxidation of polyunsaturated fatty acids (PUFAs) in membrane lipids is one frequently cited mechanism (Aitken et al, 1989, 1993b,c). Spermatozoa are particularly susceptible to lipid peroxidation because of the high concentration of PUFAs in their plasma membrane. As a consequence of lipid peroxidation, the plasma membrane loses PUFAs with the associated production of lipid hydroperoxides, alkoxyl and peroxyl radicals. These radicals promote the lipid peroxidation chain reaction and ultimately lead to the production of cytotoxic aldehydes such as malondialdehyde and 4-hydroxynonenol (4HN; reviewed by Aitken, 1995). The high concentration of

PUFAs in the sperm membrane is required to give the plasma membrane the fluidity it needs for sperm motility and participation in the membrane fusion events associated with fertilization, as well as the structural integrity required for viability. Loss of integrity can also lead to an increase in membrane permeability and a loss in the capacity to regulate the intracellular concentrations of ions involved in the control of sperm movement.

In our study and in previous studies of de Lamirande and Gagnon (1992a,b), the decline in sperm motility that occurred in the presence of the X-XO free radical–generating system was not accompanied by a detectable increase in lipid peroxidation. These results indicate that sperm motility may be a more sensitive indicator of oxidative stress and is therefore inhibited before any measurable increase in lipid peroxidation. Therefore, sperm motility may be affected by ROS through a mechanism of action separate to that of lipid peroxidation. In somatic cell systems, it has been proposed that H_2O_2 causes perturbations in important biochemical functions, including increased formation of oxidized intracellular sulfhydryls, rapid decrease in ATP levels, and a consequent depression of glycolytic flux. These processes occur before any loss of plasma membrane integrity or increased lipid peroxidation (Hyslop et al, 1986, 1988). Accordingly, de Lamirande and Gagnon (1992a) suggested that the inhibition of sperm motility after incubation with ROS was caused by a depletion of ATP. These authors subsequently confirmed this hypothesis by demonstrating a significant reduction in sperm ATP levels in the first 1 hour after ROS treatment (de Lamirande and Gagnon, 1992b). These authors proposed that sperm immobilization was due to a decreased phosphorylation of axonemal proteins required for sperm movement. Further investigation indicated that ROS inhibited one or more enzymes of oxidative phosphorylation, glycolysis, or both, thus limiting ATP generation by the sperm cell. In the experimental systems that induce oxidative stress based upon X-XO, it would appear that hydrogen peroxide might be more likely to affect intracellular enzyme systems because of its higher membrane permeability. Alternatively, enzyme inhibition could be induced indirectly by products of lipid peroxidation, especially malondialdehyde and 4HN. Low concentrations of these substances have been shown to inhibit a large number of cellular enzymes and functions, anaerobic glycolysis, DNA, RNA, and protein synthesis (Comporti, 1989). It is also possible that the assay for lipid peroxidation used in this study was not sensitive enough to detect small changes in lipid peroxidation that may have affected sperm cell function, such as in the mitochondrial membrane or mitochondrial enzymes.

Reactive oxygen species may also adversely affect sperm motility via alterations in mitochondrial function. Mitochondrial membrane potential has been used as a measure of mitochondrial function and is linked to a host of mitochondrial functions, including ATP synthesis, import of mitochondrial proteins, calcium homeostasis, and metabolite transport. The carbocyanine dye, JC-1, was used in our study to investigate mitochondrial membrane potential. No change in mitochondrial membrane potential was detected after incubation with the X-XO system at a concentration that significantly decreased motility. Armstrong et al (1999) also reported that low concentrations of H_2O_2 caused inhibition of sperm motility and a reduction in sperm ATP levels without any elevation in lipid peroxidation or significant decline in the mitochondrial membrane potential. Armstrong et al (1999) propose that the metabolic site of action of H_2O_2 in human spermatozoa is not mitochondrial membrane-dependent oxidative phosphorylation, as proposed by de Lamirande and Gagnon (1992a,b). They suggest that the role of H_2O_2 in the inhibition of sperm movement occurs because of perturbations of metabolic sites upstream of mitochondrial membrane-dependent oxidative phosphorylation; further experimentation is required to determine where the exact site of ROS injury occurs. Therefore, the specific action of ROS on the sperm cell responsible for inhibition of sperm motility has yet to be determined.

