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Influence of Various Antioxidants on Microscopic-Oxidative Stress Indicators and Fertilizing Ability of Frozen-Thawed Bull Semen

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Abstract

Cryopreservation is associated with the production of reactive oxygen substances (ROS), which lead to lipid peroxidation of sperm membranes, resulting in a loss of sperm motility, viability and fertility. The aim of this study was to determine effects of the antioxidants of oxidized glutathione (GSSG), reduced glutathione (GSH) and bovine serum albumin (BSA) on standard semen indicators (motility, acrosome and total abnormalities, HOST), endogenous antioxidant enzyme activities and fertilizing ability of frozen-thawed bull semen.

Eighteen ejaculates from each of 3 Holstein bulls were collected using an artificial vagina and 9 replicates of the ejaculates were diluted with a Bioxcell®-based extender supplemented with antioxidants, including BSA (5 mg/ml), GSH (2 mM), GGSG (2 mM), and an extender containing no antioxidants (control). Insemination doses (1.5×10^7 sperm/0.25 ml straw) were prepared for the insemination of cows at observed oestrus. Supplementation with antioxidants led to lower percentages of acrosome damage (4.0 ± 0.5%, 4.4 ± 0.5%, 4.0 ± 0.3%, respectively) and total abnormalities (10.3 ± 0.7%, 9.7 ± 0.8%, 10.4 ± 0.6%), compared to the controls (6.5 ± 0.6 and 14.9 ± 1.1% $P < 0.01$). Pregnancy rate after insemination was highest (72.2%) in the group which was given BSA ($P < 0.05$). There were no significant differences among groups in GSH and glutathione peroxidase (GSH-PX) enzyme activities. Superoxide dismutase (SOD) activities (0.05 ± 0.005, 0.03 ± 0.005, 0.05 ± 0.009 μkat/g protein, respectively) in all of the experimental groups with antioxidants were lower than the control group (0.11 ± 0.024 μkat/g protein, $P < 0.001$). Furthermore, BSA increased ($P < 0.001$) the activity of catalase (CAT, 304.23 ± 114.69 μkat/g protein), following the freezing-thawing process.

Bull semen, freezing, artificial insemination, antioxidant activities, lipid peroxidation

Semen cryopreservation has allowed specific opportunities for the conservation and widespread dissemination of valuable genetic resources through sperm banks, the guarantee of a constant commercial supply of semen, and collaboration in breed improvement programs by means of artificial insemination (AI) (Holt et al. 1997). Some of the problems encountered using AI in pigs, sheep and exotic species include decreased motility, membrane integrity and fertilizing potential of spermatozoa during cryopreservation, premature activation in the female genital tract, and altered membrane responses to physiological stimuli of sperm that survive the freezing-thawing process (Viswanath and Shannon 2000). It is well known that phospholipids in the sperm plasma membrane undergo peroxidation, which results in the formation of reactive oxygen substances (ROS, namely, superoxide anion radical, hydrogen peroxide) and lipid hydroperoxides (Alvarez and Storey 2005). Excessive generation of ROS by immature and abnormal spermatozoa, and sperm processing (e.g., extending, freezing-thawing process), accompanied by low scavenging and antioxidant concentrations in seminal plasma and semen extender will induce oxidative stress (Sikka 2004). According to Trinchero et al. (1990), frozen-thawed bull spermatozoa are more easily peroxidized than fresh spermatozoa. Additionally, cryopreservation of bull semen has

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been shown to decrease sperm intracellular antioxidants such as GSH and superoxide dismutase (SOD).

