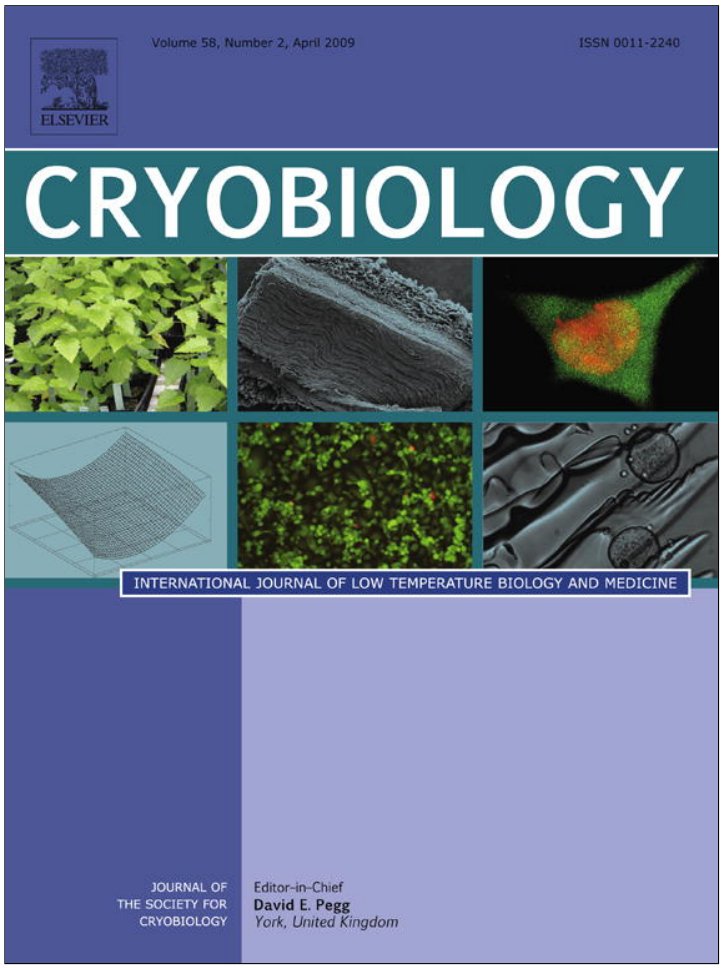

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The influence of cysteine and taurine on microscopic–oxidative stress parameters and fertilizing ability of bull semen following cryopreservation ☆

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ABSTRACT

Oxidative stress significantly damages sperm functions such as motility, functional integrity, endogenous antioxidant enzyme activities and fertility due to lipid peroxidation induced by reactive oxygen species (ROS). The aim of this study was to determine the effects of antioxidants such as taurine and cysteine in Bioxcell® extender on standard semen parameters, fertilizing ability, lipid peroxidation (LPO) and antioxidant activities comprising reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) after the cryopreservation/thawing of bull semen. Nine ejaculates for each bull were included in the study. Three groups, namely taurine (2 mM), cysteine (2 mM), and control, were designed to analyze the antioxidants in Bioxcell®. Insemination doses were processed so that each 0.25-ml straw contained 15×10^6 sperm.

The addition of cysteine led to higher motility, compared to the other groups ($P < 0.001$). Cysteine showed a greater protective effect on the percentages of acrosome damage and total abnormalities in comparison to the other groups ($P < 0.001$). No significant differences were observed in hypo-osmotic swelling test (HOST), following supplementation with antioxidants during the freeze–thawing process. No significant difference was observed in non-return rates among groups. In biochemical assays, the additives did not show effectiveness on the elimination of malondialdehyde (MDA) formation and maintenance of GSH and GSH-Px activities, when compared to controls. CAT activity (35.1 ± 8.1 kU/g) was demonstrated to be significantly higher upon the addition of 2 mM taurine ($P < 0.001$), while the level of MDA increased, indicating oxidative stress in this group. SOD activity (21.4 ± 2.9 U/g protein) was significantly elevated in the group with cysteine, compared to the other groups ($P < 0.001$).

