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Influence of fetuin and hyaluronan on the post-thaw quality and fertilizing ability of Holstein bull semen ☆

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ABSTRACT

It was determined that fetuin and hyaluronan supplementation did not provide any significant effect on the post-thaw subjective and CASA motility percentages and sperm motion characteristics, in comparison to the controls ($P > 0.05$).

Sperm acrosome and total abnormalities were similar in all groups ($P > 0.05$). Groups M (hyaluronan + fetuin) and H (hyaluronan) displayed a higher rate of sperm membrane integrity, compared to that of Group C (control) ($P < 0.01$). According to the results of the comet assay, the lowest percentage of sperm with damaged DNA was achieved in Group H, when compared to all of the experimental groups ($P < 0.01$). Furthermore, all of the additives resulted in a lower rate of sperm with damaged DNA than that of the controls, and thus, reduced DNA damage ($P < 0.01$). For pregnancy rates, there were no significant differences between the extender groups ($P > 0.05$).

MDA formation was found to be lower in Groups M and F ($P < 0.01$). In Group M, SOD activity was determined to have significantly increased (23.61 ± 5.62 U/ml) compared to the other groups ($P < 0.01$). All experimental groups had a GSH-Px activity higher than that of the control group ($P < 0.01$).

Introduction

Two major systems of sperm storage (liquid and frozen) have been developed for preservation. The preservation of sperm generally requires a reduction or arrest of the metabolism of sperm cells, and thereby, prolongs their fertile life [39,59]. Cryopreservation produces cold shock and oxidative attack on the sperm membrane, both of which decrease the survival and fertilizing ability of sperm, and thus, lead to sperm death and a negative impact on the preservation of semen for AI [27,38]. The cold shock of spermatozoa is partly associated with oxidative attack and the generation of reactive oxygen species (ROS). The ROS-induced damage of spermatozoa involves an oxidative attack on the bis-allylic methylene group of sperm membrane phospholipids, which leads to lipid peroxidation (LPO) [23,34,55,58]. On the other hand, low concentrations of ROS play a key role in the physiological control of mammalian sperm functions, including fusogenic and kinetic functions, capacitation and hyperactivation processes [4,6,10,32,37]. Mammalian semen possesses an antioxidant system comprising taurine, reduced glutathione (GSH), glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) to prevent the peroxidative damage of ROS [1,7,11,13,60]. This antioxidant capacity of spermatozoa may be insufficient to prevent LPO and maintain sperm functions during the freeze-thawing process [9,52]. The protective antioxidant systems in sperm are primarily of cytoplasmic origin and sperm cells discard most of their cytoplasm during the terminal stages of differentiation. Thus, mammalian sperm lack a significant cytoplasmic component, which contains the sufficient antioxidants to counteract the damaging

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effects of ROS and LPO. For this reason, sperm are susceptible to LPO during cryopreservation and thawing [52]. Additives with antioxidative properties may reduce this impact of ROS-induced damage caused by LPO [25]. In recent years, antioxidants such as cysteamine, trehalose and cysteine have been found to improve post-thaw sperm motility and antioxidant capacity [2,18,19,47]. Hyaluronan (which is an essential component of the extracellular matrix), a non-sulfated glycosaminoglycan and an additive with antioxidant properties, contributes to cell recognition, cellular adhesion and growth regulation. It is involved in important physiological functions such as the post-thaw motility, capacitation ability and antioxidant capacity of the sperm cell [19,26,31]. Fetuin is both 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 [50] and equine oocytes [24]. Similar to bovine serum albumin (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} [35]. The aim of this study was to investigate the effects of hyaluronan and fetuin on the post-thaw parameters and fertilizing ability of bull sperm.

Materials and methods

Chemicals

The antioxidants (fetuin and hyaluronan) and chemicals used in this study were obtained from a local supplier of Sigma–Aldrich Chemical Co. (Ankara, Turkey).

