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RESEARCH ARTICLE

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Effects of Various Antioxidants on Cryopreserved Bull Sperm Quality [1] [2]

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Summary

The objective of this study was to assess the effects of antioxidant supplement (A), fetuin (F), aminoacid (AS) and cysteine (CY) on the sperm parameters, plasma membrane integrity, chromatin damage and antioxidant activities after freeze-thawing. Ejaculates were split into five aliquots and extended to a final concentration of 15x106 spermatozoa/ml with the Tris base extender containing 0.5 ml A, 2 mg/ml F, 13% AS, 5 mM CY and no additive (C). The extended samples were cooled slowly to 4°C and then frozen using a digital freezing machine. Thereafter, the straws were plunged into liquid nitrogen at -196°C. Frozen straws were thawed individually at 37°C for 30 s to analyse progressive motility and sperm motion characteristics as well as membrane integrity. Biochemical assays were performed in a spectrophotometer using commercial kits. Chromatin damage was evaluated by Comet Assay. A, F, AS and CY did not show better result on the percentages of post-thaw sperm motilities. CY exhibited the greatest value of plasma membrane integrity ($P<0.05$). Total abnormalities were greater in C and F (17.5±0.57%; 15.5±1.98%, respectively; $P<0.05$). F had greater chromatin damage results ($P<0.05$). GPx activity was affected by type of antioxidant, notably CY yielded the lowest results when compared to the other groups ($P<0.05$). In conclusion, although using antioxidants does not have any influence on the sperm motility after thawing, A, AS and CY cause reduction at abnormal spermatozoa; CY exhibits the greatest cryoprotective activity on plasma membrane integrity and F caused an increase at chromatin damage.

Keywords: *Antioxidant activity, Bull sperm, DNA integrity, Oxidative stress, Sperm freezing*

Dondurulmuş Boğa Sperması Kalitesi Üzerine Değişik Antioksidanların Etkileri

Özet

Bu çalışmanın amacı, sperma sulandırıcısına ilave edilen antioksidan suplementi (A), fetuin (F), aminoasit (AS) ve sisteinin (CY) spermanın dondurma çözündürme sonrası spermatolojik parametreler, plazma membran bütünlüğü, kromatin hasarı ve antioksidan aktivite üzerine etkilerini değerlendirmekti. Ejakülatlar 5 eşit parçaya ayrıldı ve mililitrede 15x106 spermatozoa olacak şekilde biri kontrol ve diğerleri 0.5 ml A, 2 mg/ml F, 13% AS ve 5 mM CY içeren Tris bazlı sperma sulandırıcısı ile sulandırıldı. Sulandırılan spermalar 4°C'de 4 saat süre ile ekülibre edildi ve otomatik sperma dondurma cihazı kullanılarak donduruldu. Dondurulan spermalar sıcak su banyosunda 37°C'de 30 saniye süre ile çözündürülerek plazma membran bütünlüğünün yanı sıra spermanın ileri yönlü hareketleri ve sperma hareket özellikleri değerlendirildi. Biyokimyasal analizler ticari kit kullanılarak spektrofotometrede yapıldı. Kromatin hasarı Comet Testi ile değerlendirildi. A, F, AS ve CY; ileri yönlü spermatozoa hareketi yönünden herhangi bir iyileştirici yönde sonuç göstermedi. Plazma membran bütünlüğü yönünden CY diğer gruplarla karşılaştırıldığında en yüksek değeri gösterdi ($P<0.05$). Toplam anormal spermatozoa oranı diğer gruplara oranla en yüksek C ve F (%17.5±0.57; %15.5±1.98, sırasıyla) gruplarında belirlendi ($P<0.05$). F en yüksek kromatin hasarını oluşturdu ($P<0.05$). GPx aktivitesi antioksidan tipinden etkilendi, diğer gruplarla karşılaştırıldığında özellikle CY en düşük sonuçları verdi ($P<0.05$). Sonuç olarak, kullanılan antioksidanlar dondurma çözündürme sonrası sperma motilitesi üzerine herhangi bir olumlu etki göstermemesine rağmen A, AS ve CY anormal spermatozoa oranının düşmesine neden oldu. CY plazma membran bütünlüğü yönünden en yüksek korumayı sağlarken, F kromatin hasarının artmasına neden oldu.