Introduction

Frozen bull semen has been widely used in artificial insemination (AI). But freezing and thawing processes lead to the generation of reactive oxygen substances (ROS) that impair post-thaw motility, viability, intracellular enzymatic activity, fertility and sperm functions [4,53,55]. Sperm cells contain high concentrations of polyunsaturated fatty acids (PUFA), and therefore are highly susceptible to lipid peroxidation (LPO), which leads to a subsequent loss of motility, membrane integrity, fertilizing capability and metabolic changes of sperm [3,21]. During cryopreservation PUFA in the sperm plasma membrane undergo peroxidation, which results in the formation of ROS [5,35]. In bovine semen, ROS are generated primarily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction [50]. Low concentrations of ROS are physiologically involved in the maintenance of the fertilizing ability and capacitation/acrosome reaction of spermatozoa. But excessive ROS impair sperm function and enzymatic activity [12,29].

Spermatozoa and seminal plasma possess an antioxidant system comprising taurine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) to prevent oxidative damage. However, this anti-oxidant capacity in sperm cells, due to the small cytoplasmic component that contains anti-oxidants to scavenge oxidants, is limited. Thus, mammalian spermatozoa may, however, be insufficient in preventing LPO during the freeze–thawing process [10,37,47].

In recent years, cysteine and taurine, as anti-oxidants in the semen extender, have been used in the cryopreservation of boar [27], bull [15,22,52], human [1,38], ram [17,18] and goat sperm [9] to improve the post-thaw motility, viability, membrane integrity and fertility of spermatozoa. Amongst compounds of the epididymal and oviduct fluids, sulfonic amino acids (e.g., taurine) are important nonenzymatic scavengers that play an important role in the protection of spermatozoa against ROS, in case of exposure

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to aerobic conditions and the freezing/thawing process [7,22,31]. Mammalian cells can only utilize cysteine, which has been shown to penetrate the cell membrane easily, enhancing intracellular glutathione biosynthesis both in vitro and in vivo [42,39]. Cysteine has been shown to prevent loss of motility, viability and membrane integrity during sperm liquid storage or in the frozen state [19,49]. Additionally, it has improved porcine oocyte maturation and fertilization in vitro [33].

The improvement of semen cryopreservation technologies requires in-depth knowledge of the properties of the current extender. There is a lack of information regarding the effectiveness of commercial Bioxcell® extender on sperm functions, LPO and antioxidant activities. This research is the first evaluation of the influence of certain antioxidants such as cysteine and taurine in commercial Bioxcell® extender. We investigated the effects of adding cysteine and taurine to extended semen prior to cryopreservation on standard semen parameters, fertilizing ability, lipid peroxidation (LPO) and antioxidant activities (GSH, GSH-Px, CAT, and SOD) during the cryopreservation process.

Materials and methods

Animals and semen collection

The animals (3 and 4 years of age) were housed at the Lalahan Livestock Central Research Institute (Ankara, Turkey), and maintained under uniform feeding and housing conditions. A total number of 30 ejaculates (10 ejaculates for each bull) were collected from the bulls with the aid of an artificial vagina twice a week, according to AI standard procedures. Only ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/ml were used. Nine ejaculates for each bull were included in the study. Immediately after collection, the ejaculates were immersed in a warm water bath at 34 °C until their assessment in the laboratory. Semen assessment was performed in approximately 20 min.

Semen processing

The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals and sperm concentration was determined by means of an Accucell photometer (IMV, L'Aigle, France). Sperm motility was estimated using phase-contrast microscopy (200×). A commercial extender (Bioxcell®) was used as the base freezing extender. Each ejaculate was split into three equal experimental groups and diluted to a final concentration of 60×10^6/ml spermatozoa with the base extender containing cysteine (2 mM), taurine (2 mM) and no additive (control). Diluted semen samples were loaded into 0.25-ml French straws and cooled down to 4 °C in 2 h, frozen at a programmed rate of −3 °C/min from +4 to −10 C; −40 °C/min from −10 to −100 °C; −20 °C/min from −100 to −140 °C in a digital freezing machine (Digitcool 5300 ZB 250, IMV, France). Thereafter, the straws were plunged into liquid nitrogen. Sperm samples from three bulls were frozen for nine conditions. After 24 h, frozen straws were thawed in a 37 °C water bath for 20 s immediately before use.