Animals and semen collection

The bulls (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 36 ejaculates (12 ejaculates per animal) were collected from the animals with the aid of an artificial vagina twice a week. Only ejaculates containing spermatozoa with >80% forward progressive motility and concentrations higher than 1.0×10^9 spermatozoa/ml were included. 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 ejaculates was measured in a conical tube graduated at 0.1 ml intervals and sperm concentration was determined by using an Accucell photometer (IMV, L'Aigle, France). Sperm motility was estimated using phase-contrast microscopy (200×). Tissue culture medium (TCM-199), containing 15% (v/v) of egg yolk and 6% (v/v) of glycerol (pH 6.8), was used as the base extender (freezing extender). Prior to freezing, each ejaculate was split into four equal experimental groups and diluted to a final concentration of 60×10^6/ml spermatozoa with base extenders containing hyaluronan (H, 500 μg/ml), fetuin (F, 2.5 mg ml^{-1}), a combination of hyaluronan and fetuin (M), and no additive (C). The 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 from +4 to −10 °C; −40 °C/min from −10 to −100 °C; and −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. The trial was replicated nine times. After a minimum of 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.

A computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used to analyse sperm motion characteristics. The 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) with a TCM-199 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) 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), VSL (straight linear velocity, μm/s), VCL (curvilinear velocity, μm/s), ALH (amplitude of lateral head displacement, μm), and LIN (linearity index, %). For each evaluation, 10 microscopic fields were analyzed to include at least 300 cells.

Assessment of sperm abnormalities

For the assessment of sperm abnormalities, at least three drops of each sample were added into Eppendorf tubes containing 1 ml of Hancock solution (62.5 ml of formalin (37%), 150 ml of sodium saline solution, 150 ml of buffer solution and 500 ml of double-distilled water) [49]. 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 under a phase-contrast microscope (magnification 1000×, oil immersion).

Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, based on coiled and swollen tails. This was performed by incubating 30 μl of semen with 300 μl of a 100 mOsm hypo-osmotic solution (9 g of fructose + 4.9 g of sodium citrate per liter 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 sperm were evaluated (magnification 1000×) with bright-field microscopy. Sperm with swollen or coiled tails were recorded [22,45].

Assessment of sperm DNA damage

Diluted semen samples were centrifuged at 300*g* for 10 min at 4 °C. The seminal plasma was removed and the remaining sperm cells were washed with phosphate buffer solution (PBS) to yield a concentration of 1×10^5 spermatozoa/cm^3 [8].

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 thoroughly 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. The 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 a freshly prepared cold lysis buffer containing 2.5 M NaCl, 100 mM Na_2–EDTA, 10 mM Tris, 1% Triton X-100, 10% dimethyl sulfoxide (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 proteinase K (Sigma) and placed into the lysis buffer. The slides were removed from the lysis buffer, drained and placed into 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 neutralizing solution of 0.4 M Tris, pH (7.5), in order to remove the alkali and detergents. After neutralization, the slides were stained with 50 μl of 2 μl/ml ethidium bromide and covered with a cover slip. All steps were performed under dim light to prevent further DNA damage [33,51].

The images of 50 randomly chosen nuclei were analyzed by CASP. Observations were made at a magnification of 400× using a fluorescent microscope (Olympus, BX51, Japan). Damage was detected on the basis of the presence of a tail of fragmented DNA, which had migrated from the sperm head, causing a 'comet' pattern, whereas whole sperm heads, without a comet, were not considered to be damaged.

Biochemical parameters

Biochemical assays were performed on the samples immediately after they were thawed. An aliquot (0.5 ml) of semen from each sample was centrifuged at 800*g* for 10 min at 4 °C in order to separate the cells from the diluted seminal plasma. Afterwards, the sperm pellet was separated and washed by means of resuspension in PBS and recentrifugation at 800*g* for 20 min at 4 °C (three times). After the last centrifugation, 1 ml of deionized water was added to the sperm pellet for the biochemical analyses. The SOD and GSH-Px activities and malondialdehyde (MDA) levels of the samples were measured by using an UV–VIS recording spectrophotometer (UV-2100S, Shimadzu Co., Kyoto, Japan).

