Assessment of the cryopreservation of equine spermatozoa in the presence of enzyme scavengers and antioxidants

Julie Baumber, PhD; Barry A. Ball, DVM, PhD; Jennifer J. Linfor, MS

Objective—To evaluate the effect of the addition of enzyme scavengers and antioxidants to the cryopreservation extender on characteristics of equine spermatozoa after freezing and thawing.

Sample Population—2 ejaculates collected from each of 5 stallions.

Procedure—Equine spermatozoa were cryopreserved in freezing extender alone (control samples) or with the addition of catalase (200 U/mL), superoxide dismutase (200 U/mL), reduced glutathione (10mM), ascorbic acid (10mM), α-tocopherol (25, 50, 100, or 500µM or 1mM), or the vehicle for α-tocopherol (0.5% ethanol). After thawing, spermatozoal motility was assessed via computer-assisted analysis and DNA fragmentation was assessed via the comet assay. Spermatozoal mitochondrial membrane potential, acrosomal integrity, and viability were determined by use of various specific staining techniques and flow cytometry.

Results—The addition of enzyme scavengers or antioxidants to cryopreservation extender did not improve spermatozoal motility, DNA fragmentation, acrosomal integrity, viability, or mitochondrial membrane potential after thawing. Superoxide dismutase increased DNA fragmentation, likely because of the additional oxidative stress caused by the generation of hydrogen peroxide by this enzyme. Interestingly, the addition of the vehicle for α-tocopherol resulted in a significant decrease in live acrosome-intact spermatozoa.

Conclusions and Clinical Relevance—The addition of antioxidants to the cryopreservation extender did not improve the quality of equine spermatozoa after thawing, which suggests that the role of oxidative stress in cryopreservation-induced damage of equine spermatozoa requires further investigation. Our data suggest that solubilizing α-tocopherol in ethanol may affect spermatozoal viability, consequently, water-soluble analogues of α-tocopherol may be preferred for future investigations. (Am J Vet Res 2005;66:772–779)
decreased following cryopreservation, compared with that of fresh preparations. Furthermore, freeze-thawing of equine spermatozoa and cryopreservation of bovine spermatozoa have been associated with an increase in ROS generation. Consequently, cryopreservation may subject spermatozoa to oxidative stress. Indeed, some researchers report an increase in lipid peroxidation following cryopreservation of spermatozoa.

The addition of enzyme scavengers or antioxidants to spermatozoal preparations in vitro has been successful at countering the effects of oxidative stress on spermatozoal motility, viability, and lipid peroxidation, spermatozoont-oocyte fusion, and spermatozoal DNA fragmentation. Catalase also had a protective effect on the motility of equine spermatozoa from ROS-induced DNA fragmentation. Catalase also had a protective effect on the motility of equine spermatozoa when ROS were generated by neutrophils or by the xanthine-xanthine oxidase system, and lipid peroxidation of equine spermatozoa could be reduced by the addition of α-tocopherol or sodium ascorbate. Accordingly, the addition of an antioxidant to semen extenders has been implicated for cryopreservation to minimize the potential effects of oxidative stress during the freeze-thaw process. The purpose of the study reported here was to evaluate the effect of the addition of enzyme scavengers and antioxidants to semen extender used for cryopreservation on the quality of equine spermatozoa after thawing; motility, DNA fragmentation, viability, acrosomal integrity, and mitochondrial membrane potential of the spermatozoa were used to assess spermatozoal quality.

**Materials and Methods**

Horses—Five mature stallions of light horse breed (Thoroughbreds and Arabians; mean age ± SD, 13.4 ± 2.8 years) were included in the study. Stallions were housed at the University of California, Davis. Semen was collected from these stallions on a regular basis, and those samples had normal seminal parameters.

**Semen collection and cryopreservation**—Semen was collected by use of an artificial vagina from each of the 5 stallions, filtered, and mixed with prewarmed (37°C) extender containing ticarcillin (1 mg/mL) to 50 × 10^6 cells/mL. All ejaculates contained at least 2 billion spermatozoa and had an initial progressive motility of > 50%. After centrifugation at 400 × g for 15 minutes, pellets of spermatozoa were resuspended in freezing extender (INRA 82 + 2% glycerol) at a concentration of 200 × 10^6 cells/mL. The extended semen was loaded into 0.5 mL polyvinyl chloride straws that were then sealed with polyvinyl chloride sealing powder. Straws were frozen with a programmable freezer by use of a freezing curve as follows: temperature change from 20°C to 5°C at a rate of −0.5°C/min; temperature change from 5°C to −15°C at a rate of −10°C/min; and temperature change from −15°C to −50°C at a rate of −25°C/min. Straws were thawed (30 seconds at 37°C) for post-thaw analysis after at least 1 week of storage at −190°C.

