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Survival and In Vitro Fertility of Boar Spermatozoa Frozen in the Presence of Superoxide Dismutase and/or Catalase

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ABSTRACT: In the present study the potential benefit of reactive oxygen species (ROS)–scavenging enzymes superoxide dismutase (SOD) and catalase (CAT) when cryopreserving boar spermatozoa was evaluated. Pooled ejaculate sperm-rich fractions collected from 3 fertile boars were frozen in a split design, after being extended in a conventional freezing extender (control) or the same extender supplemented with SOD (150 or 300 IU/mL, experiment 1), CAT (200 or 400 IU/mL, experiment 2), or SOD + CAT in combination (150 + 200 or 300 + 400 IU/mL, experiment 3). Irrespective of the concentration used, SOD and CAT, alone or in combination, significantly improved postthaw sperm survival, in terms of total sperm motility (assessed with CASA) and viability (assessed with a triple stain; propidium iodide/R123/fluorescein isothiocyanate–labeled peanut agglutinin). Moreover, CAT alone, at a concentration of 400 IU/mL, or in combination with SOD, at concentrations of 200 and 400 UI/mL, improved the ability of frozen-thawed spermatozoa to produce embryos in vitro (zygote cleavage and blastocyst formation as end points). Additional data of ROS generation (luminol- and lucigenin-dependent chemiluminescence) and membrane lipid peroxidation (malondialdehyde [MDA] production) indicated that SOD and CAT reduced postthaw ROS generation by boar spermatozoa, without any influence on MDA production.

Key words: Antioxidants, cryopreservation, sperm, porcine.
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In recent years, successful fertility results after artificial insemination (AI) in sows with cryopreserved spermatozoa have been reported (Ericksson et al, 2002; Roca et al, 2003). However, these fertility levels are not high enough for an extensive use of frozen-thawed spermatozoa in commercial pig breeding. One of the primary reasons for the current lower farrowing rates and smaller litter size compared with freshly ejaculated and liquid-extended spermatozoa is the reduced capacity of boar spermatozoa to survive the freezing and thawing process. Even using the best-known cryopreservation techniques, normally less than half of the cells retain motility postthaw.

Cryopreservation of spermatozoa affects primarily the plasma membrane, with the damage varying in magnitude among species, owing to the structural complexity it presents. Species differences in the susceptibility of their spermatozoa to cooling, freezing, and thawing process are thus largely attributable to compositional variations of the sperm plasma membrane. The major susceptibility is related, in part, to the content of polyunsaturated fatty acids (White, 1993), which are susceptible to undergoing lipid peroxidation in the presence of the reactive oxygen species (ROS). The lipid peroxidation cascade is initiated when spermatozoa are attacked by ROS, which results in a loss of unsaturated fatty acids from the plasma membrane and a corresponding decline in the survival and fertilizing ability of these spermatozoa (Aitken, 1995). Boar spermatozoa are particularly susceptible to oxidative attack by ROS because they contain large amounts of polyunsaturated fatty acids (White, 1993).

Sperm oxidative damage is the result of an improper balance between ROS generation and scavenging activities. The scavenging potential of the ejaculate is normally maintained by adequate levels of antioxidants present in the seminal plasma (SP). When boar ejaculates are cryopreserved, spermatozoa are concentrated so that they can be adequately extended in cryoprotecting fluids, and removal of SP is customary. Spermatozoa lose, in consequence, the antioxidant protection that SP provides, leaving them vulnerable to oxidative attack. In addition, it is now generally accepted that the cryopreservation process induces the formation of ROS (Watson, 2000). In domestic animals, defective and dead spermatozoa have been identified as a major source of ROS generation during cryopreservation (Bailey et al, 2000). Consequently, during cryopreservation, boar spermatozoa are especially open to oxidative damage by the concomitant actions of their relatively high proportion of polyunsaturated fatty acids, the removal of the scavenging capability of SP be-

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fore freezing, and the generation of ROS by defective and dead spermatozoa, ultimately leading to membrane lipid peroxidation and membrane damage.

One way of overcoming this cascade could be the incorporation of antioxidants into the freezing extenders. To date, limited information is available on the application of antioxidants to prevent cryodamage of boar spermatozoa. Only 2 recent reports addressed the successful use of α-tocopherol (Peña et al, 2003) and butylated hydroxytoluene (Roca et al, 2004) as additives to extenders for freezing boar spermatozoa.

Seminal plasma is a powerful source of antioxidants (Saleh and Agarwal, 2002), and evidence suggests that as long as spermatozoa are suspended in SP, they are protected from oxidative damage. The most common ROS with potential implications in sperm oxidative damage include the superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). In order to counteract the toxic effect of these ROS, the SP contains the enzymes superoxide dismutase (SOD) and peroxidases, such as catalase (CAT). The SOD converts the O_2^- to H_2O_2, and the CAT then converts H_2O_2 to H_2O and O_2, eliminating the potential ROS toxicity (Aitken, 1995).

In view of the above, the present study evaluated the potential protective effect of SOD, CAT, or both, 2 oxygen species–scavenging enzymes normally present in boar SP, against cryopreservation-induced damage to boar spermatozoa. Spermatozoa were monitored postthaw for their survival and ability to produce blastocysts in vitro.

Materials and Methods

Reagents and Media

All chemicals were of analytical grade. Unless otherwise stated, all media components were purchased from Sigma Chemical Co (St Louis, Mo) and were made up under sterile conditions (HH48, Holten LaminAir, Denmark) with purified water (18 MΩ ·cm; Elgastat UHQPS, Elga Ltd, England).

