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Survival and Fertility of Boar Spermatozoa After Freeze-Thawing in Extender Supplemented With Butylated Hydroxytoluene

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ABSTRACT: This study evaluated the protective effect of butylated hydroxytoluene (BHT), a lipid-soluble antioxidant, against cryopreservation injuries to boar spermatozoa. In experiment 1, the lowest BHT concentrations able to reduce lipid peroxidation in boar spermatozoa were determined. Nine BHT concentrations (ranging from 0.025 to 3.2 mM) were evaluated, and the lowest ($P < .05$) production of malondialdehyde (MDA), as an indicator of lipid peroxidation, was obtained when BHT ranged from 0.2 to 1.6 mM. In experiment 2, sperm survivability was evaluated when BHT was added to a postthaw freezing extender by measuring the degree of sperm lipid peroxidation (using MDA production) and by measuring parameter such as motility, plasma membrane and acrosome integrity, and cell apoptosis. The ability of thawed spermatozoa to fertilize in vitro–matured oocytes and of embryos to develop to the blastocyst stage in vitro was also assessed. Pooled sperm-rich fractions collected from 3 mature Pietrain boars were frozen in 0.5-mL straws after dilution with lactose-egg yolk-glycerol-Orvus ES Paste extender supplemented with 0, 0.2, 0.4, 0.8, and 1.6 mM BHT. Postthaw sperm survival, evaluated 30 and 150 minutes after thawing, was higher in BHT-treated spermatozoa, being significant ($P < .05$) when the freezing extender was supplemented with 0.2, 0.4, and 0.8 mM BHT. The addition of BHT to the freezing extender resulted in a significant ($P < .05$) decrease in the MDA concentration in thawed spermatozoa, irrespective of the level of BHT used. BHT had no effect on oocyte cleavage rates, but the development to blastocyst was improved for embryos derived from spermatozoa frozen in extender supplemented with 0.4 mM BHT (16% vs 29% of blastocysts per total oocytes; $P < .05$). In conclusion, under the conditions tested in the present study, the addition of BHT to the freezing extender improved the overall efficiency of thawed boar spermatozoa.

Key words: Antioxidants, cryopreservation, sperm, porcine.

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In spite of the important potential applications of frozen-thawed spermatozoa in the pig industry, it is not generally used for artificial insemination (AI) programs on pig farms. The reason is the lower farrowing rate and litter size obtained after insemination with frozen-thawed semen compared with fresh or cooled semen (for a recent review, see Johnson et al, 2000).

Various circumstances are associated with the low fertility of frozen-thawed boar spermatozoa after AI (for recent reviews, see Holt, 2000; Watson, 2000), which include the damage that the spermatozoa undergo during the freeze-thaw process, which lowers their fertilizing capacity. The success of preservation by freezing depends on the susceptibility of sperm cells to low temperature, since boar spermatozoa are especially susceptible to cold shock (White, 1993). This problem is related to the lipid composition of the membrane, because membranes of boar spermatozoa have a relatively high proportion of polyunsaturated fatty acids, which decreases significantly during the cryopreservation process, indicating lipid peroxidation (Cerolini et al, 2001). Lipid peroxidation has been correlated to exposure of spermatozoa to reactive oxygen species (ROS). It has been demonstrated that spermatozoa that undergo freeze-thaw cycles produce ROS (Alvarez and Storey, 1992). Moreover, excessive ROS formation by spermatozoa during the cryopreservation process has been associated with a decrease in the function of thawed spermatozoa (Chatterjee and Gagnon, 2001). One way to overcome the detrimental effects of ROS on subsequent sperm performance after thawing could be to add antioxidant compounds to the freezing extender to block or prevent oxidative stress.

A variety of antioxidants have been tested to either scavenge ROS directly or counter the effects of ROS toxicity in the semen of a variety of mammalian and avian species. Among them, butylated hydroxytoluene (BHT), a synthetic phenolic antioxidant, has been widely used as a food additive (Williams et al, 1999). BHT has been tested with varying degrees of success to either preserve

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liquid spermatozoa in the turkey tom (Donoghue and Donoghue, 1997) and stallion (Ball et al, 2001) or minimize cold shock damage in bull (Hammerstedt et al, 1976) and ram spermatozoa (Watson and Anderson, 1983). BHT has also been successfully tested to minimize cold shock damage in boar spermatozoa (Pursell, 1979; Bamba and Cran, 1992). In spite of these promising results, the use of BHT in freezing extenders is not commonplace, and no results are available that evaluate the potential effect of BHT on frozen-thawed boar spermatozoa. The goal of the present study was to evaluate the potential protective effect of BHT on cryopreservation injuries to boar spermatozoa. Postthaw sperm survival and their ability to produce embryos in vitro that develop to the blastocyst stage were the criteria used to assess the potential protective effect of BHT.