The antioxidant system comprising GSH, GSH-PX, catalase (CAT), and SOD has been described as a defense functioning mechanism against the lipid peroxidation (LPO) of semen, and is important in maintaining sperm motility and viability (Gadea et al. 2004). This antioxidant capacity in sperm cells may, however, be insufficient in preventing LPO during the freeze-thawing process. In that regard, mammalian spermatozoa lack a substantial cytoplasmic component, which contains antioxidants that counteract the damaging effects of ROS and LPO (Peris et al. 2007). Thiols such as GSH and GSSG are a large class of antioxidants. It is well known that GSH is able to react with many ROS directly and is a co-factor for GSH-PX which catalyses the reduction of toxic H_2O_2 and hydroperoxides (Bilodeau et al. 2001). The oxidized form of glutathione, GSSG, is partially regenerated from GSH by the action of the enzyme glutathione reductase, and secreted from the cell when the capacity of glutathione reductase is exceeded (Meister 1994). Mammalian cells can only utilize thiol compounds such as reduced and oxidized glutathione, which have been shown to penetrate the cell membrane easily, enhancing intracellular glutathione biosynthesis both *in vitro* and *in vivo*, and protecting the membrane lipids and proteins by direct radical-scavenging properties (Sagara et al. 1993; Mazor et al. 1996). Furthermore, bovine serum albumin (BSA) is known to protect sperm motility and the integrity of the plasma membrane and acrosome against temperature shock during the freezing-thawing process. It may also help sperm in surviving in the reproductive tract of the cow prior to fertilization (Chen et al. 1993; Uysal and Bucak 2007).

Materials and Methods

Animals and semen collection

Semen samples from three Holstein bulls, aged 3-4 years, from the Artificial Insemination Station belonging to Lalahan Livestock Central Research Institute (Ankara, Turkey) were used in this study. In total 66 ejaculates were collected twice weekly from the bulls with the aid of an artificial vagina. Only ejaculates containing spermatozoa with > 80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/ml were used. Eighteen ejaculates for each bull were included in the study. The first and second ejaculates from the same bull were combined. 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. The study was replicated nine times.

Semen processing and evaluation

The antioxidants used in this study, including BSA (A9647), GSH (G6013), and GGSG (G2140) were obtained from Sigma-Aldrich chemicals, and Bioxcell® extender was purchased from IMV Technologies (Şark Kemikal Ltd., Istanbul, Turkey). The volume of ejaculates was measured in a conical tube (0.1 ml graduations) and sperm concentration was determined with 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 four equal experimental groups and diluted to a final concentration of 6.0×10^7/ml spermatozoa, with the base extender containing BSA (5 mg/ml), GSH (2 mM), GGSG (2 mM) and no antioxidants (control). These antioxidants were added directly to the base extender. Diluted semen samples were loaded into 0.25-ml French straws and cooled 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 (-196 °C). After 24 h, frozen straws were thawed in a 37 °C water bath for 20 s, immediately before use.

Progressive motility was assessed using a phase-contrast microscope (× 200 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 microscopic fields for each semen sample. The mean of the three successive estimations was recorded as the final motility score (Bearden and Fuquay 2000).

For 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 sodium saline solution, 150 ml buffer solution and 500 ml double distilled water (Schafer and Holzman 2000). One drop of this mixture was put on a slide and covered with a cover slip. The percentages of acrosome and total abnormalities (acrosomal abnormality, detached heads, abnormal mid-pieces and tail defects) were determined by counting 400 spermatozoa under the 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 were examined with a phase-contrast microscopy (× 400 magnification) and deemed to have either swollen or coiled tails (Bucket et al. 1997)

A total of 198 cows were artificially inseminated with frozen samples with antioxidants and control samples, each comprising semen from one bull. Each cow was artificially inseminated in the uterine body with 0.25 ml of semen (total sperm dose of 1.5×10^7 sperm). Pregnancy rate was determined at 59 days post-insemination by transrectal palpation.

Biochemical assays were performed in sperm samples immediately after thawing and by 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 (Dandekar et al. 2002).

The concentrations of malondialdehyde (MDA), as indices of LPO in sperm samples, were measured using the thiobarbituric acid reaction, in accordance with the method described by Yoshioka et al. (1979). 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 measured as nmol/l.