The basic medium used for sperm extension was Beltsville thawing solution (BTS, composed of 205 mM glucose, 20.39 mM NaCl, 5.4 mM KCl, 15.01 mM $NaHCO_3$, and 3.35 mM EDTA; Johnson et al, 1988), containing kanamycin sulfate (50 μg/ml). The basic medium used for sperm cryopreservation was a lactose-egg yolk (LEY) extender composed of 80% (vol/vol) β-lactose solution (310 mM in water), 20% (vol/vol) egg yolk, and 100 μg/mL kanamycin sulfate. The medium used for the collection and washing of oocyte-cumulus complexes was Dulbecco phosphate-buffered saline (DPBS) medium composed of 136.89 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO4, and 1.46 mM $CaCl_2$-$2H_2O$. The oocyte maturation medium was the bovine serum albumin (BSA)–free North Carolina State University-23 (NCSU-23) composed of 108.73 mM NaCl, 4.78 mM KCl, 1.7 mM $CaCl_2$-$2H_2O$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$-$7H_2O$, 25.07 mM $NaHCO_3$, 5.55 mM D-glucose, 1 mM L-glutamine, 7 mM taurine, 5 mM hypotaurine, 75 μg/mL potassium penicillin G, and 50 μg/mL streptomycin sulfate (NCSU-23; Petters and Wells, 1993) and supplemented with 10% (vol/vol) porcine follicular fluid, 0.1 mg/mL cysteine, 10 ng/mL epidermal growth factor (EGF), 10 IU/mL eCG (Folligon, Intervet International BV, Boxmeer, the Netherlands), and 10 IU/mL of hCG (Chorulon, Intervet International). The fertilization medium was a modified Tris-buffered medium (TBM) composed of 20 mM Tris (Trizma Base), 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl_2$-$2H_2O$, 11 mM D-glucose, and 5 mM sodium pyruvate (TBM; Abeydeera and Day, 1997), supplemented with 2 mM caffeine and 0.2% BSA (fraction V; A-7888, initial fractionation by cold alcohol precipitation). The embryo culture medium was NCSU-23 with 0.4% BSA.

Stock solutions of SOD (S-5395; 1.5×10^5 IU/mL) and CAT (C-9322; 2×10^5 IU/mL) were prepared according to the instructions provided by the manufacturer (Sigma). After preparation, stock solutions were preserved at −80°C until used.

Semen Processing and Sperm Cryopreservation

Ejaculates were collected weekly from 3 fertile Pietrain boars. Sperm-rich ejaculate fractions were obtained using the gloved-hand method and extended (1:1, vol/vol) in BTS. After collection, semen characteristics (total sperm numbers per ejaculate, subjective sperm motility, and acrosome integrity) were microscopically evaluated, using standard laboratory techniques (Martin Rillo et al, 1996), and only ejaculates with >75% motile spermatozoa and >80% normal acrosomes were used.

Spermatozoa were cryopreserved using the straw freezing procedure described by Westendorf et al (1975) as modified by Thurston et al (1999). Briefly, extended semen (1:1, vol/vol in BTS) was cooled to 17°C over 3 h. After centrifugation at $800 \times g$ for 10 minutes (Megafuge 1.0 R, Heraeus, Germany), the pellets were diluted in LEY (pH 6.2 and 330 ± 5 mOsm/Kg) to a concentration of 1.5×10^9 cells/mL. After further cooling to 5°C in 90 minutes, the spermatozoa were resuspended with LEY-glycerol-orvus ES paste (LEYGO) extender (92.5% LEY + 1.5% Equex STM [Nova Chemical Sales Inc, Scituate, Mass] and 6% glycerol, vol/vol; pH 6.2 and 1650 ± 15 mOsm/Kg) to a final concentration of 1×10^9/mL. The resuspended, cooled spermatozoa were packed into 0.5 polyvinyl chloride–French straws (Minitüb, Tiefenbach, Germany), and frozen using a controlled-rate freezer (IceCube 1810, Minitüb) as follows: cooled to −5°C at 6°C/min, from −5°C to −80°C at 40°C/min, held for 30 seconds at −80°C, then cooled at 70°C/min to −150°C, and finally plunged into liquid nitrogen.

Thawing and Postthaw Sperm Survival Evaluation

Thawing of the frozen spermatozoa was done in a circulating water bath at 37°C for 20 seconds. Thawed spermatozoa from 2 straws were extended with BTS (1:2, vol/vol; 37°C) and incubated in a water bath at 37°C for 150 minutes. Spermatozoa were assessed for motility, kinematic parameters, and viability.

Sperm motility and kinematic parameters were objectively evaluated using a computer-aided sperm analysis (CASA) system (sperm class analyzer [SCA]; Microptic, Barcelona, Spain). The incubated frozen-thawed spermatozoa were re-extended in BTS to a concentration of 20×10^6/mL. For each evaluation, a 4 μL sperm sample was placed in a Makler counting chamber

(Sefi Medical Instruments, Haifa, Israel) and 3 fields were analyzed at 39°C to assess a minimum of 100 spermatozoa per sample. The proportion of total motile spermatozoa (% TMS), of rapidly progressive motility spermatozoa (% RPMS, >50 μm/s), average path velocity (VAP, μm/s), linearity (% LIN), straightness of the average path (% STR), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz) were determined.

Sperm viability was evaluated in terms of plasma membrane integrity, mitochondrial membrane potential, and acrosomal integrity. These characteristics were analyzed simultaneously using a modification of a triple fluorescent procedure described by Graham et al (1990), which includes DNA-specific fluorochrome propidium iodide (PI), the mitochondria-specific fluorochrome rhodamine-123 (R123), and the acrosome-specific fluorochrome fluorescein isothiocyanate–labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA). Three hundred spermatozoa were counted under 1000× magnification (Eclipse E800; Nikon, Tokyo, Japan), using BV-2A filter (400 to 440 nm excitation filter, 455 nm emission, 470 nm barrier filter). Four staining patterns could be discerned: viable sperm with intact acrosome (no staining of the head, the tail midpiece stained green), viable sperm with acrosome reacted (both the tail midpiece and the acrosome region stained green), nonviable sperm with intact acrosome (sperm head stained red), and nonviable sperm with acrosome reacted (sperm head stained red and the acrosome region stained green). Values were expressed as percentage (%).