Anahtar sözcükler: *Antioksidan aktivite, Boğa sperması, DNA bütünlüğü, Oksidatif stres, Sperma dondurma*

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INTRODUCTION

Cryopreservation has been an invaluable technique for helping viability of spermatozoa [1,2]. However, the biggest obstacle in the preservation of semen is the occurence of lethal and sub-lethal damage on sperm structure during cryopreservation, causing poor fertility of preserved sperm [3]. Reactive oxygen species (ROS) have been generated by the cryopreservation processes. Spermatozoa have a high content of unsaturated fatty acids in their membranes, and have a lack of significant cytoplasmic component containing antioxidants [4]. Therefore, they are highly susceptible to oxidative injury and poorly equipped to fight ROS attack [5]. When ROS are produced excessively, they may display damaging effects on sperm motility [6,7], plasma membrane integrity [8], DNA integrity [9] and fertilizing capability [10].

Mammalian cells involve antioxidant systems to cope with oxidative stress and damage [11]. These antioxidant systems in sperm may be insufficient to prevent a decrease in motility and maintain sperm functions during freeze-thawing process [12]. Antioxidants have become increasingly important for the protection or management of oxidative stress and can be used as useful tools to protect from oxidative damage [6]. Cysteine (CY) is a ROS scavenger which stimulates glutathione synthesis and it prevents the loss of sperm functions during the freeze-thawing process [11]. Aminoacid (AS) is present at the extra-cellular level and improves sperm motility, acrosomal integrity and fertilizing capability after the freeze-thawing process [13,14]. Fetuin (F), which is a microheterogeneous protein, appears in fetal calf serum [15]. Several antioxidant agents have been tested *in vitro* and *in vivo* studies and there have been appeared beneficial effects [16,17] and contradictory results as well [18,19]. Most of these studies have suggested that further studies are required in order to obtain more concrete results.

Thus, the objective of this study was to assess the effects of antioxidant supplement (A), F, AS and CY on the sperm parameters, plasma membrane integrity, chromatin damage and antioxidant activities after freeze-thawing.

MATERIAL and METHODS

Animals and Semen Collection

Three Holstein bulls (3-4 years of age) with good quality semen characteristics (>80% forward progressive motility and concentrations of at least $1.0x10^9$ spermatozoa/ml) were selected to be the semen source. The bulls were clinically proven to be free from any general or genital diseases and were maintained at the Livestock Central Research Institute (Ankara, Turkey). Ejaculates were collected from the bulls with the aid of an artificial vagina twice a week. The ejaculates were pooled to increase the semen volume for replication and to eliminate variability among the evaluated samples. The pooled semen sample was immersed in a water bath at 35.5°C until it could be assessed for total and progressive motility as well as sperm concentration. This study was replicated eight times for each group. The experimental procedures were approved by the Animal Care Committee of the Faculty of Veterinary Medicine, Istanbul University.

Semen Processing

The antioxidants used (antioxidant supplement A1345, fetuin from calf serum F2379, BME aminoacid solution B6766, L-Cysteine C-7352) were obtained from Sigma-Aldrich Chemical Co., USA. The total semen volume was determined from the graded collection tube soon after collection, and its concentration was determined using an Accucell photometer (IMV Technologie, L'Aigle, France). Progressive motility was evaluated subjectively using a phase contrast microscope (200x, Olympus BX43, Tokyo, Japan) at 37°C. A Tris-based extender (T) (30.7 g of Tris, 16.4 g of citric acid, 12.6 g of fructose, 20% v/v egg yolk, glycerol 6% (v/v) and 1000 ml of distilled water at a pH of 6.8) was used as the base for the experimental extenders. Pooled ejaculates were split into five aliquots and diluted to a final concentration of $15x10^6$ spermatozoa/ml with the base extender containing A (0.5 ml), F (2 mg/ml), AS (%13), CY (5 mM) and no additive (control; C). The extended samples were cooled slowly to 4°C and equilibrated for 4 h. They were then loaded into 0.25 ml French straws and frozen using a controlled rate freezer (Digitcool 5300 ZB 250, IMV, France) at 3 programmed rates: -3°C/min from +4°C to -10°C, -40°C/min from -10°C to -100°C, and -20°C/min from -100°C to -140°C. Thereafter, the straws were plunged into liquid nitrogen at -196 °C.