**Experiment 1**—In experiment 1, samples of spermatozoa were frozen in freezing extender according to one of the following treatments: treatment 1, extender alone (control samples); 2, extender with catalase (from Aspergillus niger, 200 U/mL); 3, extender with superoxide dismutase (SOD) from bovine erythrocytes, 200 U/mL; 4, extender with reduced glutathione (free acid, 10 mM); 5, extender with α-tocopherol (10 mM) 6, extender with α-tocopherol (1 mM) + sodium ascorbate (100 mM); 7, extender with α-tocopherol (1 mM) + α-tocopherol (10 mM) + ascorbic acid (10 mM); 8, extender with α-tocopherol (1 mM) + α-tocopherol (10 mM) + ascorbic acid (10 mM) + tocopherol (ie, 0.5% ethanol). Experiment 1 was replicated for 2 ejaculates from each of 5 stallions.

To assess spermatozoal motility after thawing, thawed spermatozoa were diluted to a concentration of 25 × 10^6 cells/mL in prewarmed extender and incubated at 37°C. Spermatozoal motility was determined after 15 and 30 minutes of incubation. At least 200 cells/sample in a minimum of 4 microscopic fields were analyzed via computer-assisted spermatozoal analysis involving microcomputer slides of a fixed chamber depth and cover glasses. Instrument settings for the computer-assisted spermatozoal analysis were as follows: frame rate, 60 Hz; frames acquired, 300; minimum contrast, 40; minimum cell size, 8 pixels; low average path velocity cutoff, 5 µ/s; low straight-line velocity cutoff, 2 µ/s; progressive average path velocity threshold, 30 µ/s; progressive straightness threshold, 70%; static intensity limits, 0.69 to 1.65; static size limits, 0.4 to 2.07; and static elongation limits, 11 to 99.

After thawing, spermatozoal mitochondrial membrane potential, viability, and acrosomal integrity were also assessed. Thawed semen samples were extended to 25 × 10^6 cells/mL in a prewarmed (37°C) modified Tyrode’s albumin-lactate-pyruvate medium supplemented with 0.1% polyvinyl alcohol (TALP). Samples were then divided into 2 aliquots; the first aliquot was used immediately for the analysis of spermatozoal mitochondrial membrane potential and determination of spermatozoal viability and acrosomal integrity. The second aliquot was maintained at room temperature (approx 22°C) for 4 hours before analysis of spermatozoal mitochondrial membrane potential and determination of spermatozoal viability and acrosomal integrity. The experiment was replicated for 2 straws from each ejaculate. Replicate 1 was run in numerical order (treatment 1 to 8) and replicate 2 in reverse numerical order (treatment 8 to 1).

Spermatozoal mitochondrial membrane potential was determined via staining with 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolyl carboxylic acid (JC-1). Stock solutions of 1.53 mM JC-1 were prepared in dimethyl sulfoxide. A 0.5 mL aliquot of each sample was stained with 1.0 µL of JC-1 stock solution (final concentration, 2.0 µM) for 30 minutes at 37°C. After 30 minutes, a 100-µL aliquot of each sample was diluted 1:1 in prewarmed TALP containing 2.0 µM JC-1 and immediately assessed via flow cytometry. Data were expressed as a ratio of red fluorescence to green fluorescence, with red and green fluorescence of a sample representing spermatozoa with a high or low mitochondrial membrane potential, respectively.

Acrosomal integrity and viability were determined by dual labeling with fluorescein isothiocyanate-isothiocyanate-conjugated Pisum sativum agglutinin and ethidium-1 homodimer, respectively. The use of ethidium-1 homodimer for determination of equine spermatozoal viability was validated in preliminary experiments and was found to be comparable to the use of propidium iodide (data not shown). A 100-µL aliquot of each treatment was stained with 1.0 µL of fluorescein isothiocyanate-conjugated P. sativum agglutinin (final concentration, 0.05 mg/mL) for 10 minutes at 37°C, followed by staining with 100 µM ethidium-1 homodimer for an additional 5 minutes. Samples were then diluted 1:4 in prewarmed TALP and immediately assessed via flow cytometry.

