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Antioxidative effects of cysteamine, hyaluronan and fetuin on post-thaw semen quality, DNA integrity and oxidative stress parameters in the Brown Swiss bull

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Summary

The aim of this study was to compare the effectiveness of antioxidants including cysteamine (2.5, 7.5 mM), hyaluronan (0.25, 1 mg ml^{-1}) and fetuin (5, 10 mg ml^{-1}) in the freezing of Brown Swiss bull semen. The best percentages of CASA motilities were achieved with 10 mg ml^{-1} of fetuin and 2.5 mM of cysteamine. For sperm morphology, 10 mg ml^{-1} of fetuin and 2.5 mM of cysteamine had better protective effects ($P < 0.001$). The results of hypo-osmotic swelling test showed that the percentage values of membrane integrity in all the groups, excluding that supplemented with 5 mg ml^{-1} of fetuin, were higher than those of the control group ($P < 0.001$). Results obtained for the DNA damage of sperm cells demonstrated that 0.25 mg ml^{-1} of hyaluronan, and 2.5 and 7.5 mM of cysteamine led to lower rates of spermatozoa with damaged DNA, compared with the control group ($P < 0.001$). The maintenance of superoxide dismutase and glutathione peroxidase antioxidant activities following freeze-thawing with 2.5 and 7.5 mM of cysteamine and 10 mg ml^{-1} of fetuin was demonstrated to be at a higher level in comparison with the control group ($P < 0.001$). Malondialdehyde formation was found to be lower in the groups supplemented with 0.25 mg ml^{-1} of hyaluronan and 7.5 mM of cysteamine after the freeze-thawing process ($P < 0.001$).

Introduction

Cryopreservation procedures lead to cold shock, ice crystal formation, oxidative stress, osmotic changes and lipid-protein reorganisations within the cell membrane, which affect normal sperm functions and result in loss of motility, viability, fertilising ability, deterioration of acrosomal and plasma membrane integrity and structural damage to DNA (Watson, 1995; Aitken *et al.*, 1998; Bailey *et al.*, 2000; Vishwanath & Shannon, 2000; Medeiros *et al.*, 2002).

Oxidative stress is known as one of the major causes of adverse effects on sperm physiology, through the induction of peroxidation in the sperm plasma membrane. The detrimental effect of oxidative stress on spermatozoa is known to arise from the generation of reactive oxygen species (ROS) (Aitken & Fisher, 1994; Sharma & Agarwal, 1996; Agarwal *et al.*, 2008). ROS are physiologically generated by spermatozoa. The physiological levels of ROS play a crucial role in reproductive processes such as sperm–oocyte interactions (De Lamirande *et al.*, 1997), implantation and early embryonic development (Sakkas *et al.*, 1998), sperm capacitation, acrosome reaction, maintenance of fertilising ability, and stabilisation of the mitochondrial capsule in the mid-piece in cattle (Agarwal *et al.*, 2008; Desai *et al.*, 2009; Gonçalves *et al.*, 2010).

However, an imbalance between ROS generation and scavenging activity is known as oxidative stress (Agarwal *et al.*, 2003), which triggers the loss of membrane integrity, enzyme inactivation, DNA damage and cell death and finally links with infertility (Halliwell, 1994; Sharma & Agarwal, 1996).

Reactive oxygen species cause DNA base oxidation (Dizdaroglu *et al.*, 1991; Jaruga *et al.*, 1994) and DNA strand breaks (Hughes *et al.*, 1996; Twigg *et al.*, 1998a). The transmission of genetic information to future generations depends on sperm DNA integrity. DNA integrity has become an important indicator of sperm fertility (Hughes *et al.*, 1999). Excessive ROS generation has been associated with impaired DNA integrity as well as other impaired sperm quality parameters, which in return decrease the capability of the spermatozoa to fuse with the oocyte and thereby result in infertility (Aitken *et al.*, 1991; Sharma & Agarwal, 1996; Potts *et al.*, 2000). Spermatozoa contain various antioxidants associated with ROS scavenging, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Geva *et al.*, 1996; Garrido *et al.*, 2004). GPx is a selenium-containing antioxidative enzyme, which plays a major role in scavenging excessive free radicals against lipid peroxidation attack and acts directly as an antioxidant. Owing to its vital role in sperm maturation, the lack of GPx may reduce fertilising capacity (De Lamirande & Gagnon, 1993; Vernet *et al.*, 1997). Another ROS scavenger found in seminal plasma is SOD, which is derived primarily from the prostate gland and helps prevent oxidative damage to spermatozoa (Ball *et al.*, 2000; Baumber & Ball, 2005). Malondialdehyde (MDA) is an indicator of oxidative stress and end product of lipid peroxidation. High levels of MDA cause detrimental damage to sperm functions (Geva *et al.*, 1996). As demonstrated in recent studies, the addition of various antioxidants to the semen extender protects spermatozoa against the detrimental effects of ROS and improves post-thaw sperm functions such as motility, viability and fertility (Bilodeau *et al.*, 2001; Pena *et al.*, 2004; Sariozkan *et al.*, 2009; Bucak *et al.*, 2010a).