The GSH content of sperm was measured using the method of Beutler et al. (1963). Samples were precipitated with 50% trichloracetic acid and centrifuged at 1000 *g* for 5 min. The reaction mixture contained 2 ml of supernatant, 8 ml phosphate buffer and 1 ml DTNB (55' dithiobis-2-nitrobenzoic acid). The solution was incubated at room temperature for 5 min and then read at 412 nm on a spectrophotometer. Values of GSH were expressed as nmol/l.

The GSH-PX activity was determined according to the method of Paglia and Valentine (1967). 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-mentioned 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 into $NADP^+$, between the 2nd and 4th min after the initiation of the reaction, using a cuvette with a 1-cm light path. Calculations were based on a standard curve prepared with various concentrations of NADPH. The GSH-PX activity was expressed as nmol/NADPH/min/g protein.

Determination of SOD was done as described by Sun and Zigman (1978). A mixture 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 400mmol/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 $CuCl_2$. Absorbance was recorded at 560 nm in a spectrophotometer, with SOD activity expressed as as µkat/g protein.

The method described by Goth (1991) was used for the determination of CAT activity in sperm samples. Aliquots (0.2-ml) of 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. Under these conditions, one unit of CAT decomposes 1 µmol of hydrogenperoxide per min. 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 µkat/g protein.

Statistical analysis

The study was replicated nine times. Values of semen characteristics, including motility, acrosome abnormality, total abnormalities and HOST were transformed to the arcsin scale to minimize heterogeneity of data. Results were expressed as mean ± SEM. Means were analyzed by one-way analyses of variance, followed by Tukey's post-hoc test to determine significant differences between groups. In the analysis, to avoid inflating the degrees of freedom, account was taken of ejaculates nested within bulls. All analyses were conducted using the SPSS/PC computer programme (Version 12.0, SPSS, Chicago, IL, USA). Differences with values of $P < 0.05$ were considered significant. Pregnancy rates were evaluated with chi-square test.

Results

Semen characteristics of frozen-thawed bull semen are shown in Table 1. Antioxidants maintained post-thaw sperm morphology and acrosome integrity ($P < 0.01$) compared to the control group. However, there were no significant differences in membrane integrity and post-thaw sperm motility following the supplementation of the freezing extender with antioxidants.

Table 1. Percentages of motility, acrosome, total abnormalities and HOST in frozen-thawed bull spermatozoa (means % ± SEM)

Group	Motility	Acrosome abnormality	Total abnormalities	HOST
BSA	44.07 ± 2.98^{a}	4.00 ± 0.47^{a}	10.30 ± 0.69^{a}	47.00 ± 2.20
GSSG	50.56 ± 1.17^{ab}	4.41 ± 0.49^{a}	9.74 ± 0.83^{a}	45.52 ± 1.78
GSH	52.59 ± 1.61^{b}	4.00 ± 0.32^{a}	10.41 ± 0.57^{a}	46.59 ± 1.62
Control	49.81 ± 1.23^{ab}	6.52 ± 0.57^{b}	14.93 ± 1.07^{b}	43.70 ± 1.96
P	*	**	***	-

Different superscripts (a and b) within the same column demonstrate significant differences
(* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)
-: $P > 0.05$

Pregnancy rates are shown in Table 2. The addition of BSA (5 mg/ml) had a significant positive effect, more pronounced than the addition of GGSG and the control group in protecting the fertilizing ability of bull sperm ($P < 0.05$).

Table 2. Pregnancy rates 59-d after insemination with frozen-thawed bull semen

Group	n	Pregnancy rate (%)
BSA	54	72.2^{b} (39/54)
GSH	38	68.4^{ab} (26/38)
GSSG	47	53.2^{a} (25/47)
Control	59	54.2^{a} (32/59)

Different superscripts (a and b) within the same column demonstrate significant differences
($P < 0.05$)

The LPO concentration and antioxidant enzymatic activity in spermatozoa are given in Table 3. The concentrations of MDA did not change significantly with the addition of the antioxidants compared to the control group after thawing, except for the addition of 2 mM GGSG ($P < 0.01$). The SOD activities in all of the experimental groups with antioxidants remained significantly lower, compared to the control group ($P < 0.001$). The addition of BSA increased the activity of CAT, compared to the other treatments, following the freezing-thawing process ($P < 0.001$). There were no significant differences among groups GSH-PX and GSH enzyme activities ($P > 0.05$).

