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Sperm Characteristics and DNA Integrity of Iberian Red Deer (*Cervus elaphus hispanicus*) Epididymal Spermatozoa Frozen in the Presence of Enzymatic and Nonenzymatic Antioxidants

MARÍA R. FERNÁNDEZ-SANTOS[*,†], FELIPE MARTÍNEZ-PASTOR[*,‡], VANESA GARCÍA-MACÍAS[‡], MILAGROS C. ESTESO[*,†], ANA J. SOLER[*,†], P. PAZ[‡], L. ANEL[‡] AND JOSÉ J. GARDE[*,†]

From the [*] Biology of Reproduction Group, Department of Game Resources (IDR), Castilla-La Mancha University (UCLM), Albacete, Spain; the [†] National Wildlife Research Institute (IREC), UCLM-CSIC-JCCM, Albacete, Spain; and the [‡] Animal Reproduction Group, Faculty of Veterinary Medicine, León University, León, Spain.

Correspondence to: José Julián Garde, Instituto de Investigación en Recursos Cinegéticos (IREC), Campus Universitario sn, 02071-Albacete, Spain (e-mail: Julian.Garde{at}uclm.es).

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Abstract

The main goal of this study was to investigate the potential protective effects of enzymatic and nonenzymatic antioxidants on cryopreservation injuries to red deer epididymal spermatozoa. In Experiment 1, the effects on sperm freezability of the enzymatic antioxidants catalase, superoxide dismutase, and a combination thereof were studied. In Experiment 2, sperm cryoresistance was evaluated when different nonenzymatic antioxidants, such as vitamin E, vitamin C, and butylated hydroxytoluene (BHT), were added to the freezing extender. Sperm quality was judged in vitro by microscopic assessments of individual sperm motility (SMI), viability, and acrosome (ie, spermatozoa with normal apical ridges; % NAR) and membrane (by means of the HOS test) integrity. To address fully these topics, we incorporated a new set of functional sperm tests for mitochondrial function, membrane phospholipid disorder, and sperm chromatin stability. Samples were evaluated after freezing and thawing, and after a 2-hour period of incubation at 37°C. The present study demonstrates that the addition of enzymatic antioxidants to freezing extenders improves sperm viability after cooling, and improves sperm motility, acrosome integrity, and mitochondrial status ($P < .05$) after thawing. After a 2-hour incubation period at 37°C in the presence of enzymatic antioxidants, an improvement in membrane integrity ($P < .05$) was observed. However, when

nonenzymatic antioxidants were present in the freezing diluents, no positive effects on thawed sperm parameters were noted. The chromatin stability test did not show significant differences between the treatments. We conclude that enzymatic antioxidants should be present in the early steps of cryopreservation of epididymal spermatozoa from red deer, so as to improve motility and acrosome integrity.

Key words: Cryopreservation, fertilization, gamete biology, reproductive technology

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The processes of cooling, freezing and thawing generate physical and chemical stresses on the sperm membranes, thereby reducing sperm viability and fertilizing ability. One of the deleterious effects of cryopreservation is the generation of reactive oxygen species (ROS) (Alvarez and Storey, 1992; O'Flaherty et al, 1997; Chatterjee and Gagnon, 2001). Indeed, it has been demonstrated for human (Alvarez and Storey, 1992), bull (O'Flaherty et al, 1997), and mouse spermatozoa (Mazur et al, 2000) that cryopreservation is associated with oxidative stress. Moreover, the freezing and thawing of spermatozoa increase the generation of ROS (Chatterjee and Gagnon, 2001), resulting in DNA damage (Lopes et al, 1998), cytoskeletal alterations (Hinshaw et al, 1986), inhibition of sperm-oocyte fusion (Aitken et al, 1989), and effects on the sperm axoneme that lead to loss of motility (De Lamirade and Gagnon, 1992). Thus, it is possible that the cell death and loss of cell function that occur during or following a freeze-thaw cycle involve oxidative stress (O'Flaherty et al, 1997; Bailey et al, 2000; Bilodeau et al, 2000). One way to overcome the detrimental effects of ROS on sperm performance after thawing could be to add antioxidants to the freezing extender, to block or prevent oxidative stress.

Sperm mature as they transit from the testis to the cauda epididymides, and many of the changes occur on the surface. The high polyunsaturated fatty acid content of the sperm plasma membrane makes the sperm especially vulnerable to ROS attack (O et al, 2006). The microenvironment in the male reproductive tract has to provide enzymatic and nonenzymatic strategies to protect the spermatozoa from excessive oxidative stress, so as to preserve sperm function and sperm DNA integrity (Vernet et al, 2004). When the levels of ROS overwhelm the antioxidant defense system, oxidative stress occurs. At this point, the risk of undergoing peroxidative damage by ROS, produced by either neutrophils that are present in the semen or the sperm cells themselves (Gagnon et al, 1991; Sikka et al, 1995), is still low. This condition does not exclude the need for a protective mechanism against oxidation, which is indeed present in the sperm cells (Gu and Hecht, 1996), as well as in the epididymal fluid (Hinton et al, 1995). Moreover, the secretions of the male accessory sex glands (seminal plasma) provide many antioxidants, which contribute to protecting the spermatozoa in the postejaculatory phase. Thus, male accessory sex gland secretions play a major role in protecting sperm against oxidative stress (Chen et al, 2003). Apart from providing sperm with a nutritious medium for gamete transfer, these glands secrete antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, as well as free radical scavengers, such as vitamins C and E, hypotaurine, taurine, uric acid, and albumin (O et al, 2006).