Cysteamine belongs to a large class of antioxidants known as thiol compounds. It increases the synthesis of both glutathione and other potent antioxidant enzymes in the embryo. It has been demonstrated that the addition of cysteamine to the *in vitro* maturation medium improves embryonic development and quality (De Matos *et al.*, 1995) and increases the intracellular GSH content of bovine oocytes (De Matos *et al.*, 1995, 2002). Generally, cysteamine doses ranging from 0.5 to 20 mM have been tested in research on semen freezing extenders and *in vitro* culture media (Balasubramanian and Rho, 2007; Bucak *et al.*, 2007; Lojkic *et al.*, 2012).

It was shown that cysteamine at a dose of 10 mM increased the number of blastocyst-stage embryos (Balasubramanian & Rhob, 2007; Lojkic *et al.*, 2012). Additionally, cysteamine at a dose of 5 mM preserved post-thaw motility at higher percentages and increased vitamin E levels after cryopreservation in ram semen (Bucak *et al.*, 2007). Hyaluronan, a nonsulphated glycosaminoglycan, is an essential component of the extracellular matrix (Erlinger, 1995). It mediates sperm functions such as sperm motility (Vines *et al.*, 2001; Ghosh *et al.*, 2002) and capacitation (Suzuki *et al.*, 2002). It has a stabilising effect on decreased motility in thawed semen (Sbracia *et al.*, 1997). Pena *et al.* (2004) successfully used hyaluronan doses of 0.5–1 mg ml^{-1} for boar semen cryopreservation, and Uysal *et al.* (2007) used a dose of 1 mg ml^{-1} in bull semen as a freezing extender. Fetuin is a major glycoprotein component of foetal calf serum (FCS) and a protease inhibitor, and has been shown to inhibit zona pellucida hardening during the *in vitro* maturation of mouse (Schroeder *et al.*, 1990) and equine oocytes (Dell'Aquila *et al.*, 1999). Similar to BSA, fetuin is a commercially available protein, which improves sperm motility. It was reported that at a dose of 3 mg ml^{-1}, fetuin had no detectable effect on sperm motility, but induced forward motility at a dose of 80 mg ml^{-1} (Jaiswal *et al.*, 2010).

The aim of this study was to compare the effectiveness of the different doses of three antioxidants including cysteamine, hyaluronan and fetuin in the freezing of Brown Swiss bull semen, and to determine the effect of these additives on sperm parameters, motility characteristics, MDA levels and SOD and CAT activities following the freeze-thawing process.

Materials and methods

Chemicals

The antioxidants (fetuin, cysteamine and hyaluronan) and other chemicals used in this study were obtained from Sigma–Aldrich Chemical Co. (Interlab Ltd., Ankara, Turkey).

Animals and semen collection

Ejaculates from three Brown Swiss bulls (3 and 4 years of age) were used in the study. The bulls, belonging to the Lalahan Livestock Central Research Institute (Ankara, Turkey), were maintained under uniform feeding and housing conditions. A total number of 45 ejaculates (15 ejaculates for each bull) were collected from the bulls with the aid of an artificial vagina twice a week, according to AI standard procedures. However, 36 ejaculates (12 ejaculates for each bull) containing spermatozoa with

>80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa ml^{-1} were used. Immediately after collection, the ejaculates were immersed in a warm water bath at 34 °C until their assessment in the laboratory.