Forward and side scatter of the population of equine spermatozoa to be analyzed were determined according to the method of Gravance et al. On the basis of forward and side scatter, 10,000 gated events/sample were analyzed; the sample running rates were approximately 200 to 500 events/s. Fluorescent probes were excited at a wavelength of 488 nm by
use of an argon ion laser. Fluorescence was detected by use of a filter setup that included dichroics at 560 and 640 nm and filters of 530 and 585 nm to quantify the population of spermatozoa with green or red fluorescence, respectively.

Fragmentation of the DNA of spermatozoa in samples after thawing was determined via the neutral single-cell gel electrophoresis (comet) assay, which was modified to measure DNA damage in equine spermatozoa. For this assay, spermatozoa are embedded in agarose; samples undergo cell lysis, DNA decondensation, electrophoresis, neutralization, and DNA staining with ethidium bromide. The cells are then examined via fluorescent microscopy.

Figure 1—Photomicrographs (A through E) obtained via epifluorescence microscopy of equine spermatozoa after processing via a single-cell gel electrophoresis (comet) assay and staining by ethidium bromide. Relative changes in spermatozoal DNA fragmentation are represented by an increasing size of the comet tail and were assigned a grade of 0 (no comet tail; A), 1 (B), 2 (C), 3 (D), or 4 (large comet tail; E). Scale bar applies to all panels; bar = 20 µm.
Spermatozoa with intact nuclei appear in the comet assay to have compact and brightly fluorescent heads. In contrast, strand breaks in damaged spermatozoa allow DNA migration during electrophoresis and a trail of DNA can be seen behind the head, giving the appearance of a comet.

All 8 treatments could not be run simultaneously in the electrophoresis tank; consequently, treatments were divided into 2 groups (treatments 1 to 5 and treatments 1, 6, 7, and 8). After subjecting spermatozoa to the comet assay protocol as described by Linfor and Meyers,61 nuclei of spermatozoa were examined by use of an epifluorescence microscope (excitation = 510 nm; emission = 595 nm). Images of sperm nuclei were digitized by use of a video camera and image analysis software. Digitized images of spermatozoal nuclei (100/slide) were subsequently evaluated for each treatment by an examiner (JB) who was unaware of treatment groups. Spermatozoa with comet tails were assessed subjectively and assigned a damage grade31,63: grade 0, no comet (ie, no damage) or grade 1, 2, 3, or 4, large comet (ie, extensive damage; Figure 1). The individual grade scores for 100 spermatozoa from each treatment were converted into a composite score by multiplying the number of spermatozoal nuclei by the corresponding numerical score. Thus, the composite damage score could range from 0 (all spermatozoa undamaged) to 400 (all spermatozoa maximally damaged).

Experiment 2—In experiment 2, samples of spermatozoa were frozen in freezing extender according to one of the following treatments: treatment 1, extender alone (control samples); 2, extender with α-tocopherol (25 µM); 3, extender with chemical agents (250 µM); 4, extender with α-tocopherol (50 µM); 5, extender with chemical agents (100 µM); 6, extender with α-tocopherol (0.5 mM); 7, extender with vehicle control for α-tocopherol (ie, 0.5% ethanol). Experiment 2 was replicated for 2 ejaculates from each of 3 stallions. After thawing, spermatozoal motility, mitochondrial membrane potential, viability, and acrosomal integrity were analyzed as in experiment 1.

Statistical analyses—Data were analyzed by a mixed-model ANOVA, and individual means were compared by use of a Tukey honestly significantly difference test performed using computer software. Results are presented as least squares means ± SEM of combined data obtained at 15 and 30 minutes after thawing.

Figure 2—Total (black bars) and progressive (white bars) motility (%) of spermatozoa collected from 5 stallions after cryopreservation in freezing extender alone (control) or extender containing antioxidants and subsequent thawing. Cryopreservation treatments were as follows: 1, control samples; 2, extender with catalase (from Aspergillus niger, 200 U/mL); 3, extender with superoxide dismutase (from bovine erythrocytes, 200 U/mL); 4, extender with reduced glutathione (10 mM); 5, extender with ascorbic acid (10 mM); 6, extender with α-tocopherol (1 mM); 7, extender with α-tocopherol (1 mM) and ascorbic acid (10 mM); and 8, extender with vehicle control for α-tocopherol (ie, 0.5% ethanol). Values represent least squares means ± SEM of combined data obtained at 15 and 30 minutes after thawing. Mean values with different superscript letters are significantly (P < 0.05) different.