Table 3. Malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT) levels in frozen-thawed bull semen

Group	MDA (nmol/l)	GSH (nmol/l)	SOD (µkat/g protein)	GSH-PX (nmol/NADPH/ min/g protein)	CAT (µkat/g protein)
BSA	1.88 ± 0.45^{a}	1.39 ± 0.24^{b}	0.05 ± 0.005^{a}	3.90 ± 1.17	304.23 ± 114.69^{b}
GSH	1.37 ± 0.21^{ab}	0.57 ± 0.19^{b}	0.03 ± 0.005^{a}	5.99 ± 0.52	165.53 ± 28.84^{a}
GSSG	2.43 ± 0.25^{b}	1.19 ± 0.14^{b}	0.05 ± 0.009^{a}	3.25 ± 1.15	131.69 ± 31.17^{a}
Control	0.70 ± 0.13^{a}	1.33 ± 0.23^{b}	0.11 ± 0.024^{b}	2.90 ± 0.63	104.02 ± 26.34^{a}
P	**	*	**	-	***

Different superscripts (a and b) within the same column demonstrate significant differences
(* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)
-: $P > 0.05$

Discussion

Cryopreservation causes irreversible damage to sperm organelles, and changes in membrane fluidity and enzymatic activity, associated with a reduction in sperm motility, viability and fertilizing ability (Alvarez and Storey 1989; Hammerstedt 1993).

Glutathione (l-g-glutamyl-l-cysteinylglycine) is a tripeptide thiol compound that has many important functions in cell physiology and metabolism, including the protection of the cell from oxidative stress, synthesis of protein and DNA and gamete cell fertilization (Nasr-Esfahani and Johnson 1992). In cattle, BSA is found in reproductive tract fluids, and helps to preserve sperm motility and acrosome integrity during the semen freezing process (Yamashiro et al. 2006).

In the present study, GSH and GGSG had a slight promoting effect on sperm motility, while ROS increased. In terms of post-thaw motility, the current results agree with previous studies on boar (Gadea et al. 2004), bull (Bilodeau et al. 2001; Foote et al. 2002), and ram semen (Bucak et al. 2008), where no improvement was detected in sperm motility in the presence of GSH. However, motility was not affected by the formation of LPO. This was contradictory to studies in humans (de Lamirande and Gagnon 1992), mice (Baiardi et al. 1997), and horses (Baumber et al. 2000) where deterioration of sperm motility was observed in the precence of free radicals. However, our results seemed lower than the findings of Chatterjee et al. (2001), in that the addition of GGSG to the freezing extender reduced by 35% the loss of motility of spermatozoa undergoing a freezing-thawing cycle. These contradictory results may be due to the differences in extender composition and glutathione concentration. In this study, BSA (5 mg/ml) protected sperm morphology and fertilizing potential of bull spermatozoa, with a significant antioxidant property as a result of increased activity of CAT, against cryodamage after the freeze-thawing process.

The axosome and associated dense fibres of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. It is well established that ROS can induce axonemal and mitochondial damage, resulting in the immobilization of sperm (Aitken and Clarkson 1987; Peris et al. 2007). Based on the current results, it appeared that GSH, GSSG and BSA had protective effects on sperm morphological integrity of the head, middle and tail pieces, promoting axonemal and mitochondrial stability.

Sperm cells and seminal plasma contain ROS scavengers, including the enzymes SOD, GSH-PX and catalase, which convert superoxide (O_2^-) and peroxide (H_20_2) radicals into 0_2 and H_20. SOD and catalase also remove (O_2^-) generated by NADPH-oxidase in living cells (Alvarez and Storey 1989; Jeulin et al. 1989). However, GSH-PX removes peroxyl radicals from various peroxides, including H_2O_2 to improve sperm motility (Irvine 1996). In this study, the addition of antioxidants did not prevent LPO or maintain GSH, SOD and GSH-PX levels. Only catalase activity was higher in the group with BSA, compared to the other groups.