Measurement of Membrane Lipid Peroxidation

Membrane lipid peroxidation was estimated by the end-point generation of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) test (Esterbauer and Cheeseman, 1990). Briefly, extended spermatozoa (250×10^6 cells in 1 mL) were mixed with 1 mL of cold 20% (wt/vol) trichloroacetic acid to precipitate proteins. The precipitate was pelleted by centrifugation ($1500 \times g$ for 10 minutes), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (wt/vol) TBA in a boiling water bath at 100°C for 10 minutes. After cooling, the absorbance was determined by spectrophotometry (UNICAM PU 8610 Kinetics spectrophotometer; Philips, Eindhoven, Holland) at 534 nm. The results were expressed as a simple concentration of MDA (pmol/10^8 cells).

Measurement of ROS in Thawed Sperm Suspensions

The chemiluminescence methods using luminol (amino-2,3-dihidro-1,4-phthalazinedione; A-8511) and lucigenin (bis-N-methylacridinium nitrate; M-8010) described by Aitken et al (1992) were used with modifications. Briefly, 250 μM of luminol (4 μL of 25 mM aqueous solution, Na salt, freshly prepared) or lucigenin (4 μL of 25 mM aqueous solution) were added to each tube containing 400 μL of frozen-thawed spermatozoa (30×10^6 spermatozoa/mL) and mixed. Horseradish peroxide (7.5 μL, 19 IU/mL; type VI, P-8375) was used to enhance interaction of luminol and hydrogen peroxide. Levels of ROS were assessed by measuring the luminol- and lucigenin-dependent chemiluminescence with a luminometer (Multi-Biolumat LB 9505 C; Berthold Technologies, Bad Wildbad, Germany) in the integrated mode at 37°C for 30 minutes. A positive control of luminol assay was prepared by adding hydrogen peroxide (0.5–50 μM) to 400 μL of LEYGO plus BTS (1:36, vol/vol). The sensitivity and specificity of the lucigenin assay were determined by using xanthine (0.05–0.2 mM) and xanthine oxidase (0.05 U/mL). A negative control was prepared by adding luminol or lucigenin to 400 μL of LEYGO plus BTS (1:36, vol/vol). Mean ROS generation was calculated and expressed as the logarithm of photon counts integrated for 30 minutes.

In Vitro Embryo Production

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 75 μg/mL potassium penicillin G and 50 μg/mL streptomycin sulfate maintained at 25°C to 30°C. Oocytes were aspirated from medium-sized follicles (3 to 6 mm in diameter) with an 18-gauge needle fixed to a 10-mL disposable syringe. Oocytes surrounded by a compact cumulus mass and having evenly granulated cytoplasm were washed 3 times in maturation medium, and 45–50 oocytes were transferred into each well of a Nunc 4-well multidish containing 500 μL of pre-equilibrated maturation medium previously covered with warm mineral oil and cultured for 22 hours with added hormones and then for another 22 hours without hormones in 5% CO_2 in air at 39°C. After the completion of in vitro maturation, cumulus cells were removed with 0.1% hyaluronidase in NCSU-23 and washed 3 times with fertilization medium. After washing, batches of 30 oocytes were placed in 50-μL drops of the same medium in a 35 × 10-mm Petri dish. The dishes were kept in the incubator for about 30 minutes until spermatozoa were added for fertilization. One hundred microliters of extended spermatozoa from 1 pool of 3 frozen-thawed straws were washed 3 times by centrifugation at $1900 \times g$ for 3 minutes in DPBS medium supplemented with 0.1% BSA, 75 μg/mL potassium penicillin G, and 50 μg/mL streptomycin sulfate (pH 7.2). At the end of the washing procedure, the sperm pellet was resuspended in fertilization medium, and then 50 μL of this sperm suspension was added to the medium that contained oocytes so that each oocyte was exposed to 2000 sperm cells (Gil et al, 2003). At 6 hours after insemination, oocytes were washed and transferred (30 oocytes per well) to a Nunc 4-well multidish containing 500 μL of embryo culture medium, covered with mineral oil and cultured at 39°C, 5% CO_2 in air for 7 days. At 48 and 168 hours of culture, cleavage rate (taken as evidence of fertilization) and blastocyst formation, respectively, were evaluated under a stereomicroscope.

Experimental Design

Three separate experiments, each using a completely randomized design, were performed to evaluate the effect of adding different concentrations of SOD and/or CAT on freezer extender to the postthaw boar sperm performance, as follows:

- Experiment 1, addition of 150 or 300 IU SOD/mL.
- Experiment 2, addition of 200 or 400 IU CAT/mL.
- Experiment 3, addition of 150 or 300 IU SOD/mL and 200 or 400 IU CAT/mL.