Assessment of in vitro Sperm Quality

Subjective motility was assessed using a phase-contrast microscope (100x, Olympus BX43, Tokyo, Japan). A drop of semen was placed on a pre-warmed microscope slide and was subjectively assessed at 37°C for its percentage of progressive motility. In addition to estimating the subjective sperm motility, a computer-assisted sperm motility analysis program (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA) was also used to analyse sperm motion characteristics. CASA was pre-adjusted for bovine sperm analysis. A semen sample was diluted 1:4 in Lactated Ringer solution, and the diluted semen sample was placed onto a pre-warmed 20 mm chamber slide (Leja 4, Leja Products BV, The Netherlands). The sperm motility characteristics were determined using a 10x objective microscope lens at 37°C. The following motility values were recorded: motility (%), progressive motility (%), average path velocity (VAP, µm/s), straight linear velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm/s), and beat cross frequency (BCF, Hz). A minimum of 10 microscopic fields

were analysed for each assessment, which included at least 300 cells. The hypo-osmotic swelling test (HOS test) was used to assess the functional integrity of the spermatozoal membranes. The HOS test 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 on a warm slide with a cover slip and then was examined using a phase-contrast microscope (400x, Olympus BX43, Tokyo, Japan) [20]. The number of swollen spermatozoa out of 200 was counted; swelling is characterised by a coiled tail, indicating that the plasma membrane is intact. For the evaluation of sperm abnormalities, 10 µl of each sample was added to Eppendorf tubes containing 1 ml of Hancock solution [21]. One drop of this mixture was placed on a slide and covered with a cover slip. The percentages of acrosome, head, tail and total abnormalities out of 200 oil-immersed spermatozoa were determined using a phase-contrast microscope (1000x, Olympus BX43, Tokyo, Japan).

Assessment of Choromatin Damage

Sperm choromatin damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. Semen samples were immersed in water at 37°C for 30 s and then centrifuged at 600 g for 10 min at room temperature. The seminal plasma was removed, and the remaining sperm cells were washed with phosphate buffer solution (PBS; Ca^{2+} and Mg^{2+} free) twice to yield a concentration of $1x10^5$ spermatozoa/cm^3. Each microscope slide was pre-coated with a layer of 0.65% high melting-point agarose in distilled water and thoroughly dried at room temperature. Twenty-five µl of the sperm cell suspension were mixed with 75 µl of 0.65% low melting-point agarose at 50°C, and then a drop of the mixture was placed on a pre-coated slide and covered with a cover slip. The slides were allowed to solidify for 10 min at 4°C in a moist box. Then, the coverslips 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 and 40 mM dithiothreitol (pH 10) for 1 h at 4°C. The slides were then removed from the lysis buffer, drained, and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis solution, which contained 300 mM NaOH and 1 mM EDTA (pH 13). The slides remained in the unit for 20 min to allow the DNA to unwind. Next, electrophoresis was performed at 25 V and an adjusted 300 mA for 20 min at room temperature. 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 65 µl of 20 µg/ml ethidium bromide and covered with a coverslip. All of these steps were conducted under dimmed lighting to prevent additional choromatin damage. The images of 100 randomly chosen nuclei were analysed using a fluorescent microscope at a magnification of 400 x (Zeiss, Germany). Nucleotide DNA extends under electrophoresis to form "comet tails," and the relative intensity of DNA in the tail reflects the frequency of DNA breakage. Thus, the percentage of the total DNA in the comet tail was taken as a direct measure of the DNA break frequency. Tail DNA (%) was assessed in 100 cells using the Comet Assay III image analysis system (Perceptive Instruments, UK). The analysis was performed blindly by one slide reader [22].

Biochemical Assays

Semen samples were centrifuged at 4°C and 1000 g for 15 min to separate out the spermatozoa. The pellet was washed 3 times using 0.5 ml of PBS. This final solution was homogenised 5 times by sonication in cold for 15 s for the Lipid Peroxidation Analysis (LPO), 120 µl of the homogenate was mixed with 10 µl of 0.5 mM butyl hydroxyl toluene (BHT) and stored at -80°C until analysed. The rest of the homogenate was centrifuged at 8000 g for 15 min, and the supernatant was separated and stored at -80°C for a different enzyme analysis [22]. Enzyme levels were determined using commercial kits by spectrophotometry (Cintra 303-UV, GBC, Australia). Biochemical assay kits were obtained from Sigma-Aldrich Chemical (Interlab Ltd., Ankara, Turkey)

Statistical Analysis

Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared, using the SPSS/PC computer programme (version 14.1, Chicago, IL). The test revealed that the variances were homogeneous. After that, comparisons between the groups were made using analysis of variance with Duncan post hoc test. The results are expressed as means or proportions (±S.D.). The differences were considered significant at $P<0.05$.