SOD activity was measured as described by Fitzgerald [28]. Briefly, 50 μl-samples were mixed with 825 μl of substrate solution containing 0.05 mmol/L xanthine sodium and 0.025 mmol/L 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) in a buffer solution containing 50 mmol/L CAPS (3-(cyclohex ylaminol)-1-propanesulfonic acid) and 0.094 mmol/L EDTA (pH 10.2). 125 μl of xanthine oxidase (80 U/L) was added to the mixture and the increase of absorbance was recorded at 505 nm for 3 min. SOD activity is expressed in U/ml.

GSH-Px activity was measured as described by Pleban [43]. Briefly, a reaction mixture containing 1 mmol/L Na_2EDTA, 2 mmol/L reduced glutathione, 0.2 mmol/L NADPH, 4 mmol/L sodium azide and 1000 U glutathione reductase in 50 mmol/L TRIS buffer (pH 7.6) was prepared. 20 μl 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 mmol/L hydrogen peroxide and the decrease of absorbance was recorded at 340 nm for 3 min. GSH-Px activity is expressed in U/ml.

MDA levels were estimated by the measurement of thiobarbituric acid reactive substances (TBARS) in the samples, as described by Richard [46]. After the reaction of MDA with thiobarbituric acid, the reaction product was monitored spectrophotometrically at 532 nm, using tetramethoxypropane as a standard. The results are expressed in nmol/ml.

Fertility trials

The fertilizing ability of spermatozoa was calculated on the basis of the non-return rates at 59 days. A total of 172 cows were artificially inseminated with frozen samples preserved 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.

Statistical analysis

Results were expressed as mean ± SEM. Sperm motility, motion characteristics, abnormalities and biochemical data were analyzed by analysis of variance, followed by Tukey's post hoc test to determine the 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. The groups were compared for pregnancy rate, using Pearson's chi-square test. Differences with values of $P < 0.05$ were considered to be statistically significant. Statistical analyses were performed by using the SPSS 11.5 software package.

Results

It was determined that fetuin and hyaluronan supplementation did not provide any significant effect on the post-thaw subjective and CASA motility percentages and sperm motion characteristics, in comparison to the controls (Table 1, $P > 0.05$).

Sperm acrosome and total abnormalities were similar in all groups ($P > 0.05$). Groups M and H displayed a higher rate of sperm membrane integrity, compared with that of Group C (Table 2, $P < 0.01$). According to the results of the comet assay, the lowest percentage of sperm with damaged DNA was achieved in Group H, compared to all of the experimental groups ($P < 0.01$). Furthermore, all of the additives resulted in lower percentages of sperm with damaged DNA than that of the controls, and thus, reduced DNA damage (Table 2, $P < 0.01$). For pregnancy rates, there were no significant differences between the extender groups (Table 2, $P > 0.05$).

Analysis of oxidative stress parameters

The influence of the antioxidants on MDA levels and GSH-Px and SOD activities in thawed bull sperm are set out in Table 3. MDA formation was found to be lower in Groups M and F (Table 3, $P < 0.01$). In Group M, SOD activity was determined to have significantly increased (23.61 ± 5.62 U/ml), compared to the other groups (Table 3, $P < 0.01$). All experimental groups had a GSH-Px activity higher than that of the control group ($P < 0.01$).

Discussion

The plasma membrane of mammalian spermatozoa is particularly rich in polyunsaturated fatty acids (PUFA). This predominance of PUFA renders spermatozoa highly susceptible to lipid peroxidation due to attacks from ROS. These attacks ultimately lead to the impairment of sperm functions through oxidative stress and the production of cytotoxic aldehydes such as MDA [3,7,10]. In this study, fetuin and a combination of fetuin and hyaluronan were shown to prevent MDA formation. However, hyaluronan was determined not to play a role in the prevention of MDA formation, as previously reported in a study carried out on ram semen [19].

Oxidative damage may be prevented by the inclusion of antioxidants in the semen extender prior to storage [18,20,21]. This study

Table 1
Mean (±S.E.) bovine sperm motility and motion characteristics in semen supplemented with additives following freeze-thawing.