The concentrations of SOD and/or CAT used in the present experiments were the lowest concentrations that improved the postthaw sperm survival in a preliminary experiment (unpub-

Table 1. *Effect of the addition of superoxide dismutase (SOD) in the freezing extender on the postthaw boar sperm motilty, kinematic parameters, and viability and in their ability to develop embryos in vitro*

Postthaw Sperm Parameters*	Treatment SOD, IU/mL			SEM	Probability, *P*
	0 (Control)	150	300		
Motility and kinematic parameters					
TMS, %	48.71[a]†	58.19[b]	61.23[b]	2.94	<.01
RPMS, %	14.47	15.89	18.26	1.83	NS
VAP, μm/s	32.71	31.85	33.59	1.51	NS
LIN, %	62.83	62.53	61.06	1.53	NS
ALH, μm	1.28	1.24	1.28	0.09	NS
BCF, Hz	7.50	7.39	7.87	0.23	NS
Viable sperm with intact acrosome, %	51.98[a]	59.58[b]	62.87[b]	2.89	<.01
In vitro embryo production					
Inseminated oocytes, No.	210	210	210		
Cleaved zygotes, No. (%)	101 (48.10)	94 (44.76)	82 (39.05)	4.8	NS
Blastocyst formation, No. (%)	49 (23.33)	33 (15.71)	31 (14.76)	2.6	NS
% cleaved reaching blastocyst	48.51	35.11	37.80	5.2	NS

* TMS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa (>50 μm/s); VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement; and BCF, beat cross frequency. Pooled data from 7 replicates. Motility and viability value are the mean of the 2 separate determinations at 30 and 150 minutes postthawing in samples incubated for 150 minutes at 37°C.

† Different superscript letters denote significant differences ($P < .05$).

lished data). In all experiments, treatments were compared to a control sample, without SOD or CAT additives.

Each experimental day, stock solutions of the 2 oxygen species–scavenging enzymes were thawed and added to LEY and LEYGO extenders to get a final concentration of 600 and 400 IU/mL to SOD and CAT, respectively. After the centrifugation step during the cryopreservation process, the supernatant was removed and the sperm pellet was split into aliquots. Each aliquot was extended with different proportions of LEY and LEYGO supplemented or not with SOD and/or CAT to get the appropriate final enzyme concentrations. The extended aliquots were then cryopreserved according to the protocol described above.

Effects were reflected by data on sperm cryosurvival, recorded as sperm motility and viability, at 30 and 150 minutes postthawing, and by data on in vitro fertility and embryo development. In addition, data of ROS generation levels and of membrane lipid peroxidation (as MDA production) were also recorded at 30 and 150 minutes postthaw.

Statistical Analyses

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc, Chicago, Ill). Data from 7 replicates from each experiment were analyzed by analysis of variance (ANOVA) using the mixed-procedure. Data of postthaw sperm survival, ROS generation, and MDA levels were examined according to a statistical model that includes the fixed effects of antioxidant concentration and postthaw evaluation times, and the random effect of semen pool. There were no interactions ($P > .05$) between antioxidant concentration and postthaw evaluation times for any of all parameters evaluated. Therefore, means were averaged throughout postthaw evaluation times (30 and 150 minutes) to evaluate the main effect of antioxidant concentration. Percentages of postthaw sperm survival were subjected to arcsine transformation before analysis. Data of embryo development (cleaved zygotes, blastocyst rate, and proportion of cleaved zygotes that developed to the blastocyst stage) were examined according to a statistical model that includes the fixed effects of antioxidant concentrations and the random effect of semen pool. Embryo development data were modeled according to the binomial model of parameters, as described by Fisz (1980), before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when P was less than .05. Results are presented as least-squares means ± SEM.

Results

Experiment 1: Effect of Addition of SOD

The addition of SOD, 150 and 300 IU/mL, in sperm extender for freezing significantly ($P < .01$) increased the postthaw rates of total sperm motility compared with the control group. However, SOD did not affect ($P > .05$) either the proportion of rapid progressive motility spermatozoa or of any of the sperm kinematic parameters evaluated. Addition of SOD in freezing extender improved ($P < .01$) the postthaw percentage of viable spermatozoa with intact acrosome (Table 1).

Data from in vitro embryo production (Table 1) clearly showed that the addition of SOD in the freezing extender had no significant ($P > .05$) effects either on rates of oocyte cleavage or on blastocyst formation, or in the proportion of cleaved zygotes that reached the blastocyst stage.

Levels of ROS generation and MDA production are provided in Figure 1. Luminol-mediated chemiluminescence showed significantly lower ($P < .05$) levels of ROS

in thawed spermatozoa that were frozen with SOD than in controls. Lucigenin-mediated chemiluminescence was unaffected ($P > .05$); all postthaw sperm samples showed low ROS levels. Production of MDA on frozen-thawed spermatozoa was unaffected ($P > .05$) by the presence of SOD on the freezing extender.

Experiment 2: Effect of Addition of CAT

The addition of CAT (200 and 400 IU/mL) to the freezing extender significantly ($P < .01$) increased postthaw rates of total sperm motility and rapid progressive motility compared with controls. In relation to sperm motion kinetics, only VAP was affected by the presence of CAT in the freezing extender, being significantly ($P < .05$) higher in spermatozoa frozen in the presence of CAT. The addition of CAT to the freezing extender also significantly improved ($P < .01$) the postthaw percentage of viable spermatozoa with intact acrosome (Table 2).

Data from in vitro embryo production (Table 2) revealed an improvement ($P < .01$) of cleavage rates and blastocyst formation when spermatozoa were frozen in the presence of 400 IU/mL of CAT compared with controls or the 200 IU/mL CAT groups. However, addition of CAT to the freezing extender did not affect ($P > .05$) the proportion of cleaved zygotes that developed to blastocyst stage.

Levels of ROS generation and MDA production are provided in Figure 2. Luminol-mediated chemiluminescence showed a significantly lower ($P < .05$) level of ROS in thawed spermatozoa frozen with CAT compared with controls. Levels of MDA on thawed spermatozoa were unaffected ($P > .05$) by the presence of CAT in the freezing extender.