Figure 3—Fragmentation of DNA (as determined by the comet assay) of spermatozoa collected from 5 stallions after cryopreservation in freezing extender alone (control) or extender containing antioxidants and subsequent thawing (A and B). Spermatozoal comets were graded from 0 (no comet or no damage) to 4 (large comet or extensive damage), and the individual grade scores for 100 spermatozoa from each treatment were converted into a composite score by multiplying the number of sperm nuclei by the corresponding numerical score. The percentage contribution of each grade to the composite score is represented within each bar. Values represent least squares means ± SEM of combined data obtained at 15 and 30 minutes after thawing. See Figure 2 for key.
imer and fluorescein isothiocyanate-conjugated Pisum sativum integrity were determined via staining with ethidium-1 homod-pherol (ie, 0.05% ethanol). Spermatozoal viability and acrosomal extender with tocopherol (50 µM); 2, extender with α-tocopherol (25µM); 3, extender with α-tocopherol (100µM); 5, extender with α-tocopherol (0.5mM); 6, extender with α-tocopherol (1mM); and 7, extender with vehicle control for α-tocopherol (ie, 0.05% ethanol). Spermatozoal viability and acrosomal integrity were determined via staining with ethidium-1 homodimer and fluorescein isothiocyanate-conjugated Plasm sativum agglutin, respectively, and analyzed via flow cytometry. See Figure 2 for remainder of key.

Discussion

Cryopreservation of human,64 trout,65 and equine31 spermatozoa has been reported to result in an increase in DNA fragmentation relative to fresh spermatozoa. There is some concern that fertilization with DNA-damaged spermatozoa may result in paternal transmission of defective genetic material with adverse consequences for embryonic development.66-70 Spermatozoa are unable to repair DNA damage because they do not contain functional repair enzymes71,72; therefore, prevention of DNA damage to those cells would be beneficial. In human spermatozoa, several antioxidants are known to decrease DNA fragmentation resulting from oxidative stress in vitro; these antioxidants include glutathione,28,53 ascorbic acid,26,52 hypotaurine,28,53 N-acetylcholine,28 α-tocopherol,28,52 and urate.52 In our experience, catalase and glutathione prevent equine spermatozoa from ROS-induced DNA fragmentation.73 In the present study, the addition of antioxidants to the cryopreservation extender did not lessen the extent of spermatozoal DNA fragmentation detected after thawing. Moreover, the addition of SOD resulted in a significant (P < 0.05) decrease in the percentage of live acrosome-intact spermatozoa following cryopreservation in the presence of 50 and 500µM α-tocopherol, compared with the control treatment, which were possibly related to a vehicle effect (Figures 4 and 5). Ejaculate-within-stallion differences were significant (P < 0.05) for analysis of total and progressive spermatozoal motility, mitochondrial membrane potential, acrosomal integrity, and viability.

Experiment 2—There was no significant treatment effect on total and progressive spermatozoal motility after thawing associated with the addition of α-tocopherol to the cryopreservation extender. The addition of α-tocopherol did not improve spermatozoal mitochondrial membrane potential, viability, or acrosomal integrity after thawing. However, the addi-
generated by spermatozoa. Aitken et al.3 reported a similar adverse effect on spermatozoal motility when SOD was added to human spermatozoal suspensions in vitro.

Addition of antioxidant to the cryopreservation extender did not improve spermatozoal motility, viability, acrosomal integrity, or mitochondrial membrane potential of equine spermatozoa after thawing, compared with findings in samples of spermatozoa treated with extender alone. These findings are similar to the results obtained in our laboratory when antioxidants were added to equine semen during cooled storage.5 We have previously detected a high concentration of catalase in equine seminal plasma42 and catalase activity associated with the spermatozoal plasma membrane. Catalase has also been detected in human spermatozoa.43 Therefore, catalase activity may be responsible for the metabolism of ethanol to acetaldehyde, a highly reactive species, and α-tocopherol may have protected the spermatozoal membrane from lipid peroxidation induced by this metabolite. Further research is required to delineate the effect of alcohol on spermatozoa. As an alternative to solubilizing α-tocopherol in ethanol, use of synthetic water-soluble analogs of α-tocopherol should be considered for future investigations.

References
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