Semen evaluation

Analysis of in vitro and in vivo semen parameters

Progressive motility was assessed using a phase-contrast microscope (100× magnification), with a warm stage maintained at 37 °C. A wet mount was made using a 5-μl drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in three different microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score [13].

For the evaluation of sperm abnormalities, at least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml formalin (37%), 150 ml saline solution, 150 ml buffer solution and 500 ml double distilled water [44]. One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome and total abnormalities were determined by counting a total of 400 spermatozoa under phase-contrast microscopy (1000× magnification, oil immersion).

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane. This was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution at 37 °C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. Two hundred sperms with swollen or coiled tails were recorded under phase-contrast microscopy (400× magnification) [20,41].

In fertility trials, the fertilizing ability of spermatozoa was calculated based on the non-return rates at 59 days. A total of 145 cows were artificially inseminated with frozen samples with antioxidants and control samples, each comprising the semen of one bull. The effective non-return rates (NNR) were determined at 59 days post-insemination by palpation per rectum.

Biochemical assays

Biochemical assays were performed in sperm samples immediately after thawing and with centrifuging and washing. An aliquot (500 μl) of semen from each sample was centrifuged at 800*g* for 10 min, and the sperm pellet was separated and washed by resuspending in PBS and recentrifuging (three times). After the last centrifugation, 1 ml of deionized water was added to spermatozoa. The samples were thawed prior to the analyses for LPO and antioxidant enzyme activities [23,36].

MDA concentrations

The concentrations of malondialdehyde (MDA), as indices of LPO in sperm samples, were measured using the thiobarbituric acid reaction and according to the method described by Yoshioka et al. [54]. The quantification of thiobarbituric acid reactive substances was determined by comparing the absorption with the standard curve of malondialdehyde equivalents generated by the acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The MDA concentrations were expressed as nmol/L.

Activities of GSH, GSH-Px, SOD and CAT

The GSH content of sperm was measured using the method of Beutler et al. [14]. The samples were precipitated with 50% trichloracetic acid and then centrifuged at 1000*g* for 5 min. The reaction mixture contained 2 ml of supernatant, 8 ml phosphate buffer and 1 ml DTNB (5,5′-dithiobis-2-nitrobenzoic acid). The solution was incubated at room temperature for 5 min and then read at 412 nm on a spectrophotometer. The values of GSH were expressed as mg/dl.

GSH-Px activity was determined according to the method of Paglia and Valentine [40]. The reaction mixture contained 1 ml phosphate buffer (pH 7), 100 μl 3 mmol/L NADPH + H, 100 μl 10 mmol/L EDTA, 100 μl 20 mmol/L Na–azide, 100 μl 20 mmol/L reduced glutathione, and 200 μl 2 U glutathione reductase. A 200-μl sample was added to the above mixture and this mixture was incubated for 3 min at 37 °C. The rate of GSSG formation was measured by following the decrease in the absorbance of the reaction mixture at 340 nm as NADPH was converted to $NADP^+$, between the 2nd and 4th minutes after the initiation of the reaction by employing

a cuvette with a 1-cm light path. Calculations were based on a standard curve prepared with different concentrations of NADPH. The GSH-Px activity was expressed as nmol/NADPH + H/min/g protein.

SOD was analysed with the technique of Sun and Zigman [48]. A volume of 0.4 ml sample, 0.1 ml of 0.9% NaCl and 0.4 ml of chloroform:ethanol (3:5) was centrifuged for 10 min at 2000*g*. The reaction mixture contained 20 ml 3 mmol/L xanthine, 10 ml 0.6 mmol/L EDTA, 10 ml 0.15 mmol/L NBT, 6 ml 400 mmol/L sodium carbonate, and 3 ml 1 g/L BSA for 20 tests. An aliquot (50 μl) of xanthine oxidase (20 μmol/2 ml) was added to 2.45 ml of the reaction mixture and this mixture was incubated at 25 °C for 20 min. Subsequently, the reaction was stopped by adding 1 ml of 0.8 mmol/L $CuCl_2$ to the test. The absorbance was recorded at 560 nm using a spectrophotometer. SOD activity was expressed as U/g protein.