Extending and freezing-thawing of semen leads 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 (Bilodeau et al. 2000; 2001). In this study, the group supplemented with 5 mg/ml BSA had significantly better pregnancy rates 59 days after insemination, compared to GGSG and control groups. This finding may be due to a higher CAT level compared to the other groups. The current results agree with a study previously conducted in the bull (Foote et al. 1993) with respect to the pregnancy rate in a group with BSA.

Further studies on the determination of oxidative stress, antioxidative capacity and fertility of bull semen following the freezing-thawing process are required to understand the physiological-biochemical changes that are important in semen freezing and *in vivo*/*in vitro* fertilization. The use of certain antioxidants to achieve better results in terms of motility, membrane integrity and fertility potential of cryopreserved bull sperm, is still under discussion and requires further investigations. Further studies are needed to block the loss of motility and fertility of frozen semen with effective antioxidants.

In conclusion, the addition of various antioxidants to freezing media improved the characteristics of frozen-thawed bull semen. Compared to the controls, the antioxidants

(GSH, GGSG, and BSA) provided a cryoprotective effect on the morphological integrity of frozen-thawed bull spermatozoa. Furthermore, BSA also improved pregnancy rates, as well as significantly elevating CAT activity.

Vliv různých antioxidantů na mikroskopické oxidativní stresové ukazatele a fertilizační schopnosti semene býků po zmražení a rozmražení

Kryoprezervace je spojená s produkcí reaktivních kyslíkových substancí (ROS), které vedou k lipoperoxidaci membrán spermií. Následkem je ztráta motility, životaschopnosti a fertility spermií. Cílem této studie bylo stanovit vlivy antioxidantů oxidovaného glutathionu (GSSG), redukovaného glutathionu (GSH) a bovinního sérového albuminu (BSA) na standardní indikátory kvality semene (pohyblivost spermií, abnormality akrozomální a celkové, HOST), aktivity endogenních antioxidačních enzymů a fertilizační schopnost semene po zmražení a tání.

Od tří býků plemene Holštýn byly odebráno celkem 18 ejakulátů za pomocí umělé vagíny a 9 paralelních vzorků ejakulátů bylo ředěno pomocí extenderu na bázi Bioxcell® – s doplňkem antioxidantů včetně BSA (5 mg/ml), GSh (2 mM), GGSG (2 mM) a extenderu, který neobsahoval antioxidanty (kontrola). Inseminační dávky ($1,5 \times 10^7$ spermií /0,25 ml) byly připraveny k inseminaci krav při pozorovaném estru. Obohacení antioxidanty vedlo ke snížení procenta akrozomálních poškození (4,0 ± 0, 5%, 4,4 ± 0,5 %, 4,0 ± 0,3 %) a celkového poškození spermií (10,3 ± 0,7 %, 9,7 ± 0,8 %, 10,4 ± 0,6 %) ve srovnání s kontrolami (6,5 ± 0,6 a 14,9 ± 1,1 %) , $P < 0,01$). Procento zabřeznutí bylo po inseminaci nejvyšší (72,2 %) ve skupině krav s přídavkem BSA ($P < 0,05$). Mezi skupinami nebyly nalezeny signifikantní rozdíly v aktivitě enzymů GSH a GSH-peroxidázy (GSH-PX). Aktivita superoxid dismutázy (SOD) (0,05 ± 0,005, 0,03 ± 0,005 a 0,05 ± 0,09 μkat/g) byla ve všech sledovaných skupinách s antioxidanty nižší než v kontrolní skupině (0,11 ± 0,024 μkat/g, $P < 0,001$). A konečně přídavek BSA zvýšil ($P < 0,001$) aktivitu katalázy (CAT 303,23 ± 114,69 μkat/g) po procesu zamražení a rozmražení ejakulátu.

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