Materials and Methods

Reagents and Media

All chemicals were of analytic 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, Wycombe United Kingdom).

The basic medium used for sperm dilution 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_2HPO_4, and 1.46 mM $CaCl_2$-$2H_2O$. The oocyte maturation medium was the bovine serum albumin (BSA)-free North Carolina State University-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) that was supplemented with 10% (vol/vol) porcine follicular fluid, 0.1 mg/mL cysteine, 10 ng/mL epidermal growth factor, 10 IU/mL equine chorionic gonadotropin (Folligon, Intervet International B.V., Boxmeer, the Netherlands), and 10 IU/mL human chorionic gonadotropin (Chorulon, Intervet). The fertilization medium was a modified Tris-buffered medium 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) that was 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 BHT were prepared in absolute ethanol and dried down under air in tubes before the addition of the medium. When medium was added to each tube, the content was mixed on a Vortex mixer (VELP Scientifica, Milano, Italy). The BHT stock solution was made up fresh each experimental day.

Semen Processing and Sperm Cryopreservation

Ejaculate sperm samples were collected from 3 Pietrain boars on a weekly basis. 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, acrosome integrity, and normal morphology) were microscopically evaluated, using standard laboratory techniques (Martin Rillo et al, 1996), and only ejaculate sperm samples with more than 75% motile spermatozoa and more than 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, diluted semen samples (1:1 [vol/vol] in BTS) were cooled to 17°C for 3 hours. After centrifugation at 800 × *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 1500 × 10^6 cells/mL. After further cooling to 5°C in 90 minutes, diluted 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 1000 × 10^6/mL. The resuspended and cooled spermatozoa were packed into 0.5 polyvinyl chloride-French straws (Minitüb, Tiefenbach, Germany), which were 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, and then cooled at 70°C/min to −150°C and plunged into liquid nitrogen.

Thawing and Postthaw Sperm Survival Evaluation

Thawing was carried out in a circulating water bath at 37°C for 20 seconds. Thawed spermatozoa from 2 straws were extended at 37°C with BTS (1:2 [vol/vol]) and incubated in a water bath for 150 minutes. Sperm were assessed for motility and motion characteristics, plasma membrane integrity, acrosome integrity, and early apoptosis.

Sperm motility and motion characteristics were objectively evaluated using a computer-aided sperm analysis (CASA) system (Sperm Class Analyzer [SCA], Microptic, Barcelona, Spain) and a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel). Diluted, thawed spermatozoa were rediluted 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 at 39°C, and 3 fields were analyzed to assess a minimum of 100 spermatozoa. The proportion of total motile spermatozoa (% TMS) and rapid progressive motility spermatozoa (% RPMS, >50 μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), linearity (% LIN, ratio of VSL/VCL), straightness of the average path (% STR, ratio of VSL/VAP), amplitude of lateral head displacement (ALH, μm), and dance (DNC, VCL × ALHmed, $\mu m^2/s$) were determined.

Plasma membrane integrity was evaluated using the fluores-

cent probes SYBR-14 (SY) and propidium iodide (PI) according to the manufacturer's instructions (L-7011, Live/Dead Sperm Viability Kit; Molecular Probes Europe, Leiden, the Netherlands). Briefly, 100 μL of thawed spermatozoa (30×10^6 cells/mL in HEPES buffer) was diluted with 25 nM SYBR-14 solution and 12 μM of PI solution. Samples were incubated at room temperature in the dark for 10 minutes before flow cytometric analysis (Garner and Johnson, 1995). Spermatozoa were allocated to ''intact membrane,'' ''dying,'' and ''dead'' classifications if they exhibited SY+/PI−, SY+/PI+, and SY−/PI+ staining, respectively.

Acrosome status was assessed by fluorescein isothiocyanate conjugated with peanut agglutinin (FITC-PNA) and PI staining as described by Nagy et al (2003), with slight modifications. Briefly, 100 μL of thawed spermatozoa (30×10^6 cells/mL in HEPES buffer) was diluted with 10 μL of FITC-PNA solution (1 μg/mL in bidistilled water) and 12 μM of PI solution. Samples were incubated at room temperature in the dark for 5 minutes before flow cytometric analysis. Spermatozoa were allocated to ''live with intact acrosome,'' ''live with damaged acrosome,'' ''dead with intact acrosome,'' and ''dead with damaged acrosome'' classifications if they exhibited PNA−/PI−, PNA+/PI−, PNA−/PI+, and PNA+/PI+ staining, respectively.