The interest in preserving the germplasm of wild deer species has resulted in intense efforts to enhance the recovery, evaluation, and cryopreservation of sperm from the epididymides of dead animals (Zomborszky et al, 1999; Comizzoli et al, 2001; Hishinuma et al, 2003; Soler and Garde, 2003). However, little information has been published on freezing and thawing methods for epididymal spermatozoa of this species, although there have been many studies on the freezing of ejaculated semen from stags (Asher et al, 2000). Most of the procedures used to cryopreserve epididymal spermatozoa from red deer have been modified from those developed for ejaculated semen. This approach is not very appropriate, since it is well known that there are important differences in the

physiological characteristics of epididymal and ejaculated spermatozoa, particularly with respect to their membranes properties, which affect sperm survival after freezing (Martinez-Pastor et al, 2006b). Furthermore, epididymal spermatozoa are not exposed to the complex secretions of the accessory sex glands (seminal plasma), which are recognised as the prime source of antioxidant protection (Chen et al, 2003). In this respect, we have demonstrated that red deer seminal plasma improves the cryopreservation of Iberian red deer epididymal spermatozoa (Martinez-Pastor et al, 2006a). It is necessary to improve the existing protocols for cooling and freezing red deer epididymal spermatozoa, since oxidative damage represents a serious challenge for these unprotected cells when they are outside the epididymal environment.

No studies have been published on the effects of antioxidant addition to freezing diluents on red deer epididymal spermatozoa freezability. Several antioxidants have been tested that either scavenge ROS directly or counter the effects of ROS toxicity in the semen of a variety of mammalian and avian species; these include vitamin E (Brigelius-Flohe and Traber, 1999), vitamin C (O et al, 2006), butylated hydroxytoluene (BHT) (Donoghue and Donoghue, 1997), catalase (Roca et al, 2005), and superoxide dismutase (Berlinguer et al, 2003; Roca et al, 2005).

The main objective of the present study was to evaluate the effect of antioxidant supplementation of the freezing extender on epididymal sperm parameters during the cooling and freezing phases of the cryopreservation process. We investigated the efficacies of enzymatic (catalase, superoxide dismutase, and combination thereof) and nonenzymatic (vitamin C, vitamin E and BHT) scavenging agents.

To address fully these topics, we incorporated new functional sperm tests for mitochondrial function, membrane phospholipid disorder, and sperm chromatin status. Mitochondrial status plays an important role because of its relationship with the energetic status of the cell and motility, and it is related to fertility (Kasai et al, 2002). Therefore, we used the fluorescent dye JC-1, which has been used successfully to estimate mitochondrial membrane potential in sperm (Garner et al, 1997). In addition, merocyanine 540 has been shown to stain cell membranes more intensely when the membrane lipids are in a higher state of disorder (Williamson et al, 1981). Thus, we have used this technique to evaluate the membrane status with regard to phospholipid disorder in sperm cells. Finally, another important factor for sperm fertility is the integrity of the nuclear chromatin. This can be evaluated using a test in which in situ DNA denaturation is performed (Evenson et al, 1980). This test has been used for fertility estimations, as well as for the detection of problems during spermatogenesis (Januskauska et al, 2001). This knowledge is critical to understanding how sperm DNA is affected by oxidative stress during cryopreservation, since DNA damage may be responsible for lower fertility rates and higher embryonic mortality (Chen et al, 2002).

Materials and Methods

Chemicals

Except when stated otherwise, all of the chemicals were acquired from Sigma Chemical Co (Madrid, Spain).

Stags and Preparation of Testes

For this study, we used spermatozoa that were recovered from the epididymides of mature stags (6.7 ± 0.3 years [mean ± SE]; range, 4.5–10 years of age], which were legally culled and hunted in their natural habitat. The weight of the stags was 153.9 ± 3.4 kg (range, 130–201 kg). The stags

were shot during the rutting season (September to October). Gamekeepers collected the entire male genitalia and recorded the time of death. Hunting was performed in accordance with the harvest plan of the game reserve, which follows the Spanish Harvest Regulation, Law 2/93 of Castilla-La Mancha, which in turn conforms to European Union legislation.

Immediately upon removal, the testes with attached epididymides were placed in plastic bags, and the bags were transported to the laboratory at room temperature (approximately 22 °C) within 2 hours after removal of the tissues. The samples were processed as soon as they arrived at the laboratory. The elapsed time between animal death and sperm recovery ranged from 3 to 6 hours, which is an adequate and reliable time interval for evaluating sperm parameters, as decreases in the quality of sperm traits begin to take place 12 hours after the death of a male (Soler and Garde, 2003). For the collection of epididymal spermatozoa, the testes and epididymides were removed from the scrotal sac. The cauda epididymides, which included 5–10 cm of the proximal ductus deferens, were separated and transferred to 35-mm plastic dishes (Nunc, Roskilde, Denmark).