Semen processing

The volume of the 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 Tris-based extender (Trizma base 254 mM, citric acid monohydrate 78 mM, D (-) fructose 70 mM, egg yolk 15% (v/v), glycerol 6% (v/v), pH 6.8) was used as the base extender (freezing extender). Each ejaculate was split into seven equal parts and diluted to a final concentration of 60×10^6 ml^{-1} spermatozoa with a Tris-based extender containing cysteamine (2.5 and 7.5 mM), hyaluronan (0.25 and 1 mg ml^{-1}), fetuin (5 and 10 mg ml^{-1}) and no additive (control). The cysteamine (Balasubramanian & Rhob, 2007; Bucak *et al.*, 2007; Lojkic *et al.*, 2012), hyaluronan (Pena *et al.*, 2004; Uysal *et al.*, 2007) and fetuin (Jaiswal *et al.*, 2010) doses tested in this study were determined according to previous research. Diluted semen samples were loaded into 0.25-ml French straws, cooled down to 4 °C in 2 h and frozen at a programmed rate of −3 °C min^{-1} from +4 to −10 °C; −40 °C min^{-1} from −10 to −100 °C; −20 °C min^{-1} from −100 to −140 °C in a digital freezing machine (Digitcool 5300 ZB 250; IMV). Thereafter, the straws were plunged into liquid nitrogen. After at least 24 h, the frozen straws were thawed in a 37 °C water bath for 20 s immediately before use.

Evaluation of sperm parameters

Analysis of subjective and CASA motilities

Subjective 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 onto 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. In addition to estimating subjective sperm motility, a computer-assisted sperm motility analysis (CASA, version 12 IVOS; Hamilton-Thorne Biosciences, Beverly, MA, USA) was also performed to analyse sperm motility characteristics. CASA was set up as follows: phase contrast; frame rate – 60 Hz; minimum contrast – 70; low and high static size gates – 0.6 to 4.32; low and high intensity gates – 0.20 to 1.92; low and high elongation gates 7 to 91; default cell size – 10 pixels; default cell intensity – 80. Thawed semen was diluted (5 μl semen +95 μl extender) in a Tris-based extender (without egg yolk and glycerol) and evaluated immediately after dilution. A 4-μL sample of diluted semen was put onto a pre-warmed chamber slide (Leja 4, Leja Products, Luzernestraat B.V., Holland, the Netherlands), and sperm motility characteristics were determined with a 10× objective at 37 °C. The following motility values were recorded: motility (%), progressive motility (%), total sperm motility, VAP (average path velocity, μm s^{-1}), VSL (straight linear velocity, μm s^{-1}), VCL (curvilinear velocity, μm s^{-1}), ALH (amplitude of lateral head displacement, μm), STR (straightness, %) and LIN (linearity index, %). For each evaluation, 10 microscopic fields including at least 300 cells were analysed.

Assessment of sperm abnormalities

For the assessment of sperm abnormalities, of each sample, at least three drops were added into 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 & Holzmann, 2000). One drop of this mixture was put onto a slide and covered with a cover slip. The percentages of sperm acrosome and total abnormalities were determined by counting a total of 200 spermatozoa by phase-contrast microscopy (magnification 1000×, oil immersion).

Hypo-osmotic swelling test

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails. This was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution (9 g fructose + 4.9 g sodium citrate per litre of distilled water) 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 spermatozoa were evaluated (magnification 1000×) with bright-field microscopy. Spermatozoa with swollen or coiled tails were recorded (Revell & Mrode, 1994; Buckett *et al.*, 1997).

Assessment of sperm DNA damage

Diluted semen samples were centrifuged at 300 ***g*** for 10 min at 4 °C. The seminal plasma was discarded, and the remaining sperm cells were washed with (Ca^{2+} and Mg^{2+} free) PBS to yield a concentration of 1×10^5 spermatozoa cm^{-3} (Arabi, 2005).

Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which is generally performed under high alkaline conditions. Firstly, each microscope slide was pre-coated with a layer of 1% normal melting point agarose in PBS and dried at room temperature. Next, 100 μl of 0.7% low melting point agarose at 37 °C was mixed with 10 μl of the cell suspension and poured on top of the first layer. Slides were allowed to solidify for 5 min at 4 °C in a moist box. The cover slips were removed, and the slides were immersed in freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100, 10% DMSO and 40 mM dithiothreitol (pH 10) for 1 h at 4 °C. Then, the slides were incubated overnight at 37 °C in 100 μg ml^{-1} proteinase K (Sigma-Aldrich Co., St. Louis, MO, USA) and placed in lysis buffer. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, containing 300 mM NaOH and 1 mM EDTA, (pH 13), for 20 min to allow the DNA to unwind. Electrophoresis was performed for 20 min at 8 °C at 12 V and was adjusted to 250 mA. Subsequently, the slides were washed with a neutralising solution of 0.4 M Tris, pH (7.5), to remove the alkali and detergents. After neutralisation, the slides were stained with 50 μl of 2 μl ml^{-1} ethidium bromide and covered with a cover slip. All steps were performed under dim light to prevent further DNA damage (Haines *et al.*, 1998; Singh & Stephens, 1998). The images of 50 randomly chosen nuclei were analysed by CASP (critical assessment of protein structure prediction) (Labbe *et al.*, 2001).

Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, BX51, Tokyo, Japan). Damage was detected by a tail of fragmented DNA that migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were not considered to be damaged.

Biochemical assays

Superoxide dismutase, GPx activities and MDA levels were measured in the samples on a UV-VIS Recording Spectrophotometer (UV-2100S; Shimadzu Co., Kyoto, Japan).

Superoxide dismutase activity was measured using the method described by Fitzgerald *et al.* (1992). Briefly, 50-μl samples were mixed with 825 μl of substrate solution containing 0.05 mM xanthine sodium and 0.025 mM 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) in a buffer solution containing 50 mM CAPS (3-(cyclohexylaminol)-1-propanesulphonic acid) and 0.094 mM EDTA (pH 10.2). One hundred and twenty-five micro litre xanthine oxidase (80 U l^{-1}) was added to the mixture, and the increase in absorbance was followed at 505 nm for 3 min. SOD activity was expressed as U ml^{-1}.

Glutathione peroxidase activity was measured using the method described by Pleban *et al.* (1982). Briefly, a reaction mixture containing 1 mM Na2EDTA, 2 mM reduced glutathione, 0.2 mM NADPH, 4 mM sodium azide and 1000 U glutathione reductase in 50 mM TRIS buffer (pH 7.6) was prepared. Twenty micro litre of the samples and 980 μl of the reaction mixture were mixed and incubated for 5 min at 37 °C. The reaction was initiated by adding 8.8 mM of hydrogen peroxide, and the decrease in absorbance was recorded at 340 nm for 3 min. GPx activity was expressed as U ml^{-1}.

Malondialdehyde level was estimated by the measurement of thiobarbituric acid-reactive substances (TBARS) in the samples by the method described by Richard *et al.* (1991). After the reaction of MDA with thiobarbituric acid, the reaction product was followed spectrophotometrically at 532 nm, using tetramethoxypropane as a standard. The results were expressed as nmol ml^{-1}.

Statistical analysis

The study was replicated six times. Results were expressed as the mean ± SEM. Sperm motility, motility characteristics, abnormalities and biochemical data were analysed by analysis of variance, followed by Tukey's *post hoc* test to determine significant differences between the groups. The groups were compared for DNA damage, using analysis of variance and Tamhane's T2 multiple-range test as a *post hoc* test. Differences with values of $P < 0.05$ were considered to be statistically significant. Statistical analyses were performed using the SPSS 11.5 package program (SPSS Inc., Chicago, IL, USA).

Results

Sperm parameters (percentages of motility, sperm motility characteristics, acrosome and total abnormalities, plasma membrane integrity and DNA damage)

The effects of cysteamine, hyaluronan and fetuin on the sperm motility and motility characteristics and morphological abnormalities, membrane integrity and damaged DNA of frozen-thawed bull semen are presented in Tables 1 and 2. The samples cryopreserved in supplemented freezing extenders had higher percentages of CASA motility and CASA total motility compared with the control group ($P < 0.001$). However, the best percentages of CASA motility and CASA total motility were achieved with 10 mg ml^{-1} of fetuin (52.6 ± 2.9% and

Table 1 Mean (±SE) sperm motility and motion characteristics in semen supplemented with different antioxidants of Brown-Swiss bovine semen following freeze–thawing