Early apoptosis was detected using Annexin V conjugated with FITC (A-FITC; Annexin V-FITC Apoptosis Detection Kit) as described by Peña et al (2003a). Briefly, 100 μL of thawed spermatozoa (30×10^6 cells/mL in HEPES buffer) was diluted with 5 μL of Annexin V-FITC solution and 10 μM of PI solution. Samples were incubated at room temperature in the dark for 15 minutes before flow cytometric analysis. Spermatozoa were allocated to ''live,'' ''early apoptotic,'' ''late apoptotic,'' and ''dead'' classifications if they exhibited A−/PI−, A+/PI−, A+/PI+, and A−/PI+ staining, respectively.

Immediately prior to analysis by flow cytometry, stained sperm samples from the 3 fluorescent probes were rediluted to 6×10^6 cells/mL in HEPES buffer. Analyses were carried out on a Becton Dickinson FACSort flow cytometer (San Jose, Calif) equipped with an argon-ion laser (488 nm, 15 mW). The green fluorescence (FITC) was measured using a 530/30-nm bandpass filter, and the red fluorescence (PI) was measured using a 650-nm LP filter, except in the plasma membrane integrity assessment, where a 585/30-bandpass filter was used. Debris was gated out on the basis of the forward scatter and side scatter dot plot by drawing a region enclosing the cell population of interest. In addition, events with scatter characteristics similar to sperm cells but without reasonable DNA content (very weak SYBR-14 or PI staining) were gated out. Subpopulations were divided by quadrants, and the frequency of each subpopulation was quantified. A minimum of 10 000 spermatozoa were collected and analyzed at a flow rate of approximately 800 cells/s.

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, diluted spermatozoa (250×10^6 cells in 1 mL) were mixed with 1 mL of cold 20% (wt/vol) trichloroacetic acid to precipitate protein. 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 a spectrophotometer (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).

In Vitro Oocyte Maturation, Fertilization, and Embryo Culture

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–30°C. Oocytes were aspirated from medium-sized follicles (3–6 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 preequilibrated 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 diluted, thawed spermatozoa from 1 pool of 3 straws was 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; 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). Six hours after insemination, the oocytes were washed and transferred (30 oocytes per well) to a Nunc 4-well multidish containing 500 μL of embryo culture medium; they were then 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 and blastocyst formation, respectively, were evaluated under a stereomicroscope.

Experimental Design

Experiment 1: Determination of the Lowest Effective BHT Concentration to Reduce Lipid Peroxidation—The goal of this experiment was to determine the lowest BHT concentrations able to reduce lipid peroxidation in boar spermatozoa. Sperm-rich ejaculate fractions from 3 boars were pooled immediately after collection. The pooled semen samples were split into 10 aliquots and diluted in phosphate buffer to 250×10^6 spermatozoa/mL. One aliquot was used as a negative control, and 9 aliquots were subjected to incubation for 30 minutes at 37°C with the following final concentrations of BHT (B-1378): 0 (as a positive control), 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 mM. After incubation, these 9 aliquots were subjected to in vitro–induced

lipid peroxidation. To induce lipid peroxidation, diluted spermatozoa were incubated for 60 minutes at 37°C with 0.5 mM sodium ascorbate and 0.05 mM ferrous sulfate (Brzezinska-Slebodzinska et al, 1995). After incubation, MDA concentration was evaluated.

Experiment 2: Effect of BHT Supplementation on Postthaw Sperm Function—In this experiment, the effect that BHT had on sperm function was evaluated by adding various ion concentrations of BHT to the freezing extender. After the centrifugation step during the cryopreservation process, the supernatant was removed, and the sperm pellet was split into 5 aliquots. Each aliquot was extended with LEY and LEYGO supplemented with BHT at the final concentrations of 0 (as control), 0.2, 0.4, 0.8, and 1.6 mM. Extended aliquots were cryopreserved according the protocol described above.

After thawing, assessments were made of sperm survival, membrane lipid peroxidation, and the ability of spermatozoa to fertilize in vitro–matured oocytes and for embryos to develop to the blastocyst stage using the procedures previously described. Sperm survival was assessed 30 and 150 minutes after thawing, and the MDA concentration (as an indicator of lipid peroxidation) was determined 0, 30, and 150 minutes after thawing.