Experiment 3: Effect of the Combined Addition of SOD and CAT

The combined addition of SOD and CAT in the sperm freezing extender affected ($P < .01$) all the postthaw sperm survival parameters evaluated, irrespective the dose (Table 3). The SOD plus CAT combinations improved ($P < .01$) the percentages of postthaw total sperm motility and rapid progressive motility spermatozoa. In relation to the sperm kinematic parameters, VAP, ALH, and BCF were higher and LIN lower in thaw spermatozoa frozen in the presence of SOD plus CAT compared with controls. The addition of SOD plus CAT also increased ($P < .01$) the postthaw rate of viable sperm with intact acrosome compared with controls.

Data from in vitro embryo production (Table 3) showed, when compared with controls, a significantly ($P < .01$) higher proportion of zygotes cleaved and of blastocysts developed when SOD and CAT were added in combination to the sperm freezing extender. However, the presence of SOD plus CAT did not exert any beneficial

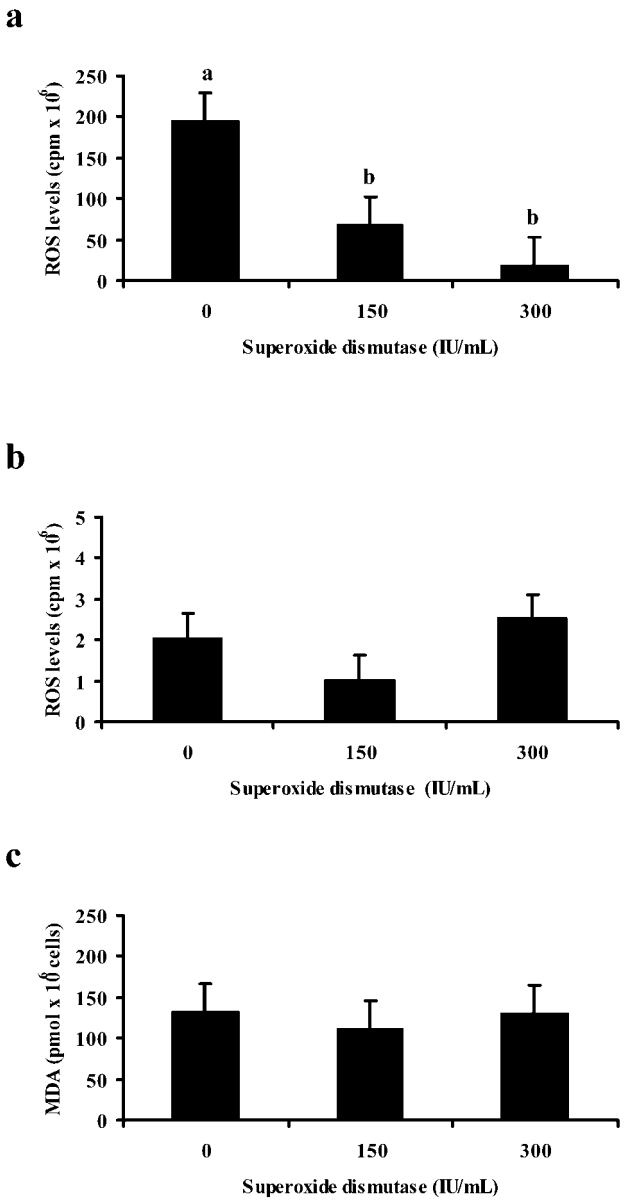

Figure 1. **(a)** Luminol-mediated chemiluminescence (mean ± SEM × 10^6 counted photons per 16 × 10^6 spermatozoa), **(b)** lucigenin-mediated chemiluminescence (mean ± SEM × 10^6 counted photons per 16 × 10^6 spermatozoa), and **(c)** concentration of malondialdehyde (MDA) in thawed boar sperm suspension frozen in the presence or absence of superoxide dismutase (SOD). Values are the least square mean ± SEM of 2 separate determinations at 30 and 150 minutes postthawing in samples incubated during 150 minutes in a water bath at 37°C. Means with different superscripts (a, b) differ ($P < .05$; $n = 7$ replicates).

effect ($P > .05$) in the proportion of cleaved zygotes that reached the blastocyst stage.

Levels of ROS generation and MDA production are provided in Figure 3. Luminol-mediated chemiluminescence showed significantly lower ($P < .05$) levels of ROS in thawed spermatozoa frozen in the presence of SOD plus CAT than in controls. Lucigenin-mediated chemiluminescence was unaffected ($P > .05$) by the presence of

Table 2. *Effect of the addition of catalase (CAT) in the freezing extender on the postthaw boar sperm motilty, kinematic parameters, and viability and in their ability to develop embryos in vitro*

Postthaw Sperm Parameters*	Treatment CAT, IU/mL			SEM	Probability, *P*
	0 (Control)	200	400		
Motility and kinematic parameters					
TMS, %	54.95[a]†	66.41[b]	66.87[b]	5.62	<.01
RPMS, %	14.59[a]	20.69[b]	21.87[b]	2.99	<.01
VAP, μm/s	31.59[a]	35.83[b]	36.06[b]	2.36	<.05
LIN, %	61.71	61.45	62.94	2.18	NS
ALH, μm	1.30	1.63	1.39	0.21	NS
14BCF, Hz	7.61	8.15	8.07	0.38	NS
Viable sperm with intact acrosome, %	56.55[a]	66.92[b]	68.85[b]	4.90	<.01
In vitro embryo production					
Inseminated oocytes, No.	210	210	210		
Cleaved zygotes, No. (%)	51 (24.29)[a]	65 (30.95)[a]	96 (45.71)[b]	3.3	<.01
Blastocyst formation, No. (%)	20 (9.52)[a]	27 (12.86)[a]	52 (24.76)[b]	2.5	<.01
% cleaved reaching blastocyst	39.22	41.54	54.17	6.4	NS

* TMS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa (>50 μm/s); VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement; and BCF, beat cross frequency. Pooled data from 7 replicates. Motility and viability value are the mean of the 2 separate determinations at 30 and 150 minutes postthawing in samples incubated for 150 minutes at 37°C.