Groups	CASA motility (%)	CASA progressive motility (%)	CASA total motility (%)	VAP (μm s^{-1})	VSL (μm s^{-1})	VCL (μm s^{-1})	ALH (μm)	STR (%)	LIN (%)
Control	30.0 ± 1.8^{d}	12.1 ± 1.2^{c}	39.1 ± 2.2^{e}	121.7 ± 2.4^{a}	85.4 ± 1.5	214.0 ± 9.0^{a}	7.8 ± 0.5^{a}	70.3 ± 1.2^{de}	44.0 ± 1.7^{bc}
Hyaluronan 0.25 mg ml^{-1}	42.1 ± 1.8^{b}	13.7 ± 2.3^{bc}	55.4 ± 1.4^{cd}	119.2 ± 5.5^{a}	87.3 ± 4.4	204.0 ± 11.8^{a}	7.7 ± 0.4^{a}	73.9 ± 1.6^{bcd}	46.3 ± 1.6^{bc}
Hyaluronan 1 mg ml^{-1}	40.1 ± 2.6^{b}	19.6 ± 1.6^{a}	52.7 ± 2.8^{d}	100.2 ± 6.5^{b}	78.8 ± 3.0	160.7 ± 15.4^{b}	6.3 ± 0.5^{b}	78.4 ± 1.6^{a}	51.1 ± 1.8^{a}
Fetuin 5 mg ml^{-1}	38.1 ± 2.3^{c}	14.6 ± 1.4^{abc}	51.6 ± 1.9^{d}	123.5 ± 5.7^{a}	84.3 ± 4.1	220.7 ± 10.5^{a}	7.9 ± 0.4^{a}	68.7 ± 0.8^{e}	41.6 ± 1.0^{c}
Fetuin 10 mg ml^{-1}	52.6 ± 2.9^{a}	14.0 ± 1.4^{bc}	64.3 ± 2.4^{ab}	124.3 ± 2.2^{a}	86.5 ± 2.7	223.5 ± 4.7^{a}	8.3 ± 0.3^{a}	71.7 ± 0.9^{cde}	43.3 ± 0.7^{c}
Cysteamine 2,5 mM	55.3 ± 2.2^{a}	17.4 ± 1.9^{ab}	69.1 ± 1.8^{a}	117.3 ± 3.6^{a}	86.6 ± 2.4	200.8 ± 9.6^{a}	7.8 ± 0.5^{a}	76.4 ± 1.9^{ab}	49.0 ± 2.3^{ab}
Cysteamine 7,5 mM	46.0 ± 2.1^{b}	18.1 ± 1.6^{ab}	59.6 ± 1.7^{bc}	114.1 ± 1.5^{a}	85.7 ± 2.3	210.9 ± 4.2^{a}	8.7 ± 0.3^{a}	75.1 ± 1.7^{abc}	43.9 ± 2.0^{bc}
P	***	*	***	**	–	***	*	***	***

'*n* = 6' refers to six replications.
Different superscripts within the same column demonstrate significant differences (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).
–, no significant difference ($P > 0.05$).

64.3 ± 2.4%) and 2.5 mM of cysteamine (55.3 ± 2.2% and 69.1 ± 1.8%). The addition of 1 mg ml^{-1} of hyaluronan (19.6 ± 1.6%) and 2.5 and 7.5 mM of cysteamine (17.4 ± 1.9% and 18.1 ± 1.6%) provided a greater post-thaw progressive motility in comparison with the control group and the other experimental groups ($P < 0.001$). For VAP, VCL and ALH, the lowest values (100.2 ± 6.5%, 160.7 ± 15.4% and 6.3 ± 0.5%) were obtained with 1 mg ml^{-1} of hyaluronan. For STR and LIN, the highest values (78.4 ± 1.6% and 51.1 ± 1.8%) were recorded for 1 mg ml^{-1} of hyaluronan ($P < 0.001$). No significant differences were observed among the groups for VSL values ($P > 0.05$). In terms of sperm acrosome and total abnormalities, 10 mg ml^{-1} of fetuin (4.3 ± 0.4% and 11.7 ± 0.6%) and 2.5 mM of cysteamine (4.9 ± 0.3% and 13.0 ± 0.7%) produced better protection levels ($P < 0.001$). The results of HOST showed that the percentage values of membrane integrity in all the groups, excluding that supplemented with 5 mg ml^{-1} of fetuin, were higher than that of the control group ($P < 0.001$). As regards the DNA damage of sperm cells, 0.25 mg ml^{-1} of hyaluronan (2.39 ± 0.21%), and 2.5 and 7.5 mM of cysteamine (2.98 ± 0.20% and 3.07 ± 0.24%) led to lower concentrations of spermatozoa with damaged DNA than that of the control group (4.88 ± 0.48%). This reduction in DNA damage was found to be statistically significant ($P < 0.001$).