Statistical Analyses

All of the data editing and statistical analyses were performed by SPSS, version 11.5 (SPSS Inc, Chicago, Ill). Data from 6 replicates in each experiment were analyzed by analysis of variance using the MIXED procedure according to a statistical model, including the fixed effect of concentration of BHT and the random effect of semen pool. In experiment 2, data of the postthaw incubation times (30 and 150 minutes for sperm survival and 0, 30, and 150 minutes for MDA production) were combined and analyzed as a complete data set. Percentages of postthaw sperm survival were subjected to an arcsine transformation before analysis. Embryo development data are presented in accordance with the binomial model of parameters as described by Fisz (1980) before analysis. When analysis of variance showed a significant effect, values were compared using the Bonferroni test and were considered significant when $P < .05$. Results are presented as means ± standard error of the mean.

Results

Experiment 1: Determination of the Lowest Effective BHT Concentration that Reduced Lipid Peroxidation

The results are summarized in Figure 1. The production of MDA by fresh boar spermatozoa after in vitro induction of peroxidation with sodium ascorbate and ferrous sulfate was significantly lower ($P < .05$) in the samples previously incubated with BHT, irrespective of the concentration tested, than in the positive control. Moreover, there were no significant differences in the levels of MDA found in the negative control (sample without in vitro induction of peroxidation) when compared with the induced samples incubated with BTH for concentrations ranging from 0.2 to 1.6 mM.

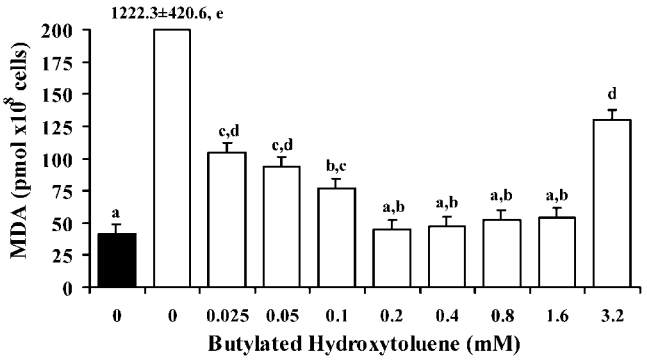

Figure 1. Mean ± SEM concentration of malondialdehyde (MDA) in fresh boar sperm suspensions after incubation (30 minutes at 37°C) with butylated hydroxytoluene and in vitro induction of lipid peroxidation (a 60-minute incubation at 37°C with 0.5 mM sodium ascorbate and 0.05 mM ferrous sulfate; (white bars) compared to the control sample without induced lipid peroxidation (black bars). Means with different superscripts (a–e) differ ($P < .05$; n = 6 replicates).

Experiment 2: Effect of BHT Supplementation on Postthaw Sperm Function

The effect of the presence of BHT in the freezing extender on postthaw sperm motility parameters is shown in Table 1. The percentages of TMS and RPMS were significantly ($P < .05$) higher in spermatozoa frozen in the presence of 0.2–0.8 mM BHT than in the control. However, BHT had no influence on the other motility parameters (VSL, VCL, VAP, LIN, STR, ALH, and DNC).

Table 2 shows the results of plasma membrane integrity and acrosome integrity tests carried out in thawed spermatozoa. The percentages of spermatozoa classified as membrane intact (SY+/PI−) and live with intact acrosome (PNA−/PI−) were significantly ($P < .05$) higher in the samples cryopreserved in the presence of BHT at concentrations ranging from 0.2 to 0.8 mM than in its absence.

The percentages of thawed spermatozoa in the 4 categories identified using the A/PI tests are presented in Table 3. The percentage of spermatozoa classified as live (A−/PI−) was significantly ($P < .05$) higher in the samples cryopreserved in the presence of BHT at concentrations ranging from 0.2 to 0.8 mM, than in the control. The percentages of apoptotic sperm (A+/PI− and A+/PI+) were not affected ($P > .05$) by the presence of BHT in the freezing extender.

Figure 2 displays the production of MDA by thawed spermatozoa. The presence of BHT in the freezing extender significantly decreased ($P < .05$) the MDA concentration in thawed semen, irrespective of the concentration of BHT.

The results of in vitro embryo development are summarized in Figure 3. BHT in the freezing extender did not improve ($P > .05$) the cleavage rates of oocytes.