† Different superscript letters denote significant differences ($P < .05$).

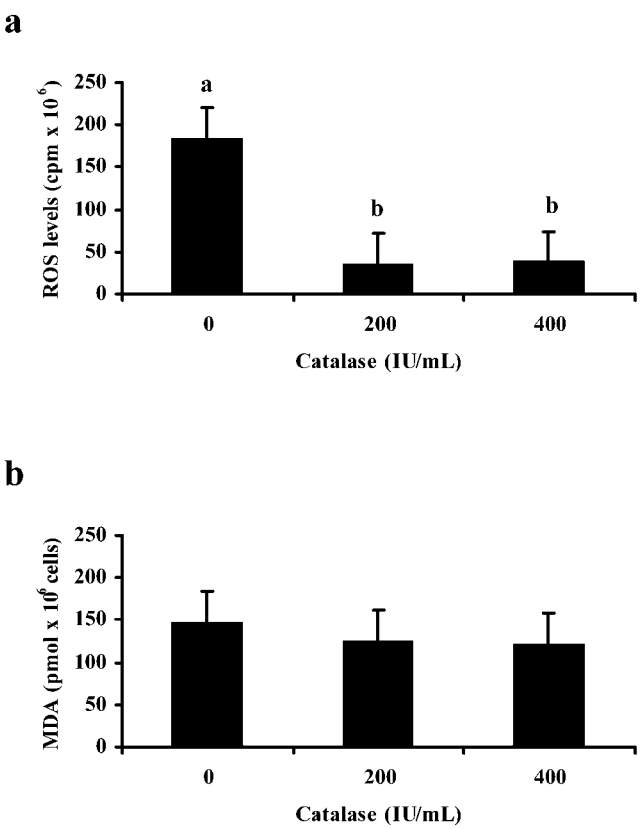

Figure 2. **(a)** Luminol-mediated chemiluminescence (mean ± SEM × 10^6 counted photons per 16 × 10^6 spermatozoa) and **(b)** concentration of MDA in thawed boar sperm suspension frozen in the presence or absence of catalase (CAT). Values are the least square mean ± SEM of 2 separate determinations at 30 and 150 minutes postthawing in samples incubated during 150 minutes in a water bath at 37°C. Means with different superscripts (a, b) differ ($P < .05$; $n = 7$ replicates).

SOD plus CAT in the sperm freezing extender, showing all postthaw sperm samples with low ROS levels. Production of MDA on thawed spermatozoa was unaffected ($P > .05$) by the presence of SOD plus CAT on the freezing extender.

Discussion

In the present study we evaluated the potential benefits of the ROS-scavenging enzymes CAT and SOD for boar sperm cryopreservation. Under the present experimental conditions, addition of these antioxidants—alone or in combination—to the freezing extender improved postthaw sperm motility and viability, irrespective of the concentration added. Moreover, the proportion of oocytes that cleaved was higher when CAT alone (400 IU/mL) or in combination (200 and 400 UI/mL) with SOD was added to the sperm freezing extender. The findings in these experiments seem to agree with those of Maxwell and Stojanov (1996), who demonstrated that the addition of CAT and SOD to the extender improves the survival and in vitro fertility of liquid stored ram spermatozoa. In spite of the above results, it is necessary to point out that the addition of SOD and CAT in extenders for preservation of mammalian semen has resulted in variable success regarding the improvement of sperm survival. Therefore, CAT had shown a controversial effect on sperm functional competence in cryopreserved bull (Foote, 1962) and chilled stored stallion (Aurich et al, 1997; Ball et al, 2001) semen. Differences in preservation protocols and extender formulations among laboratories and between species may explain, at least in part, this variability. Additionally,

Table 3. *Effect of the addition of catalase (CAT) and superoxide dismutase (SOD) in combination in the freezing extender on the postthaw boar sperm motilty, kinematic parameters, and viability and in their ability to develop embryos in vitro*

Postthaw Sperm Parameters*	Treatment CAT + SOD, IU/mL			SEM	Probability, *P*
	0 (Control)	200+150	400+300		
Motility and kinematic parameters					
TMS, %	57.82[a]†	68.43[b]	67.64[b]	3.59	<.01
RPMS, %	15.38[a]	19.80[b]	20.05[b]	2.72	<.01
VAP, μm/s	31.87[a]	34.81[b]	35.23[b]	2.19	<.05
LIN, %	59.06[a]	55.19[b]	55.56[b]	1.33	<.01
ALH, μm	1.42[a]	1.92[b]	1.83[a,b]	0.16	<.05
BCF, Hz	7.76[a]	8.75[b]	8.52[b]	0.23	<.01
Viable sperm with intact acrosome, %	58.89[a]	70.23[b]	69.98[b]	3.45	<.01
In vitro embryo production					
Inseminated oocytes, No.	240	240	240		
Cleaved zygotes, No. (%)	74 (30.83)[a]	120 (50)[b]	130 (54.17)[b]	3.3	<.01
Blastocyst formation, No. (%)	42 (17.50)[a]	76 (31.67)[b]	76 (31.67)[b]	2.8	<.01
% cleaved reaching blastocyst	56.76	63.33	58.46	4.5	NS

* TMS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa (>50 μm/s); VAP, average path velocity; LIN, linearity; ALH, amplitude of lateral head displacement; and BCF, beat cross frequency. Pooled data from 7 replicates. Motility and viability value are the mean of the 2 separate determinations at 30 and 150 minutes postthawing in samples incubated for 150 minutes at 37°C.

† Different superscript letters denote significant differences ($P < .05$).

it is possible that boar spermatozoa are more susceptible to ROS-mediated damage than bull or stallion spermatozoa.