Collection, Processing, and Initial Evaluation of Epididymal Spermatozoa

Spermatozoa were collected from the distal portion of the epididymis, as described by Soler et al (2003). The epididymal contents from both testicles of the same male were pooled for processing. After spermatozoa collection, a routine sperm evaluation was carried out. Sperm concentration was determined using a hemocytometer. The percentage individual motile sperm (motility) was noted, and the quality of motility was assessed using a scale that ranged from 0 (lowest, immobile or dead) to 5 (highest, progressive and vigorous movement). A Sperm Motility Index, in which SMI = % individual motility + (quality of motility x 20) x 0.5, was calculated following the method of Comizzoli et al (2001) for red deer semen. Acrosomal integrity was evaluated for a 1:20 dilution in 2% glutaraldehyde, 0.165 M cacodylate/HCl buffer (pH 7.3). The percentage of spermatozoa with intact acrosomes (ie, with normal apical ridges; % NAR) was assessed by phase-contrast microscopy at 400x magnification. Only those samples with initial sperm motility and NAR greater than 60–65% were used in this study.

Plasma membrane functionality was assessed by means of the hypo-osmotic swelling (HOS) test, as described by Garde et al (1998). Briefly, 5 µL of diluted sperm suspension was mixed with 50 µL of a hypo-osmotic sodium citrate solution (100 mOsmol/kg) and the mixture was incubated at room temperature (22 °C) for 30 min. The samples were then fixed in 2% glutaraldehyde/cacodylate, as described above, and evaluated under phase-contrast microscopy at 400x magnification. The sperm membrane was considered intact if the sperm tail was coiled at the end of the assay, and the results are expressed as % positive endosmosis (HOST +).

Afterwards, the sperm samples were diluted to a sperm concentration of ~400 x 10^6 sperm/mL in Fraction A of each diluent, depending on the experimental design. Fraction B had the same composition as Fraction A but it also contained glycerol to a final concentration of 6% (Fernandez-Santos et al, 2005).

Sperm Diluents

Salomon modified solution (Tris-fructose-citrate; TFC) was used as a control in the two experiments. In particular, a solution that contained Tris (27.0 g/L), citric acid (14.0 g/L), fructose (10.0 g/L), and clarified egg yolk (20%) was used as the base extender (Fernandez-Santos et al, 2005). Sperm dilution was performed in a two-step procedure at room temperature (22 °C). The base extender (Fraction A) was added to give two-fold the desired final sperm concentration, and then the second extender (Fraction B) was added at a 1:1 ratio, to produce a final concentration of ~200 x 10^6

spermatozoa/mL (Fernandez-Santos et al, 2005). Fraction B differed from the base diluent in the replacement of water (12% v/v) with the same volume of glycerol (6% v/v final concentration). This 2-step procedure for sperm dilution was employed to obtain the same final concentration of glycerol for each stag.

Clarified egg yolk (EY) was prepared as described by Holt et al (1996). Briefly, fresh hen eggs were manually broken. The yolks were separated from the albumen and were carefully rolled on a filter paper to remove the chalazes and traces of albumen that adhered to the vitelline membrane. The latter was then disrupted with a scalpel blade and the yolk was collected with a sterile syringe. The whole egg yolk was then diluted (1:3) in redistilled water and centrifuged in sterile tubes at 10 000 x *g* for 30 minutes at 5 °C, to prepare clarified egg yolk. After centrifugation, the pellet (granules) at the bottom of the tube and the water-soluble clear fraction (plasma) were saved to prepare the clarified EY-TCF medium.

Experiment 1. Effects of Enzymatic Antioxidant Supplementation on Epididymal Red Deer Spermatozoa Freezability

To explore the effect of enzymatic antioxidant supplementation during the cooling and freezing processes, superoxide dismutase (SOD) and catalase (CAT) were added to the TFC medium. Epididymal sperm samples from each stag were split into 7 aliquots. One aliquot was used as a negative control, and was diluted in EY-TCF without antioxidants. The remaining 6 aliquots were subjected to the following treatments: 400 U/mL SOD (SOD_{400}); 800 U/mL SOD (SOD_{800}); 200 U/mL CAT (CAT_{200}); 400 U/mL CAT (CAT_{400}); 400 U/mL SOD + 200 U/mL CAT (SOD_{400}/CAT_{200}); and 800 U/mL SOD + 400 U/mL CAT (SOD_{800}/CAT_{400}). Stock solutions of SOD (1.5×10^5 U/mL) and CAT (2×10^5 U/mL) in PBS were prepared daily (Roca et al, 2005). This experiment was replicated with epididymal sperm samples from 9 mature stags.