Groups	Subjective motility (%)	Progressive motility (%)	Total motility (%)	VAP (μm/s)	VSL (μm/s)	VCL (μm/s)	ALH (μm)	LIN (%)
Control	48.00 ± 2.77	13.00 ± 1.26	30.00 ± 1.73	112.98 ± 2.53	105.84 ± 2.35	153.60 ± 4.58	4.46 ± 0.10	71.40 ± 0.75
Fetuin 2.5 mg ml^{-1}	51.00 ± 4.16	9.60 ± 2.38	34.25 ± 6.52	108.70 ± 4.09	103.24 ± 4.26	146.96 ± 7.39	4.44 ± 0.27	72.00 ± 1.52
Hyaluronan 500 μg/ml	45.00 ± 1.83	10.80 ± 0.58	23.25 ± 1.44	110.80 ± 2.82	103.82 ± 2.73	149.50 ± 6.44	4.36 ± 0.27	71.60 ± 1.69
Mix	46.00 ± 2.38	6.80 ± 1.66	26.00 ± 2.35	102.38 ± 5.43	95.60 ± 5.46	138.60 ± 8.59	4.30 ± 0.27	71.00 ± 1.05
P	–	–	–	–	–	–	–	–

–: The same column shows no significant differences among proportions ($P > 0.05$).

Table 2
Mean (±S.E.) sperm abnormalities, membrane integrity, DNA damage and fertility results in semen supplemented with additives following freeze-thawing.

Groups	Acrosomal abnormality (%)	Total abnormality (%)	Plasma membrane integrity (%)	Damaged DNA (%)	Non-return rate (%) Non-returned cows/Total inseminated cows)
Control	8.20 ± 0.58	14.60 ± 1.17	43.40 ± 2.25^{c}	3.81 ± 0.22^{c}	60 (18/30)
Fetuin 2.5 mg ml^{-1}	7.00 ± 0.71	12.20 ± 0.86	48.00 ± 2.00^{bc}	2.14 ± 0.10^{a}	55 (26/47)
Hyaluronan 500 μg/ml	8.00 ± 0.71	13.20 ± 0.67	50.00 ± 2.74^{b}	1.47 ± 0.08^{b}	51 (23/45)
Mix	6.00 ± 0.45	12.00 ± 0.71	56.60 ± 0.93^{a}	1.94 ± 0.15^{a}	58 (29/50)
P	–	–	**	**	–

–: The same column shows no significant differences among proportions ($P > 0.05$).
^{a,b,c}Different superscripts within the same column demonstrate significant differences ($^{**}P < 0.01$).

Table 3
Mean (±S.E.) malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX) levels in semen supplemented additives following freeze-thawing.

Groups	MDA (nmol/ml)	SOD (U/ml)	GPX (U/ml)
Control	1.33 ± 0.04^{a}	4.85 ± 1.20^{b}	1.79 ± 0.13^{b}
Fetuin 2.5 mg ml^{-1}	1.06 ± 0.02^{b}	11.62 ± 2.02^{b}	2.23 ± 0.08^{a}
Hyaluronan 500 μg/ml	1.28 ± 0.05^{a}	10.32 ± 1.03^{b}	2.26 ± 0.06^{a}
Mix	0.91 ± 0.08^{b}	23.61 ± 5.62^{a}	2.51 ± 0.13^{a}
P	**	**	**

^{a,b}Different superscripts within the same column demonstrate significant differences ($^{**}P < 0.01$).

was undertaken to ascertain which antioxidant would provide the most effective protection against membrane damage during the freeze-thawing process. Sperm motility per se becomes crucial in facilitating the passage through the cervix and uterotubal junction. Motility is also important for the actual penetration of the cumulus cells and zona pellucida of the ovum [33]. In the present study, the addition of hyaluronan to the freezing extender did not cause any further improvement in sperm motility, or acrosome and total abnormalities. These data were similar to those reported for ram sperm by Najafi et al. [40], Bucak et al. [15] and Bucak et al. [19]. In the present study, additives provided the elimination of MDA, but they did not improve sperm motility or fertility.