In summary, hydrogen peroxide has a primary role in mediating equine spermatozoa damage due to ROS generation by the X-XO system. Catalase may therefore be an excellent candidate for future antioxidant therapy in the field of stallion sperm storage. Equine sperm motility is a more sensitive indicator of oxidative stress than are viability, acrosome integrity, lipid peroxidation, and mitochondrial membrane potential. This result also suggests that equine sperm motility may be affected by a pathway of ROS action independent of lipid peroxidation and mitochondrial membrane dependent functions, both previously reported to be responsible for ROS damage to motility in human spermatozoa.

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References

Aitken RJ. Free radicals, lipid peroxidation and sperm function. *Reprod Fertil Dev.* 1995;7:659–668.

Aitken RJ, Buckingham, D., Harkiss, D. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *J Reprod Fertil.* 1993a; 97:441–450.

Aitken RJ, Buckingham DW, West KM. Reactive oxygen species and human spermatozoa: analysis of the cellular mechanisms involved in luminol- and lucigenin-dependent chemiluminescence. *J Cell Physiol.* 1992;151:466–477.

Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. *J Reprod Fertil.* 1987;81:459–469.

Aitken RJ, Clarkson JS, Fisher S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod.* 1989;41: 183–197.

Aitken RJ, Fisher HM, Fulton N, Gomez E, KnoxW, Lewis B, Irvine S. Reactive oxygen species generation by human spermatozoa is induced by exogenous NADPH and inhibited by the flavoprotein inhibitors diphenylene iodonium and quinacrine. *Mol Reprod Dev.* 1997;47: 468–482.

Aitken RJ, Harkiss D, Buckingham DW. Analysis of lipid peroxidation mechanisms in human spermatozoa. *Mol Reprod Dev*. 1993b;35:302–315.

Aitken RJ, Harkiss D, Buckingham D. Relationship between iron-catalyzed lipid peroxidation potential and human sperm function. *J Reprod Fertil.* 1993c;98:257–265.

Aitken RJ, Harkiss D, Knox W, Paterson M, Irvine DS. A novel signal transduction cascade in capacitating human spermatozoa characterised by a redox-regulated, cAMP-mediated induction of tyrosine phosphorylation. *J Cell Sci.* 1998;111:645–656.

Aitken RJ, Paterson M, Fisher H, Buckingham DW, Van Duin M. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci.* 1995;108: 2017–2025.

Alvarez JG, Storey BT. Taurine, hypotaurine, epinephrine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. *Biol Reprod.* 1983;29:548–555.

Alvarez JG, Storey BT. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.* 1989;23:77–90.

Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. *J Androl.* 1987;8:338–348.

Armstrong JS, Rajaskaran M, Chamulitrat W, Gatti P, Hellstrom WJ, Sikka SC. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. *Free Radic Biol Med.* 1999;26:869–880.

Aurich JE, Schonherr U, Hoppe H, Aurich C. Effects of antioxidants on motility and membrane integrity of chilled-stored stallion semen. *Theriogeniology* 1997;48:185–192.

Baiardi G, Ruiz RD, Fiol de Cuneo M, Ponce AA, Lacuara JL, Vincenti L. Differential effects of pharmacologically generated reactive oxygen species upon functional activity of epididymal mouse spermatozoa. *Can J Physiol Pharmacol.* 1997;75:17533–17538.

Baker HW, Brindle J, Irvine DS, Aitken RJ. Protective effect of antioxidants on the impairment of sperm motility by activated polymorphonuclear leukocytes. *Fertil Steril.* 1996;65:411–419.

Brittigan BE, Pou S, Rosen GM, Lilleg DM, Buettner GR. Hydroxyl radical is not a product of the reaction of xanthine oxidase and xanthine. *J Biol Chem.* 1990;265:17533–17538.

Casey PJ, Hillman RB, Robertson KR, Yudin AI, Liu IK, Drobnis EZ. Validation of an acrosomal stain for equine sperm that differentiates between living and dead sperm. *J Androl.* 1993;14:289–297.

Chow CK. Vitamin E and oxidative stress. *Free Radic Biol Med.* 1991; 11:215–232.

Comporti M. Three models of free radical-induced cell injury. *Chemico-Biol Interact.* 1989;72:1–56.