Experiment 2. Effects of Nonenzymatic Antioxidant Supplementation on the Freezability of Epididymal Red Deer Spermatozoa

To examine the effect of nonenzymatic antioxidant supplementation during the cooling and freezing processes, vitamin C, vitamin E, and BHT were added to the TFC medium. Epididymal sperm samples for each stag were split into 8 aliquots. One aliquot was used as a negative control, and was diluted in EY-TCF without antioxidants. The remaining 7 aliquots were subjected to the following treatments: 0.4 mM vitamin C (Vit $C_{0.4}$); 0.8 mM vitamin C (Vit $C_{0.8}$); 3.2 mM vitamin E (Vit $E_{3.2}$); 6.4 mM vitamin E (Vit $E_{6.4}$); 0.4 mM BHT ($BHT_{0.4}$); 0.8 mM BHT ($BHT_{0.8}$); and 1.6 mM BHT ($BHT_{1.6}$). Stock solutions of vitamin C were prepared in Mili-Q water. Stock solutions of BHT and vitamin E (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Trolox) were prepared in absolute ethanol and dried down under air in tubes before the addition of the medium, following the recommendations of Roca et al (2004) and Peña et al (2004). When medium was added to each tube, the contents were mixed using a Vortex mixer. The BHT and vitamin E stock solutions were freshly made up each experimental day. This experiment was replicated with epididymal sperm samples from 10 mature stags.

Cryopreservation and Assessment of Frozen-Thawed Spermatozoa

The diluted sperm suspension in a 15-mL centrifuge tube (Iwaki, Tokyo, Japan) was placed in a beaker with water (75 mL at 5 °C) and transferred to a refrigerator set at 5 °C (Fernandez-Santos et al, 2006). After cooling, the sperm suspensions were equilibrated at 5 °C for 2 hours and after equilibration, the diluted sperm was loaded into 0.25-mL plastic straws (IMV, L'Aigle, France). The straws were immediately frozen for 10 minutes in nitrogen vapor, 4 cm (−120 °C) above the surface

of the liquid nitrogen, and then plunged into liquid nitrogen (−196 °C). The straws were retained in liquid nitrogen for a minimum period of 1 year before thawing.

Before freezing, the sperm samples were assessed for sperm motility and plasma membrane (HOST) and acrosome integrities using the methods described above. Sperm viability was also evaluated by means of the nigrosin-eosin (NE) stain, which was prepared as described by Tamuli and Watson (1994). The samples were evaluated under bright field microscopy at 400x. Live spermatozoa remained unstained, while dead cells were dull pink. The percentage of live spermatozoa is expressed as viability (%).

Frozen semen samples were thawed in a water bath (37 °C) for 30 seconds, and the contents of the straws were poured into a glass tube. Samples were evaluated for motility, viability, and acrosome and plasma membrane integrities using the methods described above. Thawed samples were incubated at 37 °C for 2 hours without dilution (that is, in the same diluent as they were frozen). At the end of this incubation period, the sperm suspensions reassessed for motility, acrosome integrity, membrane integrity, and viability, as described above. The rate that yielded information on survival after incubation of the thawed spermatozoa was calculated as follows:

$$\text{Postthaw survival rate} = (\text{value 2 hours postthawing at 37 C} / \text{value after thawing}) \times 100$$

Assessments of Phospholipid Disorder in the Plasma Membrane and Sperm Viability

Thawed spermatozoa with increased membrane phospholipid disorder were detected using the hydrophobic dye merocyanine 540 (M540; Sigma). M540 detects a decreased packaging order of the phospholipids in the outer leaflet of the lipid bilayer of the plasma membrane, as occurs, for instance, in capacitated spermatozoa (Langner and Hui, 1993), by means of an increase in orange fluorescence. Sperm samples were diluted with PBS to a concentration of 5 x 10^6 spermatozoa/mL, and 300 µl of this sample was transferred to a polypropylene tube, to which was added 1.5 µL of M540 stock solution (2.7 µM in DMSO). Viability was assessed with the LIVE/DEAD Sperm Viability Kit (Molecular Probes, Eugene, Ore), with the addition to the same tubes of 3 µL of propidium ioide (PI; 0.1 mM in water) and 1.2 µL of SYBR-14 (0.1 mM in water). The tubes were incubated at 37 °C for 30 minutes in the dark. In addition to the orange staining with M540, viable sperm were stained green by SYBR-14, and nonviable sperm were stained red by PI, whereas moribund sperm showed mixed fluorescence. This method was used to assess simultaneously changes in sperm viability and membrane phospholipid disorder (Figure 1).

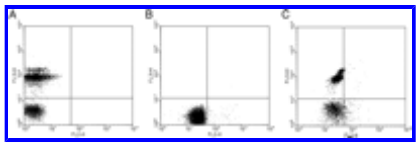

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Figure 1. Adjustment of the triple staining with SYBR-14/PI/M540 for analysis by flow cytometry. (**A**) The adjusted dot plot for the single PI staining. (**B**) The adjusted dot plot for the single M540 staining; the FL-2 level is adjusted so there is no bleeding towards FL-3. (**C**) PI vs M540 dot plot for the triple staining (SYBR-14 staining is not shown), with complete photodetector and compensation setting adjustments. The data from the lower-left quadrant of (**C**) was used in this analysis.

Assessment of Sperm Mitochondrial Status

The lipophilic cationic probe JC-1 was used to assess the mitochondrial status of the spermatozoa.