Our finding that the addition of SOD or CAT—alone or in combination—improved postthaw boar sperm survival provides indirect evidence that an oxidative stress, involving the presence of O_2^- and H_2O_2, occurs during the freezing and thawing process. This suggestion is in agreement with that of Cerolini et al (2001), who, evaluating the changes in lipid composition undergone by boar spermatozoa during cryopreservation, suggested that cryopreservation enhanced lipid peroxidation and concluded that there is a need for O_2^- and H_2O_2 scavengers. In this way, it is well established that oxidative stress plays an important role in the cryoinjury damage of mammalian spermatozoa. Alvarez and Storey (1992) demonstrated that the freeze-thaw process enhanced lipid peroxidation in human sperm. Likewise, Chatterjee and Gagnon (2001) showed evidence of ROS generation by bovine spermatozoa undergoing cooling, freezing, and thawing processes. Although the present experiments were not designed to provide evidence of whether either ROS generation or lipid peroxidation develops during cryopreservation, the levels of chemiluminescence detected by luminol in control samples of thawed spermatozoa clearly show that oxidative stress takes place during cooling, freezing, and thawing of boar spermatozoa. Moreover, the fact that luminol-dependent chemiluminescence was significantly reduced by the addition of CAT and SOD alone or in combination to the sperm freezing extender reflects that these enzymes are capable of scavenging the ROS generated during the cryopreservation process, which reduces the potential damaging effects of oxidative stress and subsequently improves the postthaw functional competence of the thawed boar spermatozoa.

In contrast to the recognition of the damaging effects of oxidative stress, it is established that low concentrations of ROS, specifically O_2^- and H_2O_2, mediate the process of capacitation, hyperactivation, and acrosome reaction (human: de Lamirande et al, 1998; bovine: O'Flaherty et al, 1999). Although a drastic fall in ROS generation was shown, no reduction in fertilization rates (taken as the proportion of zygotes that cleaved) was found when CAT and SOD—alone or in combination—were used, indicating that the physiological events involved in the capacitation and fertilization processes were unaffected by the addition of these ROS-scavenging enzymes to the freezing extender at the concentration tested. On the contrary, cleavage rates were highest when CAT, alone (400 IU/mL) or in combination (200 and 400 IU/mL) with SOD, was added to the freezing extender. The data suggest a detrimental effect of excessive H_2O_2 on the fertilizing potential of thawed boar spermatozoa, which can be reversed by adding CAT to the freezing extender. This concept may be supported by the results of Blondin et al (1997), who demonstrated a reduction in oocyte penetration rates by bovine spermatozoa exposed to artificially produced ROS, generated through the use of the hypoxanthine-xanthine oxidase system, which was reversed by the presence of CAT. Similarly, Kuribayashi and Gagnon (1996), in a study in which murine sperm suspensions were preincubated with CAT prior to in vitro fertilization, indicated that CAT maintains the sperm potential to promote embryo development.

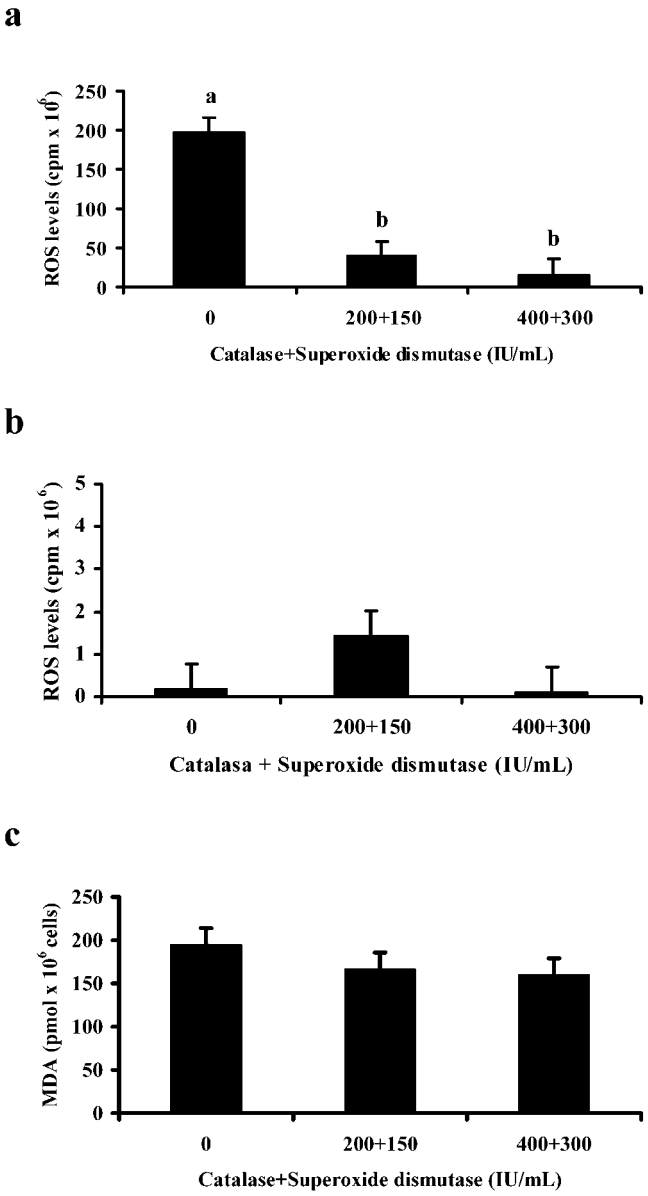

Figure 3. **(a)** Luminol-mediated chemiluminescence (mean ± SEM × 10^6 counted photons per 16 × 10^6 spermatozoa), **(b)** lucigenin-mediated chemiluminescence (mean ± SEM × 10^6 counted photons per 16 × 10^6 spermatozoa), and **(c)** concentration of MDA in thawed boar sperm suspension frozen in the presence or absence of CAT and SOD in combination. Values are the least square mean ± SEM of 2 separate determinations at 30 and 150 minutes postthawing in samples incubated during 150 minutes in a water bath at 37°C. Means with different superscripts (a, b) differ ($P < .05$; $n = 7$ replicates).