Table 1. *Mean ± SEM of motility parameters from the CASA data set of thawed spermatozoa frozen in the presence or absence of butylated hydroxytoluene; values are the mean of the 2 separate determinations at 30 and 150 minutes postthawing in samples incubated for 150 minutes at 37°C**

Motility Parameters (%)	Concentration of Butylated Hydroxytoluene (mM)				
	0	0.2	0.4	0.8	1.6
TMS	32.16 ± 2.61 B	45.44 ± 2.03 A	44.07 ± 2.56 A	44.52 ± 2.53 A	37.73 ± 2.89 AB
RPMS	18.59 ± 2.17 B	27.54 ± 1.95 A	27.19 ± 2.38 A	26.43 ± 1.93 A	20.93 ± 2.42 AB
VSL	49.42 ± 2.31	52.81 ± 1.83	51.27 ± 1.78	52.31 ± 1.61	47.99 ± 2.16
VCL	66.58 ± 2.72 AB	72.51 ± 1.89 A	70.98 ± 1.82 AB	71.22 ± 2.06 AB	64.99 ± 2.63 B
VAP	54.76 ± 2.61	58.44 ± 1.84	56.96 ± 1.86	57.55 ± 1.74	52.91 ± 2.37
LIN	70.08 ± 1.41	70.75 ± 1.04	70.98 ± 1.82	71.22 ± 2.06	70.89 ± 1.09
STR	84.16 ± 0.76	84.39 ± 0.71	85.39 ± 0.81	85.44 ± 0.59	85.42 ± 0.77
ALH	2.04 ± 0.07 AB	2.11 ± 0.05 A	2.08 ± 0.05 A	2.04 ± 0.05 AB	1.88 ± 0.06 B
DNC	161.60 ± 10.49 AB	180.45 ± 8.27 A	170.54 ± 7.51 AB	170.59 ± 9.66 AB	140.91 ± 8.81 B

* CASA indicates computer-aided sperm analysis; TMS, total motile spermatozoa; RPMS, rapid progressive motile spermatozoa (>50 μm/s); VSL, straight-line velocity; VCL, curvilinear velocity; VAP, average path velocity; LIN, linearity; STR, straightness of the average path; ALH, amplitude of lateral head displacement; and DNC, dance.

† Values in the same row with different letters (A, B) differ ($P < .05$; n = 6 replicates).

However, a higher proportion of oocytes reached the blastocyst stage ($P < .05$) when in vitro–matured oocytes were fertilized with thawed spermatozoa cryopreserved in the presence of 0.4 mM BHT compared to the control (28.8% vs 15.8%).

Discussion

In the present study, we showed that the presence of BHT in the freezing extender improved both the postthaw survival of boar spermatozoa and the ability of cleaved oocytes, derived from frozen-thawed spermatozoa, to develop to blastocysts. BHT has been recognized as a protector of spermatozoa against oxidative damage. However, the mechanism by which BHT protects the spermatozoa from cold shock and freezing stress is still uncertain. It is well known that cryopreservation induces the formation of ROS detrimental to subsequent sperm performance (Watson, 2000). It has been suggested that lipid-soluble compounds with antioxidant properties, such as BHT, can incorporate into sperm membranes and prevent the damaging activity of lipid peroxyl radicals (Aitken and Clarkson, 1988). Nevertheless, from a general point of view, the potential protective effect of BHT on stored mammalian spermatozoa is still controversial.

Table 2. *Percentage (mean ± SEM) of sperm subpopulations labeled by SYBR-14 (SY)/propidium idodide (PI) and FITC-PNA (PNA)/PI tests after flow cytometric analyses of thawed spermatozoa frozen in the presence or absence of butylated hydroxytoluene; values are the mean of the 2 separate determinations at 30 and 150 minutes postthawing in samples incubated for 150 minutes at 37°C**

Spermatozoa	Concentration of Butylated Hydroxytoluene (mM)				
	0	0.2	0.4	0.8	1.6
SY/PI assay					
Intact membrane (SY+/PI−)	45.45 ± 2.62 B†	60.93 ± 1.59 A	55.83 ± 2.46 A	58.13 ± 1.77 A	53.10 ± 2.26 AB
Dying (SY+/PI+)	3.39 ± 0.87 A	1.93 ± 0.36 B	1.67 ± 0.21 B	1.48 ± 0.17 B	1.98 ± 0.31 AB
Dead (SY−/PI+)	51.16 ± 2.56 A	35.77 ± 1.93 B	42.49 ± 2.42 B	40.40 ± 1.80 B	44.68 ± 2.35 B
PNA/PI assay					
Live with intact acrosome (PNA−/PI−)	44.07 ± 2.58 B	60.70 ± 1.67 A	56.79 ± 2.48 A	58.04 ± 1.85 A	53.51 ± 2.20 AB
Live with damaged acrosome (PNA+/PI−)	0.84 ± 0.08	0.97 ± 0.08	1.00 ± 0.10	1.04 ± 0.09	1.06 ± 0.07
Dead with intact acrosome (PNA−/PI+)	29.48 ± 1.45 A	20.25 ± 0.67 B	22.67 ± 1.34 B	20.96 ± 0.67 B	24.71 ± 1.33 B
Dead with damaged acrosome (PNA+/PI+)	25.60 ± 2.20 A	18.07 ± 1.25 B	19.55 ± 1.55 B	19.96 ± 1.48 B	20.72 ± 1.39 AB