MDA levels, SOD and GPX activities

The influence of antioxidants on MDA levels and GPX and SOD activities in thawed bull spermatozoa are set out in Table 3. Supplementation with antioxidants significantly affected SOD and GPX activities in comparison with the control group ($P < 0.001$). The maintenance of SOD and GPX antioxidant activities in the groups supplemented with 2.5 and 7.5 mM of cysteamine (27.60 ± 5.74 U ml^{-1} and 30.40 ± 4.16 U ml^{-1}, 3.45 ± 0.65 U ml^{-1} and 3.80 ± 0.52 U ml^{-1}, respectively) and 10 mg ml^{-1} of fetuin (12.42 ± 0.24 U ml^{-1} and 1.56 ± 0.02 U ml^{-1}, respectively) was demonstrated to be higher than in the control group, following the freeze-thawing process ($P < 0.001$). Malondialdehyde formation was found to be at a lower level in the groups supplemented with 0.25 mg ml^{-1} of hyaluronan (0.57 ± 0.02 nmol ml^{-1}) and 7.5 mM of cysteamine (0.21 ± 0.01 nmol ml^{-1}), compared with the other experimental groups, after the freeze-thawing process ($P < 0.001$).

Discussion

The successful cryopreservation of semen provides a high conception rate and economical benefits via artificial

Table 2 Mean (±SE) sperm abnormalities, membrane integrity and DNA damage in semen supplemented with different antioxidants of Brown-Swiss bovine semen following freeze–thawing

Groups	Acrosomal abnormality (%)	Total abnormalities (%)	Membrane integrity (%)	Damaged DNA (%)
Control	11.7 ± 0.9^{a}	23.1 ± 0.9^{a}	53.0 ± 0.7^{b}	4.88 ± 0.48^{c}
Hyaluronan 0.25 mg ml^{-1}	10.4 ± 0.5^{ab}	21.6 ± 1.3^{ab}	63.7 ± 1.1^{a}	2.39 ± 0.21^{b}
Hyaluronan 1 mg ml^{-1}	9.6 ± 0.6^{bc}	22.7 ± 0.9^{a}	62.1 ± 1.7^{a}	3.47 ± 0.24^{ac}
Fetuin 5 mg ml^{-1}	8.6 ± 0.6^{c}	19.1 ± 1.3^{b}	56.4 ± 1.4^{b}	3.59 ± 0.25^{ac}
Fetuin10 mg ml^{-1}	4.3 ± 0.4^{d}	11.7 ± 0.6^{c}	62.4 ± 2.1^{a}	3.43 ± 0.27^{ac}
Cysteamine 2.5 mM	4.9 ± 0.3^{d}	13.0 ± 0.7^{c}	63.0 ± 1.5^{a}	2.98 ± 0.20^{ab}
Cysteamine 7.5 mM	9.6 ± 0.6^{bc}	21.3 ± 1.1^{ab}	62.6 ± 1.6^{a}	3.07 ± 0.24^{ab}
P	***	***	***	***

'*n* = 6' refers to six replications.
Different superscripts within the same column demonstrate significant differences (***$P < 0.001$).