Davies KJ. Protein damage and degradation by oxygen radicals I: general aspects. *J Biol Chem.* 1987;262:9895–9901.

de Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. I. Effects on the motility of intact spermatozoa and on sperm axonemes. *J Androl.* 1992a;13:368–378.

de Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl*. 1992b;13:379–386.

de Lamirande E, Gagnon C. A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *Int J Androl.* 1993a;16:21–25.

de Lamirande E, Gagnon C. Human sperm hyperactivation and capacitation as parts of an oxidative process. *Free Radic Biol Med*. 1993b; 14:157–66.

Drobnis EZ, Zhong CQ, Overstreet JW. Separation of cryopreserved human semen using Sephadex columns, washing, or Percoll gradients. *J Androl.* 1991;12:201–208.

Gavella M, Lipovac V, Vucic M, Rocic B. Evaluation of ascorbate and urate antioxidant capacity in human semen. *Andrologia.* 1997;29:29–35.

Gomez E, Buckingham DW, Brindle J, Lanzafame F, Irvine DS, Aitken RJ. Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J Androl.* 1996;17:276–287.

Gomez E, Irvine DS, Aitken RJ. Evaluation of a spectrophotometric assay for the measurement of malondialdehyde and 4-hydroxyalkenals in human spermatozoa: relationships with semen quality and sperm function *Int J Androl.* 1998;21:81–94.

Griveau JF, Dumont E, Renard P, Callegari JP, Le Lannou D. Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *J Reprod Fertil.* 1995;103:17–26.

Griveau JF, Le Lannou, D. Effects of antioxidants on human sperm preparation techniques. *Int J Androl.* 1994;17:225–231.

Hyslop PA, Hindshaw DB, Halsey WA, Schraufstatter IU, Sauerheber RD, Spragg RG, Jackson JH, Cochrane CG. Mechanisms of oxidant-mediated cell injury. The glycolytic and mitochondrial pathways of ADP phosphorylation are major intracellular targets inactivated by hydrogen peroxide. *J Biol Chem.* 1988;262:1665–1675.

Hyslop PA, Hindshaw DB, Schraufstatter IU, Sauerheber RD, Sklar LA, Spragg RG, Cochrane CG. Intracellular calcium homeostasis during hydrogen peroxide injury to cultured P388D_1 cells. *J Cell Physiol.* 1986;129:356–366.

Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril.* 1992;57:409–416.

Jeulin C, Soufir JC, Weber P, Laval-Martin D, Calvayrac R. Catalase activity in human spermatozoa and seminal plasma. *Gamete Res.* 1989;24:185–196.

Kodama H, Yamaguchi R, Fukuda J, Kasai H, Tanaka T. Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril.* 1997;68:519–524.

Li TK. The glutathione and thiol content of mammalian spermatozoa and seminal plasma. *Biol Reprod.* 1975;12:641–646.

McCord JM, Fridovich I. The reduction of cytochrome C by milk xanthine oxidase. *J Biol Chem.* 1968;249:391–399.

Meyers SA, Overstreet JW, Liu IKM, Drobnis EZ. Capacitation in vitro of stallion spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile males. *J Androl.* 1995;16:47–54.

Niki E. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. *Am J Clin Nutr.* 1991;54(suppl):1119S–1124S.

Nissen HP, Kreysel HW. Superoxide dismutase in human semen. *Klin Wochenschrift.* 1983;61:63–65.

Padilla AW, Foote RH. Extender and centrifugation effects on the motility patterns of slow-cooled stallion spermatozoa. *J Anim Sci.* 1991;69: 3308–3313.

Ronquist G, Niklasson F. Uridine, xanthine, and urate contents in human seminal plasma. *Arch Androl.* 1984;13:63–70.

Sikka SC, Rajasekaran M, Hellstrom WJ. Role of oxidative stress and antioxidants in male infertility. *J Androl.* 1995;16:464–481.

Smith R, Vantman D, Ponce J, Escobar J, Lissi E. Total antioxidant capacity of human sperm. *Hum Reprod.* 1996;11:1655–1660.

Erratum

Quinn P. Review of media used in ART laboratories [Andrology Lab Corner]. *J Androl.* 2000;21:610–615.

Page 613, first paragraph, line 2:

The sentence should read, ‘‘Embryo development was inhibited in the presence of glucose and Pi.’’