The method described by Goth [28] was used for the determination of CAT activity in sperm samples. 0.2-ml sperm samples were incubated in 1 ml substrate (65 mmol/ml) hydrogenperoxide in 60 mmol/L sodium–potassium phosphate buffer (pH 7.4) at 37 °C for 60 s. One unit of CAT decomposes 1 μmol of hydrogen peroxide per min, under these conditions. The enzymatic reaction was terminated with 1 ml of 32.4 mmol/L ammonium molybdate and hydrogen peroxide was measured at 405 nm using a spectrophotometer. Values of CAT activity were expressed as kU/g protein.

Statistical analysis

The study was replicated nine times. Results were expressed as the mean ± SEM. Means were analyzed by analysis of variance, followed by Tukey's post-hoc test to determine significant differences in all the parameters between groups using the SPSS/PC computer programme (version 12.0, SPSS, Chicago, IL). Non-return rates were evaluated by the *Z*-test. Differences with values of $P < 0.05$ were considered to be statistically significant.

Results

Sperm parameters (percentages of motility, acrosome and total abnormalities, HOST and in vivo fertility)

The influence of certain additives on the standard semen parameters of frozen bull semen were evaluated in nine independent experiments. As set out in Table 1, the addition of cysteine led to higher motility (57.8 ± 1.2%), compared to the other groups ($P < 0.001$). Cysteine showed a greater protective effect in the percentages of acrosome damage and total abnormalities in comparison to the other groups ($P < 0.001$). No significant differences were observed in HOST, following supplementation with antioxidants during the freeze–thawing process. Fertility results based on 59-day non-returns after rectovaginal insemination are set out in Table 2. No significant differences were observed in non-return rates among groups.

Analysis of oxidative stress parameters

The effect of certain antioxidants on lipid peroxidation and antioxidant activities in thawed bull semen are set out in Table 3. The additives did not show effectiveness on the elimination of MDA formation and maintenance of GSH and GSH-Px activities, when compared to controls. CAT activity (35.1 ± 8.1 kU/g) was demonstrated to be significantly higher upon the addition of 2 mM taurine ($P < 0.001$), while the level of MDA increased, indicating an oxidative stress in this group. SOD activity (21.4 ± 2.9 U/g protein) was significantly elevated in the group with cysteine, compared to the other groups ($P < 0.001$).

Discussion

Spermatozoa are susceptible to oxidative damage under oxidative stress. This damage can be prevented by supplementation of the semen extender with antioxidants [46]. A variety of scavengers are present in spermatozoa and seminal plasma, including SOD, GSH, taurine, GSH-PX and catalase [6,30,34], which remove alkoxyl and peroxyl radicals, and thus protect sperm motility and functions against oxidative damage [2,11,32]. Additionally, the sulfonated amino acid taurine found in the seminal plasma and oviductal fluid, is one of the major non-enzymatic scavengers that plays an important role in the protection of spermatozoa against ROS and lipid peroxidation [43,45].

Table 1
Mean (±S.E.) percentages of motility, acrosome, total abnormalities and HOST in frozen–thawed bull spermatozoa.

Groups	Motility	Acrosome abnormality	Total abnormalities	HOST
Taurine	43.3 ± 2.4^{c}	6.6 ± 0.6^{b}	14.0 ± 0.7^{b}	46.6 ± 1.9
Cysteine	57.8 ± 1.2^{a}	2.6 ± 0.4^{a}	8.0 ± 0.5^{a}	48.4 ± 1.9
Control	49.6 ± 1.1^{b}	6.7 ± 0.6^{b}	15.0 ± 1.1^{b}	43.8 ± 2.1
P	***	***	***	—

Different superscripts (a–c) within the same column showed significant differences among groups.
(—) No significant difference ($P > 0.05$).
*** $P < 0.001$.