Table 3 Mean (±SE) MDA levels, GPX and SOD antioxidant activities in semen supplemented with different antioxidants of Brown-Swiss bovine semen following freeze–thawing

Groups	SOD (U ml^{-1})	GPX (U ml^{-1})	MDA (nmol ml^{-1})
Control	2.75 ± 0.24^{c}	0.38 ± 0.03^{c}	2.31 ± 0.76^{c}
Hyaluronan 0.25 mg ml^{-1}	7.88 ± 1.03^{bc}	0.98 ± 0.13^{bc}	0.57 ± 0.02^{d}
Hyaluronan 1 mg ml^{-1}	4.70 ± 0.41^{bc}	0.61 ± 0.04^{bc}	1.92 ± 0.78^{c}
Fetuin 5 mg ml^{-1}	8.13 ± 0.20^{bc}	1.02 ± 0.03^{bc}	2.30 ± 0.03^{c}
Fetuin 10 mg ml^{-1}	12.42 ± 0.24^{b}	1.56 ± 0.02^{b}	7.26 ± 0.30^{a}
Cysteamine 2.5 mM	27.60 ± 5.74^{a}	3.45 ± 0.65^{a}	3.81 ± 0.12^{b}
Cysteamine 7.5 mM	30.40 ± 4.16^{a}	3.80 ± 0.52^{a}	0.21 ± 0.01^{d}
P	***	***	***

'*n* = 6' refers to six replications.
Different superscripts within the same column demonstrate significant differences (***$P < 0.001$).

insemination. Various factors including temperature changes, ice formation, oxidative stress, membrane disintegrity and damage to the acrosome and DNA reduce post-thaw semen fertility (Halliwell, 1994; Sharma & Agarwal, 1996; Thun *et al.*, 2002).

Reactive oxygen species appear to play several roles in the maintenance and disruption of spermatozoa physiology. They can also damage many cell components and induce the oxidation of cell membrane lipids, amino acids in proteins and DNA (Agarwal *et al.*, 2004). Sperm membranes are vulnerable to ROS-induced damage due to their high content of polyunsaturated fatty acids (Aurich *et al.*, 1997; Storey, 1997). Oxidative attack to sperm phospholipid-bound polyunsaturated fatty acids induces changes in membrane fluidity and integrity that impair the fertilising potential of these cells (Jones *et al.*, 1979; Aitken & Fisher, 1994). The unfavourable effects of this process can be prevented by the use of antioxidants (Sikka, 2004).

Cysteamine, a low-molecular-weight thiol compound, reacts with cystine by sulphhydryl–disulphide interchange to form a mixed disulphide (Ishii *et al.*, 1981) and enhances glutathione synthesis (Issels *et al.*, 1988; De Matos *et al.*, 2002b). Some authors reported that cysteamine supplementation of the culture medium improved blastocyst percentages (Balasubramanian & Rhob, 2007; Silva *et al.*, 2010). In the present study, the addition of 2.5 and 7.5 mM of cysteamine and 10 mg ml^{-1} of fetuin to the semen extender provided a higher increase in SOD and GPx antioxidant enzyme activities. This was in agreement with studies performed by De Matos *et al.* (1996, 2002b), who stated that the addition of cysteamine into the maturation media resulted in an increase in GSH activity. The findings of the present study demonstrated that the addition of cysteamine to the freezing extender at concentrations of 2.5–7.5 mM could provide cryoprotection of thawed bull semen against oxidative stress-induced damage, despite the measurement of higher MDA values in the group supplemented with 2.5 mM of cysteamine. Similarly, Bucak *et al.* (2007) reported that the addition of 5 mM of cysteamine led to an increase in vitamin E levels in addition to its protective effect on sperm survival. Furthermore, Sarıözkan *et al.* (2013) demonstrated that FCS had a protective role in maintaining GPx antioxidant activity and also led to a decrease in MDA levels, compared with the control group during the liquid storage of rabbit semen.

The mechanism of hyaluronan in increasing sperm motility shows that hyaluronan receptors are located in the tail, mid-piece and head of human spermatozoa (Kornovsky *et al.*, 1994). Hyaluronan supplementation may lead to an increase in phosphorylation and ATP levels, resulting in improved flagellar function and motility (Ranghanatan *et al.*, 1994, 1995; Sbracia *et al.*, 1997). The