RESULTS

As shown in *Table 1*, using A, F, AS and CY as antioxidants did not give better results on the percentages of sperm motility assessed subjectively or by CASA after thawing. Spermatozoa frozen in which containing CY exhibited the greatest value of VAP (120.5±1.97 µm/s), VCL (207.4±3.32 µm/s) and plasma membrane integrity (48.1±0.79%) compared to other groups ($P<0.05$). Although there were no significance differences in the percentages of acrosome abnormalities among treatment groups ($P>0.05$), total abnormalities were greater in C and F (17.5±0.57%; 15.5±1.98%, respectively) than the other groups ($P<0.05$).

As shown in *Table 2*, chromatin damage depending on the type of antioxidant; F caused greater chromatin damage than the other groups ($P<0.05$).

As shown in *Table 3*, as regards to antioxidant activity; although there were no significant differences in the GSH, CAT and total antioxidant activities, GPx activity was

Table 1. *Mean (±SE) sperm values in frozen thawed bull semen*
Tablo 1. *Boğa spermasının dondurma çözündürme sonrası ortalama spermatolojik değerleri*

Analysis	Control	Antioxidant (0.5 ml)	Fetuin (2mg/ml)	Aminoacids (%13)	Cysteine (5 mM)	P
Subjective motility (%)	51.9±3.89	58.8±4.51	58.1±3.53	57.5±4.43	52.5±5.00	-
CASA motility (%)	48.3±4.34	53.1±5.56	50.8±6.59	49.4±4.52	53.3±5.03	-
Progressive motility (%)	20.8±2.40	22.4±3.79	21.4±3.55	20.5±2.43	18.1±3.73	-
VAP (µm/s)	100.8±1.19^{a}	102.6±1.78^{a}	100.6±2.05^{a}	102.0±1.86^{a}	120.5±1.97^{b}	*
VSL (µm/s)	77.1±1.16	78.6±1.16	79.5±1.30	79.5±1.19	81.5±1.87	-
VCL (µm/s)	176.0±1.79^{a}	178.5±4.70^{a}	169.5±5.23^{a}	175.6±3.81^{a}	207.4±3.32^{b}	*
ALH (µm/s)	7.9±0.12	7.9±0.24	7.6±0.31	7.7±0.51	8.5±0.24	-
BCF (Hz)	16.3±0.35^{a}	16.0±0.62^{a}	17.2±1.07^{a}	17.3±0.55^{a}	13.7±0.50^{b}	*
HOS T (%)	41.1±0.40^{a}	44.5±1.04^{b}	42.5±0.80^{ab}	41.6±0.53^{a}	48.1±0.79^{c}	*
Acrosome abnormalities (%)	5.8±0.45	4.3±0.75	4.3±0.59	3.9±0.61	3.4±0.53	-
Total abnormalities (%)	17.5±0.57^{a}	12.3±1.69^{bc}	15.5±1.98^{ab}	13.3±0.88^{bc}	9.8±0.80^{c}	*

*^{a,b,c} Different superscripts within the same row demonstrate significant differences (*P<0.05), - No significant difference (P>0.05)*

Table 2. *Mean (±SE) chromatin damage values in frozen thawed bull semen*
Tablo 2. *Boğa spermasında dondurma çözündürme sonrası oluşan ortalama kromatin hasarları*

Analysis	Control	Antioxidant (0.5 ml)	Fetuin (2mg/ml)	Aminoacids (%13)	Cysteine (5 mM)	P
Tail intensity (%)	10.7±0.49^{a}	9.7±0.66^{a}	14.6±1.34^{b}	10.6±0.83^{a}	9.6±0.41^{a}	*
Tail moment (µm/s)	3.9±0.25^{a}	4.1±0.18^{a}	7.4±1.21^{b}	4.0±0.16^{a}	2.42±0.23^{a}	*

*^{a,b,c} Different superscripts within the same row demonstrate significant differences (*P<0.05), - No significant difference (P>0.05)*