* SY indicates SYBR-14 (SYBR-14 is the component A of Live/Dead® Sperm Viability Kit; Molecular Probes Inc, Eugene, Ore). The Sperm Viability Kit includes PI. PI, propidium iodide; and FITC-PNA, fluroescein isothiocyanate conjugated with peanut agglutinin.

† Values in the same row with different letters (A, B) differ ($P < .05$; n = 6 replicates).

Table 3. *Percentage (mean ± SEM) of sperm subpopulations indentified by the Annexin V (A)/propidium iodide (PI) test after flow cytometric analyses of thawed spermatozoa frozen in the presence or absence fo butylated hydroxytoluene; values are the mean of the 2 separate determinations at 30 and 150 minutes postthawing in samples incubated for 150 minutes at 37°C**

	Concentration of Butylated Hydroxytoluene (mM)				
Spermatozoa	0	0.2	0.4	0.8	1.6
Live (A−/PI−)	47.24 ± 2.63 B	59.19 ± 1.86 A	57.47 ± 2.10 A	60.03 ± 1.71 A	54.85 ± 2.09 AB
Early apoptotic (A+/PI−)	2.68 ± 0.27	3.86 ± 0.28	3.13 ± 0.31	3.14 ± 0.24	3.09 ± 0.24
Late apoptotic (A+/PI+)	21.01 ± 1.72	18.98 ± 1.50	17.61 ± 1.36	17.88 ± 1.51	19.00 ± 1.26
Dead (A−/PI+)	29.06 ± 2.67 A	15.96 ± 1.47 B	21.12 ± 2.09 B	18.96 ± 1.72 B	23.04 ± 2.01 AB

* A indicates Annexin V (a component of Annexin V-FITC Apoptosis Detection Kit; Sigma-Aldrich Co, St Louis, Mo). The Kit includes PI. PI indicates propidium iodide.

† Values in the same row with different letters (A, B) differ ($P < .05$; n = 6 replicates).

The concept that BHT might be beneficial to mammalian and avian sperm preservation, when it is added to semen extenders, has been previously tested. However, adding BHT to stored semen has shown mixed results with respect to improving the survival of spermatozoa. Whereas BHT improved sperm survival in cold-stressed (Graham and Hammerstedt, 1992) or cryopreserved (Killian et al, 1989) bull spermatozoa and chilled turkey tom semen (Donoghue and Donoghue, 1997), it had a detrimental effect on cooled stallion spermatozoa (Ball et al, 2001). These results suggest that considerable difficulties still exist with regard to species differences and technical abilities when using BHT to preserve sperm motility and viability. In relation to boar semen, our findings, as well as those previously reported (Pursel, 1979; Bamba and Cran, 1992), demonstrate that the presence of BHT in the extender protects boar spermatozoa from damage caused by cryopreservation or cold shock. Moreover, our results indicate that the addition of BHT at concentrations ranging from 0.2 to 0.8 mM improves the motility, plasma membrane integrity, and acrosome integrity of thawed spermatozoa. Because BHT has the ability to incorporate into sperm membranes and prevent the damaging activities of the lipid peroxyl radical, the resulting effectiveness of BHT suggests that boar spermatozoa subjected to chilled storage or cryopreservation are particularly susceptible to oxidative attack of lipid peroxyl radicals. This explanation is supported by previous studies in which α-tocopherol, another powerful scavenger for lipid peroxyl radicals (Kotamraju et al, 2001), improved survival of chilled (Cerolini et al, 2000) and cryopreserved (Peña et al, 2003b) boar spermatozoa. Moreover, lipid peroxyl radicals have been shown to initiate lipid peroxidation specifically in boar sperm membranes (Comaschi et al, 1989).