According to the manufacturer (Molecular Probes), JC-1 changes reversibly its fluorescence from green (monomeric status) to orange (multimeric status) when the mitochondrial membrane potential is high. Thawed samples were diluted with PBS buffer to a concentration of 5 x 10^6 spermatozoa/mL, 300 µL of each sample was transferred to a polypropylene tube, and 1.2 µL of JC-1 stock solution (1.5 mM JC-1 in DMSO) was added. The LIVE/DEAD Sperm Viability Kit was used as described previously, to identify the events related to the spermatozoa. The tubes were incubated at 37 °C for 30 minutes in the dark. The stained sperm samples were then analyzed by flow cytometry, which identified cells with high mitochondrial membrane potential (hMMP; orange-stained cells).

Assessment of Sperm Chromatin Stability

Chromatin stability was assessed by staining with the metachromatic fluorescent dye acridine orange (AO), the use of which is based on the susceptibility of sperm DNA to acid-induced denaturation in situ. AO shifts from green (dsDNA) to red (ssDNA) fluorescence depending on the degree of DNA denaturation (Evenson et al, 1980; Januskauska et al, 2001). Samples were diluted in TNE buffer (0.01 M Tris-HCl [pH 7.4], 0.15 M NaCl, 1 mM EDTA) to a final sperm concentration of approximately 2 x 10^6 cells/mL in polypropylene tubes. Two 0.2-mL aliquots of each sample were dropped into LN_2 and then allowed to thaw at room temperature. This process was repeated two more times. Thereafter, the samples were refrozen and stored in an ultracold freezer at −80 °C until needed. For analysis, the samples were thawed on crushed ice. Acid-induced denaturation of DNA in situ was achieved by adding 0.4 mL of an acid-detergent solution (0.17% Triton X-100, 0.15 M NaCl, 0.08 N HCl [pH 1.4]). After 30 seconds, the cells were stained by adding 1.2 mL of a solution (0.1 M citric acid, 0.2 M Na_2HPO_4, 1 mM EDTA, 0.15 M NaCl [pH 6.0]) that contained 6 µg/mL AO. The stained samples were analyzed by flow cytometry just 3 minutes after AO staining.

Flow Cytometric Analysis

Samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif), which was equipped with standard optics and an Ar-ion laser that was tuned to 488 nm and running at 200 mW. Calibration was carried out using standard beads (1.0-µM Fluoresbrite Plain YG; Polysciences Inc, Warrington, Pa). Fluorescence was detected using the following filters: red fluorescence (PI and AO) with a 650 long pass filter; orange fluorescence (M540 and JC-1) with a 585/42 band pass filter; and green fluorescence (SYBR-14 and AO) with a 530/30 band pass filter. Data were collected from 10 000 events for further analysis with the CellQuest software (Becton Dickinson). Sideward and forward scatter of light were recorded so that only sperm cell-specific events were selected for analysis. The flow rate was 1500 cells/s, except in the chromatin stability analysis, where it was 200 cells/s.

Statistical Analysis

Two hundred sperm cells were assessed in each sample and for each sperm evaluation test. Statistical analyses were performed using SPSS for Windows, version 11.5 (SPSS Inc, Chicago, Ill). The most suitable GLM-ANOVA was used for each experiment. Data are expressed as least-squares means (LSmeans) ± SEM. Comparisons of means among treatments were performed using the Tukey multiple range tests. Differences were considered statistically significant at P less than .05.

The results of the DNA denaturation test depended on function α_t, which reflects the shift from green to red fluorescence and is expressed as the ratio of red cells to total (red plus green) cells. High values of α_t, indicate high levels of chromatin abnormalities. The flow cytometry data were retrieved using the WinMDI software (The Scripps Research Institute, La Jolla, Calif), and the standard deviation ($SD\alpha_t$) of α_t was calculated for each sample. Moreover, adopting the guidelines

described by Evenson et al (2002), the extent of DNA denaturation was expressed in terms of a DNA fragmentation index (DFI, formerly termed the α_t function), which is the ratio of the red to total (red plus green) fluorescence intensities. This conversion was necessary to calculate accurately the percentages of spermatozoa with undetectable DFI (formerly termed the main or normal population of cells) or detectable (moderate and high) DFI (formerly collectively termed the cells outside the main population or COMPα_t). The DFI value was derived for each sperm cell in a sample, and the detectable DFI values were calculated from the resulting DFI frequency histogram (%DFI).

Results

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Experiment 1. Effects of Enzymatic Antioxidant Supplementation on the Freezability of Epididymal Red Deer Spermatozoa

The effects of SOD and CAT on sperm motility are summarized in Table 1. After cooling and equilibration, there were no differences between the treatments, whereas after thawing, statistically significant (P = .03) differences were noted between the control and SOD_{400}. When thawed spermatozoa were incubated for 2 hours at 37 °C, a marked loss in motility was observed when the spermatozoa were incubated in control media. The best motility results were obtained for CAT_{200}, which was statistically different from the control (P = .007) and SOD_{400} /CAT_{200} (P = .01). The control was also statistically different from SOD_{800} (P = .03) after 2 hours of incubation at 37 °C.