The analysis of motility does not provide enough data for the evaluation of post-thaw sperm quality. Sperm morphology is of major importance in both the fertilizing capacity and the assessment of membrane functions. Highly motile spermatozoa may have damaged structure and functions. On the contrary, highly nonmotile cells may have an intact plasmalemma and acrosomal and morphological integrity [56,57]. The acrosome contains the enzymes required for the penetration of the spermatozoon through the ovum layers and zona pellucida during fertilization [33]. With respect to fertility results based on pregnancy rates, antioxidants do not seem to be an influential factor on the post-thaw fertilizing ability of sperm. This result is in agreement with those reported in previous studies demonstrating no improvement in fertility when certain additives were added to the freezing extender [16,48,53]. The extension and freeze-thawing of semen lead to low antioxidant activity in both the presence and absence of antioxidants. This may partly explain the lower fertility of frozen–thawed semen in comparison to fresh semen [12,13]. The current results are contradictory to those obtained in a previous study carried out in bulls [48], where sperm parameters were determined to have improved when semen was frozen with fetuin. The axosome and associated dense fibers of the middle pieces of sperm cells are covered by mitochondria, which generate energy from intracellular ATP stores. These are responsible for sperm motility [30]. Based on our results, we can hypothesize that hyaluronan and fetuin do not show any cryoprotective effect on the functional integrity of the axosome and mitochondria, and therefore, do not improve post-thaw sperm motility and abnormalities.

The HOST test assessed the resistance of the sperm plasma membrane to damage induced by the loss in permeability under the stress of swelling driven by the hypo-osmotic treatment. Thus, this provided a form of a membrane stress-test, which is particularly useful when testing the membrane-stabilising action of antioxidants [17]. In this study, the highest HOST rates were obtained when sperm samples were cryopreserved with hyaluronan and a combination of hyaluronan and fetuin. However, the findings obtained for membrane integrity in the case of cryopreservation with hyaluronan, which were found to be similiar to the results of Bucak et al. [21], are contradictory to those reported by Bucak et al. [19], who demonstrated no improvement in the post-thaw motility of ram semen. These contradictory results may be due to differences in the semen extender composition, animal species and hyaluronan concentrations used in these studies. The comet assay is a widely applied technique for measuring and analyzing DNA breakage in individual cells [42]. In several biological studies, it has also been proven to be a valid technique in evaluating the ability of antioxidants to protect the integrity of the genetic material [36,41,53]. Furthermore, some authors suggest that sperm DNA integrity is a more objective marker of sperm function as opposed to sperm parameters such as motility [44,54]. In this study, the antioxidants hyaluronan and fetuin were found to maintain DNA integrity, when compared to the controls. These results were in agreement with those reported in a previous

study on bull sperm [14], in which the protection of DNA integrity was recorded.

It is well-known that SOD is an enzymatic biological antioxidant, which scavenges ROS, such as the superoxide anion and hydroxyl radicals, and thus, controls oxidative stress in mammalian sperm [29]. Furthermore, GSH is able to directly react with many ROS and is a co-factor for GSH-Px, which catalyses the reduction of toxic H_2O_2 and hydroperoxides [12]. In this study, it is suggested that combined fetuin and hyaluronan play an antioxidative role in the prevention of MDA formation, and thus, result in higher SOD activity. We can suggest that the additives used in this study can easily scavenge cytotoxic aldehydes and improve membrane and DNA integrity. For GSH-Px, all groups displayed higher activity when compared to the controls, in contrast to a study carried out on ram semen [5], which revealed no increase in GSH-Px activity when hyaluronan was added to the freezing extender. These findings possibly indicate that minimized MDA levels and increased antioxidant enzyme activities are apparently not major factors that influence post-thaw sperm motility and non-return rates. The question regarding the inexistence of consistency among antioxidative capacities following the thawing of semen remains to be answered. Related to spermatozoal concentration, it may be possible that some antioxidant systems are destroyed during the freeze-thawing process. Changes in the preservation protocol and experimental methodology, extender formula, antioxidant concentrations and animal species-breeds may explain different behaviours of antioxidant capacities and why antioxidants prevent MDA formation [21]. In conclusion, antioxidants can improve sperm quality and DNA integrity by decreasing MDA formation and elevating antioxidant capacity, after freeze-thawing. Future studies are required to find the most appropriate antioxidants and their effective concentrations, which will improve post-thaw sperm parameters.

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