The potential effect of BHT to protect spermatozoa from stress induced by cold or frozen storage may also vary widely with the concentration used. The results of experiment 1 indicate that all doses of BHT examined (ranging from 0.025 to 3.2 mM) were effective in the reduction of in vitro–induced lipid peroxidation. This finding is in agreement with those achieved in human spermatozoa by Aitken and Clarkson (1988), who demonstrated the effectiveness of BHT in the suppression of lipid peroxidation at concentrations ranging from 0.1 to 10 mM. However, these researchers reported that the highest concentrations of BHT had a toxic effect on spermatozoa, both inducing loss of motility and affecting their

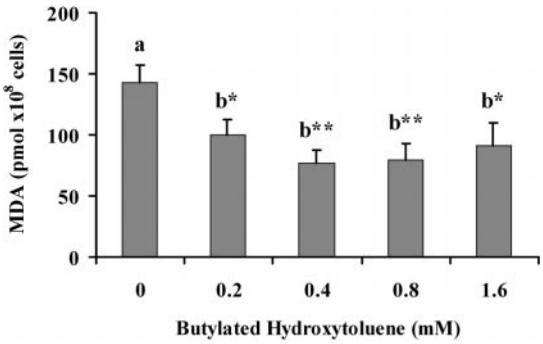

Figure 2. Mean ± SEM concentration of malondialdehyde (MDA) in thawed spermatozoa frozen in the presence or absence of butylated hydroxytoluene. Values are the mean of the 3 separate determinations at 0, 30, and 150 minutes postthawing in samples incubated for 150 minutes in a water bath at 37°C. Means with different superscripts (a, b) differ ($P < .05$; n = 6 replicates). Differences from the control (0 mM) are indicated by asterisks (*$P < .05$; **$P < .01$).

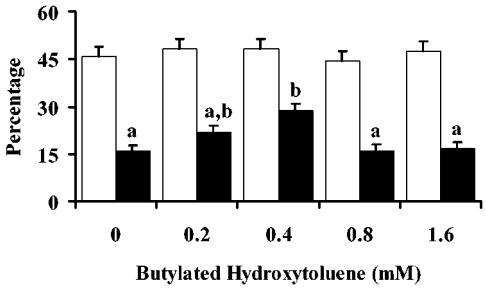

Figure 3. Percentage (mean ± SEM) of oocytes cleaved in vitro (white bars) and embryos developing to blastocysts per total oocytes (black bars) after insemination with thawed boar spermatozoa frozen in the absence (control, 0) or presence of varying concentrations of butylated hydroxytoluene. Means with different superscripts (a, b) differ ($P < .05$; n = 6 replicates).

capacity to fuse with oocytes. The controlled generation of ROS in spermatozoa is associated with some normal physiologic functions such as sperm hyperactivation, capacitation, and acrosome reaction (Aitken, 1995). An excessive addition of antioxidant substances to the semen extender can neutralize the oxidative stress induced by an excessive ROS formation, but it can also stop the normal sperm functions associated with ROS. Therefore, it is important to select the appropriate antioxidant concentrations to maintain the natural balance that exists between ROS generation and scavenging activities. At the BHT concentrations tested in the second experiment (ranging from 0.2 to 1.6 mM), there were no deleterious effects on any of the functional parameters of thawed sperm. On the contrary, when the concentrations ranged from 0.2 to 0.8 mM, the postthaw sperm survival improved. Similarly, the improvement of sperm survival observed by Pursel (1979) and Bamba and Cran (1992) in boar spermatozoa undergoing cold shock was achieved at BHT concentrations ranging from 0.05 to 2 mM. Nevertheless, at the highest concentration evaluated (1.6 mM) in the present study, BHT had no protective effect on boar spermatozoa. Likewise, Bamba and Cran (1992) observed a loss of the protective effect of BHT against the cold stress of spermatozoa when the concentrations used were more than 2 mM. It seems, therefore, that the addition of BHT to semen extenders has beneficial or detrimental effects on postthaw sperm survival depending on the concentration used, with the maximum concentration required for effectiveness being between 1 and 2 mM.

The interaction between extender components and antioxidants is another important factor to consider when evaluating the effectiveness of any antioxidant in the protection of spermatozoa from either cold shock or freeze-thaw stress. In this way, it has been reported that catalase supplementation prolongs survival of bull spermatozoa in yolk extenders but not in skim milk extenders (Foote, 1962). For frozen boar spermatozoa in the present study, we used an extender containing 20% egg yolk. The effectiveness of BHT for improving both the survival and fertility of thawed spermatozoa may be related, in part, to the cooperative interaction between BHT and egg yolk. In this respect, Graham and Hammerstedt (1992) reported that BHT analogs in diluents with no egg yolk resulted in reduced sperm motility compared with the presence of egg yolk, suggesting that egg yolk interacted synergistically with BHT analogs to protect spermatozoa from cold shock.