Table 2
Fertility results based on 59-day non-returns after rectovaginal insemination with frozen–thawed bull semen.

Groups	Total inseminated cows	Non-return rates (%)
Taurine	41	56.09 (23/41)
Cysteine	55	74.54 (41/55)
Control	49	57.14 (28/49)

The same column showed no significant differences among proportions ($P > 0.05$).

Table 3
Mean (± S.E.) malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT) levels in frozen–thawed bull semen.

Groups	MDA (nmol/ml)	GSH (mg/dl)	SOD (U/g protein)	GSH-Px (nmol/NADPH + H/min/g protein)	CAT (kU/g)
Taurine	1.5 ± 0.2^{b}	0.4 ± 0.1^{b}	2.1 ± 0.4^{a}	4.6 ± 0.5	35.1 ± 8.1^{b}
Cysteine	0.8 ± 0.2^{a}	1.1 ± 0.1^{a}	21.4 ± 2.9^{b}	3.6 ± 1.0	11.0 ± 2.6^{a}
Control	0.7 ± 0.2^{a}	1.4 ± 0.3^{a}	7.2 ± 1.8^{a}	3.0 ± 0.8	6.8 ± 1.8^{a}
P	*	**	***	—	***

Different superscripts (a and b) within the same column showed significant differences among the groups.
(—) No significant difference ($P > 0.05$).
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

In the present study, addition of taurine to the freezing extender did not cause any further improvement in semen quality parameters and, was similar to results obtained by Bucak and Uysal [19]. Current findings are in contrast with those reported by Atessahin et al. [9] and Alvarez and Storey [7] who demonstrated a marked improvement in the motility of ram and rabbit semen following the freeze–thawing process. These contradictory results may be due to the differences of semen extender composition, animal species and taurine concentration used in the study. Based on our results, taurine had a beneficial effect on endogenous antioxidant capacity, improving CAT level in the presence of ROS. This finding is in agreement with a similar study conducted on ram sperm [17].

The axosome and associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. These are responsible for sperm motility [24]. In this study, the addition of cysteine exhibited significant cryoprotective activity on certain sperm parameters such as post-thaw motility, acrosomal and overall morphological integrity, similar to the studies performed in bull [15], boar [27,49], ram [51] and goat semen [19]. Based on our results, we can hypothesize that cysteine displayed protective influence on the functional integrity of the axosome and mitochondria, improving post-thawed sperm motility.

SOD is a well-known enzymatic biological antioxidant that scavenges superoxide and peroxide and thus controls oxidative stress in mammalian sperm [8,26]. In this study, it is suggested that cysteine plays an antioxidative role in preventing MDA production, thus resulting in higher SOD activity, compared to the other groups.

Extending and freeze–thawing of semen lead to low antioxidant activity in the presence or absence of antioxidants. This may partly explain the lower fertility of frozen–thawed semen in comparison to fresh semen [15,16]. In the current study, cysteine led to higher non-return rates of inseminated cows. However, this result was not considered to be statistically important. This study agrees with studies where no improvement was observed in fertility when certain additives were added to the semen extender [22,25].

In conclusion, it may be stated that, compared to the controls, the use of antioxidants in the extender did not lead to any significant reduction of MDA production during the cryopreservation process. Antioxidants such as those used, could not improve levels of antioxidant activities, except for CAT in the group with 2 mM taurine and the SOD level in the group with 2 mM cysteine, following the thawing of semen ($P < 0.001$). Acceptable levels of standard sperm parameters such as motility and morphological integrity were provided with cysteine following the thawing of semen ($P < 0.001$). The effect of cysteine was not associated with fertility statistically, although the highest non-return rate was achieved by adding cysteine (Table 2). Further studies are needed to test the fertilizing ability of bull spermatozoa frozen with effective additives used in commercial AI.

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