Although a significant improvement in fertilization rates (taken as the proportion of zygotes that cleaved) was observed in spermatozoa frozen in the presence of CAT, alone or in combination with SOD, no differences were found when SOD alone was used. Because the SOD enzyme in the spermatozoon catalyzes the O_2^- radicals and produces H_2O_2 (Aitken, 1995), the ineffectiveness of SOD alone might be explained by the fact that its positive effect in removing excessive levels of O_2^- could be counteracted by the negative effect of the corresponding excessive production of H_2O_2. The above results also suggest that H_2O_2—rather than O_2^-—is more harmful to the fertilizing capability of thawed boar spermatozoa.

In addition to the negative effects on motility and viability, ROS can also damage the DNA in the sperm nucleus (Aitken, 1999; Baumber et al, 2003). Ahmadi and Ng (1999) demonstrated, in research with homologous (mouse) and heterologous (human spermatozoa, hamster oocytes) models, a similar fertilization rate between DNA-damaged and live, intact spermatozoa. The ability to develop to the blastocyst stage was, however, reduced in oocytes fertilized with DNA-damaged spermatozoa. Extrapolating the above findings to boar spermatozoa, the lack of influence of the ROS-scavenger enzymes SOD and/or CAT on the proportion of cleaved zygotes that reached the blastocyst stage might suggest that the addition of these enzymes in the sperm freezing extender does not provide any additional protection to subsequent in vitro embryo development. Although the results of in vitro embryo production should be taken with caution because the blastocyst rate depends much more on others factors than the fertilizing capacity of spermatozoon, the ineffective addition of SOD and/or CAT might be explained by an inefficiency of these ROS-scavenging enzymes to protect sperm DNA. The protective action of CAT and SOD on sperm DNA is still controversial. Lopes et al (1998) demonstrated that CAT was ineffective in preventing human sperm DNA damage and suggested that H_2O_2 has little effect on sperm chromatin. In contrast, CAT, and not SOD, had protective effects on the DNA of cryopreserved equine spermatozoa. Moreover, Baumber et al (2003) suggested that H_2O_2 and not O_2^- is the ROS responsible for DNA damage in equine spermatozoa, at least during the cryopreservation process. The implications of H_2O_2 and O_2^-, and the protective role of CAT and SOD, on sperm DNA integrity and its consequences on subsequent sperm ability to develop embryos in vitro or in vivo of thawed boar spermatozoa requires further investigation.

Luminol- and lucigenin-dependent chemiluminescences are the most common methods used for measuring ROS in sperm samples (Sikka, 2004). Differences in the pattern of ROS generation were evident between luminol and lucigenin in the present study. Luminol does not discriminate between intracellular and extracellular ROS generation. In contrast, the lucigenin technique detects only extracellular ROS release (McKinney et al, 1996). Although interpretation of luminol and lucigenin data is complicated by the fact that a variety of biochemical pathways can lead to the generation of the chemiluminescence signal (Aitken et al, 1992), the results obtained in the present study showed that a significantly higher level of ROS was detected with luminol compared with lucigenin

in thawed boar sperm suspensions. The high levels of chemiluminescence detected by luminol suggest that a substantial component of ROS generation during the cryopreservation process occurs inside the spermatozoa. Furthermore, because lucigenin detects mainly O_2^-, the low levels of lucigenin-dependent chemiluminescence detected suggest that extracellular O_2^- is not an important source of ROS in thawed boar spermatozoa.

It is well established that an excess of ROS causes peroxidation of plasma membrane lipids, boar spermatozoa being especially susceptible because of their high degree of polyunsaturated acids. In the present study, the levels of lipid peroxidation were determined by the measurement of MDA, a degradation product—of small molecular mass—of the peroxidative process (Aitken et al, 1993). The MDA test, assayed by the TBA reaction, remains one of the most efficacious methods for assessing levels of lipid peroxidation in sperm suspensions (Sikka, 2004). In the present study, unlike with human sperm (Gomez et al, 1998), it was not necessary to promote a lipid peroxidation chain reaction in order to generate results that fell within the detectable range of the spectrophotometer. In all thawed sperm samples, the levels of MDA were always high and not different from those previously assessed in thawed boar sperm samples (Roca et al, 2004). In addition, nonsignificant differences in MDA levels between control and CAT and/or SOD supplemented samples were evident. The lack of influence of these ROS-scavenger enzymes on MDA production together with the fact that luminol-dependent chemiluminescence was significantly reduced in samples containing these enzymes may reflect that the addition of CAT and SOD alone or in combination in the sperm freezing extender provides good protection against oxidative stress involved in the cryopreservation process, via mechanisms that do not implicate the production of MDA as a degradation product of the lipid peroxidation process. In this way, it is known that MDA is only one of the several degradation products generated during the lipid peroxidation process of sperm membranes (Gomez et al, 1998).

In conclusion, the present data suggest that the damage undergone by boar spermatozoa during the cryopreservation process is due, in part, to oxidative stress. The addition of SOD or CAT, alone or in combination, to the freezing extender reduces postthaw ROS generation, improving sperm motility and viability. Moreover, the supplementation with CAT, alone or in combination with SOD, also improves the in vitro fertilizing potential (taken as the proportion of zygotes that cleaved) of thawed spermatozoa. For these reasons, CAT, alone or in combination with SOD, could be used as a component in boar cryopreservation extenders.

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