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Table 1. *Effects of enzymatic antioxidants on epididymal sperm from red deer**

The effects of enzymatic antioxidants on sperm parameters are summarized in Table 1. After cooling and equilibration, increases (P < .05) in the percentages of viable sperm were observed for treatment with SOD_{800}, CAT_{200}, CAT_{400}, and SOD_{400} /CAT_{200} compared to the control. The membrane (positive endosmosis) and acrosome integrities (% NAR) were well-preserved for all of the diluents added before freezing, with no statistical differences between the diluents. After freezing and thawing, there were no differences between the treatments in terms of membrane integrity or sperm viability. However, there were significant differences (P < .05) between the control and SOD_{400} and SOD_{800} treatments with respect to acrosome integrity (Table 1). The control treatment rendered a significantly (P < .05) lower percentage of viable sperm after 2 hours of incubation at 37 °C (Table 1). No differences were noted for the other evaluated parameters.

The effects of adding an enzymatic antioxidant on the postthaw survival rates (ie, the ability of thawed spermatozoa to resist incubation) are summarized in Figure 2. In the case of the SMI, the best results were obtained for the CAT_{200} and SOD_{800}/CAT_{400} treatments, which were statistically different (P < .05) to the control. There were no statistical differences between the treatments with respect to either acrosome integrity (% NAR) or sperm viability. Furthermore, membrane integrity (as revealed by the HOS test) was better preserved (P < .05) by SOD_{800}/CAT_{400} treatment

than by the control, SOD_{800} or SOD_{400} /CAT_{200} treatments.

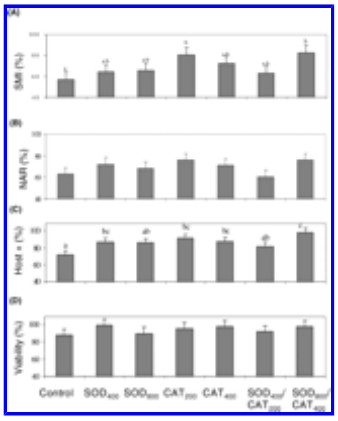

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Figure 2. Effects of enzymatic antioxidants on the postthaw survival rates of sperm. **(A)** Sperm motility index. **(B)** Acrosome integrity (% NAR) as the percentage of spermatozoa with intact acrosomes. **(C)** Membrane integrity (HOST+) as the percentage of spermatozoa with coiled tails (positive response) after the HOS test. **(D)** Sperm viability as the percentage of viable sperm. The results are expressed as LSmean ± SEM (n = 9). Bars with different letters are significantly different (*P* < .05).

After thawing, M540/PI staining did not show any differences between the control and any of the diluents that contained antioxidants. After 2 hours of incubation, there were no significant differences (*P* > .05) in terms of membrane phospholipid disorder between the treatments.

The percentages of thawed spermatozoa with hMMP were statistically higher (*P* < .05) for the CAT_{200} and SOD_{800}/CAT_{400} treatments compared to the control. For the other treatments, there were no statistical differences in hMMP. After 2 hours of incubation, there were no significant differences (*P* > .05) in hMMP frequency among the various treatments.

There were no significant differences in chromatin stability between the treatments, either after freezing and thawing (SDαt: 4.0 ± 2.1; DFI: 2.4 ± 2.8; mean ± SD) or after 2 hours of incubation at 37 °C postthawing (SDαt: 2.6 ± 0.8; DFI: 1.0 ± 1.2; mean ± SD). Therefore, enzymatic antioxidant supplementation did not significantly affect chromatin stability after cryopreservation.

Experiment 2. Effects of Nonenzymatic Antioxidant Supplementation on the Freezability of Epididymal Red Deer Spermatozoa

The effects on sperm parameters of nonenzymatic antioxidants (vitamin C, vitamin E, and BHT) after cooling and equilibration are summarized in Table 2. For the SMI, both Vit $C_{0.4}$ (77.0 ± 3.1%) and Vit $C_{0.8}$ (74.7 ± 3.1%) gave superior results, being different to the control (61.4 ± 3.1%), Vit $C_{0.4}$ (*P* = .001), and Vit $C_{0.8}$ (*P* = .004) treatments. The poorest results were obtained for the control, Vit $E_{6.4}$, and $BHT_{1.6}$. Moreover, acrosome integrity was better preserved by any treatment compared with the control (*P* < .05). Membrane integrity was preserved with all of the treatments (*P* > .05). In terms of sperm viability, Vit $C_{0.8}$ gave the best result (92.7 ± 1.2%), and this was statistically different from the control (88.9 ± 1.2%; *P* < .05). There were no other differences among the various treatments.

View this table: Table 2. *Effects of nonenzymatic antioxidants on epididymal sperm from red deer*†

After freezing and thawing, no improvement in any sperm parameter was found between the treatments and the control. Moreover, Vit $E_{6.4}$ and $BHT_{1.6}$ were deleterious for some sperm parameters, such as acrosome integrity and viability. When thawed spermatozoa were incubated for 2 hours at 37 °C, no improvement was noted for any sperm parameter between the treatments and the control (Table 2). Moreover, some treatments gave poorer outcomes than the control, whereas Vit $C_{0.4}$ gave the best outcomes for all of the sperm parameters. Nevertheless, no statistical differences were observed.