Recently, it was proposed that lipid peroxyl radicals induce an apoptotic signaling cascade in endothelial cells (Kotamraju et al, 2001). Moreover, it is claimed that apoptosis occurs as a result of cryodamage in mammalian cells (Baust, 2002). Likewise, BHT inhibited caspase-3 activation, an enzyme that is considered a committed step in several apoptotic pathways (Kotamraju et al, 2001). In the present study, sperm apoptosis was investigated using the Annexin V assay. The Annexin V and PI stain combinations are able to identify early and late apoptosis in thawed boar spermatozoa (Peña et al, 2003a). As shown in Table 3, the presence of BHT in the freezing extender did not reduce the percentage of either early or late apoptotic cells in thawed spermatozoa. It may be that caspase-3 does not play an important role in sperm apoptotic mechanisms or that there is no relationship between ROS and apoptosis in boar sperm. Further studies are needed to address these questions.

It is well established that cryopreservation induces lipid peroxidation in mammalian spermatozoa. Alvarez and Storey (1992) demonstrated that the freeze-thaw process enhanced peroxidation of human sperm membrane lipids. Likewise, Chantterjee and Gagnon (2001) showed how cooling and thawing processes caused an increase of lipid peroxidation in bovine spermatozoa. Until the present study, no data were available on the effect of cryopreservation on lipid peroxidation in boar spermatozoa. Lipid peroxides, derived from peroxidation of polyunsaturated fatty acids, are unstable and decompose to form a complex series of compounds. These include reactive carbonyl compounds, of which the most abundant is MDA. Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation in a variety of cell types, including spermatozoa (Sikka, 1996). In the present study, MDA production was used to determine the peroxidation levels of thawed spermatozoa. We showed that the presence of BHT in the freezing extender reduced MDA production in thawed spermatozoa. Since it has been demonstrated that lipid peroxyl radicals are one of the most important of the ROS involved in MDA production in boar spermatozoa (Comaschi et al, 1989), our results corroborate the hypothesis that lipid peroxyl radicals could be one of the most important ROS implicated in cryodamage to boar spermatozoa.

The investigation of in vitro fertilization and embryo development demonstrated that BHT at a concentration of 0.4 mM in the freezing extender boosted the ability of embryos derived from thawed boar spermatozoa to develop to the blastocyst stage. A possible explanation for our results is that BHT had an additional protective effect on cryopreserved spermatozoa to prevent the loss of DNA integrity. Besides membrane damage and impairment of motility, lipid peroxidation can damage the DNA in the sperm nucleus, either through oxidation of DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to MDA, resulting in strand breaks and crosslinking (Sikka, 1996). A reduction in embryonic development after fertilization with DNA-damaged spermatozoa has been described in humans (Ahmadi and Ng, 1999). Possible differences among BHT concentrations in the protective effect on sperm DNA integrity

could explain differences in the ability of thawed spermatozoa to develop embryos in vitro. Unfortunately, as DNA damage in thawed spermatozoa was not evaluated in the present study, it was not possible to check the above-mentioned hypothesis. However, if lipid peroxidation promotes sperm DNA damage and oocyte fertilization with DNA-damaged spermatozoa results in a low rate of embryo development, then the reduction of lipid peroxidation should improve development in embryos derived from spermatozoa treated with antioxidants. This hypothesis is supported in the present study where the levels of MDA, as indicators of lipid peroxidation, were lower in spermatozoa that had been freeze-thawed in the presence of BHT than in the control. Moreover, the lowest MDA levels were measured when a BHT concentration of 0.4 mM was used, which was the concentration that achieved the highest rate of embryo development in vitro. However, the implications of lipid peroxidation, as well as the protective role of BHT on sperm DNA integrity and its consequences to postthaw sperm fertility of boar spermatozoa in vitro or in vivo, require considerable further investigation.

To summarize, the data presented in this study demonstrated that the presence of BHT in the freezing extender at concentrations ranging from 0.2 to 0.8 mM improved the survival of boar spermatozoa after thawing. Furthermore, at a concentration of 0.4 mM, BHT enhanced the ability of embryos derived from thawed spermatozoa to develop in vitro. These results also suggest that the protective effect of BHT on cryopreserved spermatozoa is directly related to a reduction in lipid peroxidation. We conclude that adding BHT to the freezing extender improves the overall efficiency of utilization of thawed boar spermatozoa.

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