results of the present study showed that supplementation with 1 mg ml^{-1} of hyaluronan and 2.5 and 7.5 mM of cysteamine provided a greater post-thaw progressive motility in comparison with the other groups. This observation is similar to that previously described for frozen/thawed human (Sbracia *et al.*, 1997) and mouse semen (Bakhtiari *et al.*, 2007), where hyaluronan supplementation resulted in significant increases in sperm motility and velocity movement. In contrast, Uysal *et al.* (2007) reported that hyaluronan supplementation did not provide any improvement in the post-thaw motility of bull spermatozoa. In this study, hyaluronan addition produced a significant improvement in post-thaw CASA motility. In our study, hyaluronan yielded lower VCL, VAP and ALH values in frozen/thawed bull semen. The results obtained in the present study for CASA motility are in contrast with those reported in previous research (Pena *et al.*, 2004), indicating higher VSL and VAP and lowerALH values in boar semen. The differences between the results of the present study and previous reports may be attributed to the use of different semen extenders and animal species. In the present study, all types of supplementation of the freezing extender increased CASA motility and CASA total motility, compared with the control group ($P < 0.001$). Moreover, 10 mg ml^{-1} of fetuin and 2.5 mM of cysteamine had significant positive effects on the maintenance of CASA motility and CASA total motility of post-thaw bull spermatozoa when compared to the other groups.

Fetuin is an important ingredient of FCS, which is a constituent of most media used for the culture of animal cells. FCS is composed of a variety of proteins, which maintain cultured cells in a medium (Hayman *et al.*, 1985). FCS has been widely used in the culture of cumulus–oocyte complexes, because it is believed that FCS stabilises the expanding cumulus extracellular matrix (Simon *et al.*, 1992). It has been reported that fetuin inhibits zona pellucida hardening and allows normal fertilisation rates in oocytes cultured in the absence of serum (Schroeder *et al.*, 1990; Dell'Aquila *et al.*, 1999). Fetuin addition at concentrations of 5–10 mg ml^{-1} and cysteamine supplementation at a dose of 2.5 mM significantly decreased acrosomal and total morphological damage of frozen-thawed bull semen, in comparison with the controls ($P < 0.001$). Similarly, Sarıözkan *et al.* (2013) indicated that FCS in the semen extender protected both the plasma membrane and the acrosomal integrity of chilled rabbit spermatozoa as well as sperm motility. In the present study, supplementation with 10 mg ml^{-1} of fetuin also provided for a significantly higher percentage of CASA motility, compared with the control group. Similarly, the protective effect of fetuin on motility was shown by Jaiswal *et al.* (2010). Furthermore, in the present study, all the supplementations tested, except for fetuin at 5 mg ml^{-1}, protected the functional membrane integrity of frozen/thawed bull semen.

Apoptosis induces sperm DNA damage during cryopreservation and the thawing process (Said *et al.*, 2010). However, Thomson *et al.* (2009) suggested that increased sperm DNA damage during the freezing/thawing process results principally from oxidative stress rather than apoptosis. The Comet assay is a widely applied technique for measuring and analysing DNA breakage in individual cells (Ostling & Johanson, 1984). It has also been proven as an effective technique in evaluating the capacity of antioxidants to protect genetic material integrity in biological studies (Heaton *et al.*, 2002; Novotna *et al.*, 2007; Tuncer *et al.*, 2010). Furthermore, some authors suggest that sperm DNA integrity is a more objective marker of sperm function as opposed to sperm parameters such as motility (Twigg *et al.*, 1998b; Rajesh *et al.*, 2002).

In this study, supplementation of the semen extender with 0.25 mg ml^{-1} of hyaluronan and 2.5 and 7.5 mM of cysteamine maintained DNA integrity at a statistically significant level, compared with the control group. These results were contrary to those reported in a previous study on bovine spermatozoa (Bucak *et al.*, 2010b), indicating the protection of DNA integrity. Fetuin concentrations of 5–10 mg ml^{-1} did not provide statistically higher sperm DNA integrity levels than that of the control group. In a previous report, it was suggested that supplementation with FCS provided a significant reduction in DNA damage during the liquid storage of rabbit semen, compared with the control group (Sarıözkan *et al.*, 2013).

Conclusions

The results of this study clearly suggest that all the additives used had positive effects on the maintenance of sperm motility, and the integrities of the sperm plasma membrane, morphology, and DNA, compared with the control group. Furthermore, all the supplementations tested in the present study showed strong cryoprotective effect against cryopreservation/thawing damage induced by LPO. In particular, higher SOD and GPx antioxidant activities were achieved with the addition of 2.5–7.5 mM of cysteamine. Therefore, it can be stated that the addition of antioxidants to the freezing extender could improve post-thaw sperm parameters and may be recommended to facilitate the enhancement of sperm cryopreservation techniques.

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