M540/PI staining after cryopreservation showed no improvements in terms of lower membrane phospholipid disorder when nonenzymatic antioxidants were added to the diluent. Moreover, some treatments, such as Vit $E_{6.4}$ and $BHT_{1.6}$, significantly increased membrane phospholipid disorder compared to the control. After 2 hours of incubation, the spermatozoa fared better with the control and Vit $C_{0.4}$. The other treatments showed poorer membrane phospholipid disorder results than those obtained with the control ($P < .05$; Table 2).

With respect to mitochondrial status, there were no differences between the control and most of the treatments evaluated after freezing and thawing, with the exception of Vit $E_{6.4}$, which gave lower results than the control ($P < .05$; Table 2). After 2 hours of incubation at 37 °C, no further changes in mitochondrial status were observed (Table 2).

There were no significant differences in chromatin stability among the treatments, either after freezing and thawing ($SD\alpha t$: 4.5 ± 3.1; DFI: 2.6 ± 2.8) or after 2 hours of incubation at 37 °C postthawing ($SD\alpha t$: 4.0 ± 0.8; DFI: 2.9 ± 2.8). Therefore, nonenzymatic antioxidant supplementation did not significantly affect chromatin stability after cryopreservation.

Discussion

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In the present study, we initially evaluated the potential benefits of the ROS-scavenging enzymes catalase and superoxide dismutase for the cryopreservation of epididymal spermatozoa from red deer. Under the experimental conditions used, the addition of these antioxidants, either alone or in combination, to the freezing extender improved the sperm parameters as soon as after the cooling stage, with higher percentages of viable sperm being achieved for most of the antioxidant treatments. Moreover, SOD_{800} and CAT_{200} preserved sperm motility both postthawing and after 2 hours of incubation at 37 °C. Our results are in agreement with those reported by Bilodeau et al (2002), who showed that the addition of antioxidants to an EY-TCF extender was beneficial in terms of the motility of frozen-thawed spermatozoa, even in absence of an external source of oxidative stress.

After freezing and thawing, acrosome integrity was better preserved when the freezing diluent was supplemented with either of the 2 concentrations of SOD. Berlinguer et al (2003) have demonstrated the effects of SOD in prolonging in vitro viability and delaying the onset of the acrosome reaction of cryopreserved spermatozoa from European mouflon. This delay is essential for maintaining

functional properties, since the acrosome must remain undamaged to enable binding to the zona pellucida and to respond to the appropriate signals from oocytes (Wassarman, 1988). Maxwell and Stojanov (1996) have also demonstrated delayed membrane destabilization of ram spermatozoa following the addition of antioxidants to the freezing medium. As previously shown by other investigators (O'Flaherty et al, 1997; De Lamirade and Gagnon, 1998), one of the most frequently studied effects of SOD is the delayed onset of sperm capacitation and consequently, the delayed onset of the physiological acrosome reaction. Therefore, it is likely that SOD inhibits capacitation, possibly because O^{-}_{2} mediates this physiological process.

Comparisons of the postthaw survival rates for the various treatments revealed that sperm samples frozen in presence of certain antioxidants gave the highest survival rates, especially when the sperm were frozen with SOD_{400}, CAT_{200}, CAT_{400}, and SOD_{400}/CAT_{400}. These findings agree with those obtained by other authors for different species. Thus, Maxwell and Stojanov (1996) have demonstrated that the addition of CAT and SOD to the extender improves the survival of liquid-stored ram spermatozoa. In addition, Roca et al (2005) have reported improvements in sperm motility and viability when these antioxidants were added to the freezing extender used for boar spermatozoa. The evidence from our experiments that the addition of CAT and SOD improve most of the postthaw parameters indicates that oxidative stress occurs during the freezing and thawing processes.

In the present study, we used M540 as a probe to explore whether or not an antioxidant could influence sperm membrane phospholipid disorder. Our results show that the percentage of M540-unstained cells not only did not increase, but it significantly decreased compared to the control when antioxidants were added to the diluent either upon thawing or after incubation at 37 °C for 2 hours. This is in agreement with the idea that the adverse effects of ROS on spermatozoa are more likely to be caused by direct oxidation of proteins and membrane permeabilization than by disturbance of lipid packaging (Christova et al, 2004). Nevertheless, since cryopreservation per se leads to deleterious effects on sperm, it may disguise the real effects of ROS. In fact, it is well established that oxidative stress plays an important role in the cryoinjury damage suffered by mammalian spermatozoa (Chatterjee and Gagnon, 2001). Alvarez and Storey (1992) have demonstrated that the freeze-thaw process enhances lipid peroxidation in human sperm. Chatterjee and Gagnon (2001) have demonstrated the same phenomenon for bovine sperm. During cryopreservation, spermatozoa are particularly prone to oxidative damage due to the generation of ROS by defective and dead spermatozoa (Bailey et al, 2000), which ultimately leads to membrane lipid peroxidation and membrane damage.

Our results also reveal the protective effects of enzymatic antioxidants (CAT_{200} and SOD_{800}/CAT_{400}) on mitochondrial function. The mitochondrion contains the machinery for oxidative energy production and seems to be the cellular structure that is most sensitive to freezing and thawing (Cummins et al, 1994). These procedures cause changes in mitochondrial function that are reflected in sperm motility. Indeed, the postthaw survival rates indicated protective effects on sperm motility of CAT_{200} and SOD_{800}/CAT_{400}, which were the 2 best treatments in terms of preserving the mitochondrial machinery.

One of the important findings to emerge from this study is that the addition of nonenzymatic antioxidants to the freezing extender does not have positive effects on the freezability of red deer epididymal sperm. We observed no effect of vitamin C on sperm parameters after thawing. However, we report that the use of vitamin E or BHT has negative effects on sperm cryopreservation. Thus, we found that Vit $E_{6.4}$ caused deteriorations in all the sperm parameters after thawing, whereas Vit

$E_{3.2}$ caused deteriorations in acrosome integrity and membrane stability. One possible explanation is that a high concentration of vitamin E may act as an oxidation stimulator rather than as an antioxidant (Cao and Cutler, 1993). In this regard, many studies have noted the detrimental effects of vitamin E (either α-tocopherol or Trolox) supplementation on sperm motility in fresh human semen (Donnelly et al, 1999) and liquid ram semen (Upreti et al, 1997), whereas vitamin E had little or no effect on chilled equine semen (Aurich et al, 1997) or thawed human semen (Askari et al, 1994). However, discrepant results have been observed for bovine semen with α-tocopherol, whereby high concentrations (1000 µg/mL) of α-tocopherol decreased the lipid peroxidation of thawed semen (Breininger et al, 2005). The oxidant activity of vitamin E may be prevented by vitamin C (Carr et al, 2000), since the combination of vitamin E, which is a lipophilic antioxidant, with vitamin C, which is a hydrophilic antioxidant, detoxifies lipids from peroxides (Schwenke and Behr, 1998). Thus, this combination may be beneficial for sperm preservation.

BHT at 1.6 mM in the diluent not only did not improve the sperm parameters but worsened some of them. BHT addition to stored sperm has shown mixed results with respect to the survival of spermatozoa. Whereas it improves cold-stressed (Graham and Hammerstedt, 1992) and cryopreserved (Killian et al, 1989) bull spermatozoa and chilled turkey semen (Donoghue and Donoghue, 1997), it has a detrimental effect on cooled stallion spermatozoa (Ball et al, 2001). Moreover, in agreement with our results, Roca et al (2004) have demonstrated that the presence of 1.6 mM BHT in the freezing extender is of no benefit for boar spermatozoa (Roca et al, 2004). Likewise, Bamba and Cran (1992) have observed loss of the protective effect of BHT on cold stress suffered by spermatozoa when the concentrations used were higher than 2 mM.

To our knowledge, no other researchers have investigated the DNA damage caused to Iberian red deer epididymal spermatozoa by cryopreservation processes. These samples may be more vulnerable to oxidative stress, since they are not exposed to seminal plasma, which is the major source of antioxidant enzymes in the ejaculate and which has the important function of protecting the integrity of the sperm DNA from the oxidative stress experienced in the uterine environment (O et al, 2006). Nevertheless, we did not found any differences in DNA status among the various treatments. This appears to be in agreement with the results of Baumber et al (2005), who demonstrated that the addition of antioxidants to the cryopreservation extender used for equine spermatozoa did not improve spermatozoal motility, acrosome integrity, viability, mitochondrial membrane potential or DNA integrity after thawing. Moreover, SOD increased DNA fragmentation, proabably as a result of the additional oxidative stress caused by the generation of hydrogen peroxide by this enzyme.

We have shown that the addition of catalase and superoxide dismutase to red deer epididymal spermatozoa preserves sperm motility, acrosome integrity, membrane stability, and mitochondrial function after thawing. However, the nonenzymatic antioxidants used in the present study, vitamin C, vitamin E, and BHT, caused deteriorations in most of sperm parameters after thawing. Only vitamin C showed any positive effect after the cooling process. In the present study, we show for the first time the effect of antioxidants on epididymal spermatozoa, with only 1 previous study on rat epididymal samples, although in that instance, antioxidants were supplemented in the diet instead of being applied directly to the sperm (Jervis and Robaire, 2004). It is difficult to compare our results with those reported previously using ejaculated sperm, as it is well known that there are important differences in the physiological characteristics of epididymal and ejaculated spermatozoa, which may affect sperm cell cryoresistance (Martinez-Pastor et al, 2006b).

In summary, we conclude that enzymatic antioxidants should be added during the early steps of cryopreservation of epididymal spermatozoa. Considering the different mechanism of action of the

different antioxidants, future research should be aimed not only at improving antioxidant supplementation, but also at identifying the protective mechanism of seminal plasma, in order to apply this type of protection to epididymal samples. In addition, combinations of enzymatic and nonenzymatic antioxidants deserve attention, because of the synergistic antioxidant effects. These enhancements may contribute to the management and conservation not only of red deer, but also of other cervids.

Footnotes

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