Table 3 *Mean (±SE) glutathione peroxidase (GPx), lipid peroxidase (LPO), reduced glutathione (GSH), catalase (CAT) and total antioxidant activities in frozen thawed bull semen*
Tablo 3. *Boğa spermasında dondurma çözündürme sonrası ortalama glutatyon peroksidaz (GPx), lipit peroksidaz (LPO), Redükte glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Analysis	Control	Antioxidant (0.5 ml)	Fetuin (2mg/ml)	Aminoacids (%13)	Cysteine (5 mM)	P
GPx (mU/ml-10^{9}cell/ml)	14.9±0.64^{c}	14.8±0.18^{c}	15.4±0.53^{c}	12.8±0.83^{b}	10.8±0.62^{a}	*
LPO (µm/ml-10^{9}cell/ml)	0.5±0.27	0.7±0.23	0.5±0.29	0.6±0.28	0.44±0.28	-
GSH (µm/ml-10^{9}cell/ml)	37.7±10.86	37.5±10.16	27.1±9.23	27.2±7.50	20.7±1.46	-
CAT (µm/ml-10^{9}cell/ml)	18.9±4.94	17.5±4.91	7.7±1.35	13.6±2.49	15.7±4.73	-
Total antioxidant activities (mmol/trolox/ml-10^{9} cell/ml)	15.2±3.30	14.2±3.27	7.7±0.90	11.6±1.66	13.0±3.15	-

*^{a,b,c} Different superscripts within the same row demonstrate significant differences (*P<0.05), - No significant difference (P>0.05)*

affected by the type of antioxidant, notably CY yielded the lowest results in comparison with the other groups (P<0.05).

DISCUSSION

The axoneme and associated dense fibers of the mid-pieces in sperm, which are responsible from the motility, are covered by mitochondria that generate energy by oxidative phosphorylation [23,24]. Large amounts of ROS can impair the sperm motility [9,25]. This study showed that using antioxidants do not give better results on the sperm's motility after thawing. However, their abnormal spermatozoa rates are lower than C except F. Spermatozoa in T containing CY exhibited the greatest percentages of plasma membrane integrity. Consistent with our study, Tuncer et al.[26] demonstrated that adding antioxidant to freeze the spermatozoa do not have any marked effects on the subjective and CASA motilities. Bucak et al.[18], reported that adding antioxidant to freeze the spermatozoa has positive effect on plasma membrane integrity but this situation does not prevent ROS formation and has no effect on total antioxidant activity. It has been suggested that using of glutathione and cysteine may improve the spermatozoa viability and functional integrity [17]. In contrast with our findings, it has been proposed that the antioxidants can be used successfully to improve boar [27], dog [28], goat [29] and rainbow trout [30] sperm motility. This study also contradicts the previous bull sperm study which cysteine (2.5 mM) was supplemented. Sperm motility and

acrosomal integrity rates were increased but these results did have no effects on fertility rates [31]. Bucak et al. [16] showed that bull sperm samples in which 7.5 mM carnitine and 7.5 mM inositol had been added, caused an increase on the spermatozoa motility. Their subjective motilities (61.9±1.3%; 51.3±1.6%) were similar, but CASA (41.6±2.9%; 34.9±2.0%) and progressive motilities (12.8±1.4%; 13.3±1.5%) were lower than those obtained in our study. Parallel to our findings, it was revealed that bull sperm samples with addition of cysteine (5 and 10 mM) and GSH (0.5 and 2 mM) did not have any further improvement on GPx activity and motility [19]. This study's progressive and CASA motilities were also similar with our findings, but plasma membrane integrity was greater and abnormal spermatozoa rate was lower than the values obtained in our study. Based on our results, we can hypothesize that the difference between the findings can be related with using different antioxidants or different doses of the same antioxidants or different antioxidant capacity of spermatozoa in the testing stage.

The excessive ROS production causes a damage on plasma membrane and DNA integrity of spermatozoa [9,32]. Chromatin damage, which is formed after dilution, freezing and thawing of sperm, can be prevented with the addition of antioxidants [33]. Chromatin damage on spermatozoa effects potantial embryo growing negatively [34] by reducing the fertilization rate [35]. In contrast to the results obtained, it has been shown that antioxidants added to sperm extender reduce the chromatin damage [36]. However, the antioxidants that we have used in our study did not provide any marked improvement at DNA integrity and F usage caused an increase in chromatin damage. These contradictory results can be hypothesized that chromatin damage may be related not only with oxidative damages but also with osmotic damages too.

Spermatozoa and seminal plasma involves ROS scavengers, including the enzymes such as SOD, GPx, and CAT [4,37]. In this study, it is stated that using antioxidant has no effect on the antioxidant activity and does not cause further improvement on motility values. Similar with our findings, Kasimanickam et al.[10] reported that there is no relationship between the antioxidant activity (GPx, LPO, SOD), DNA integrity and plasma membrane integrity. In a study on the sperm of ram and goat, it is found that using antioxidants does not have positive effect on LPO, GSH and GPx activities [18, 38]. Different from our findings, it has been reported that GSH activity is decreased 80% while freezing and thawing of the bull sperm [6]. In another study, it is also reported that this potential reduction of GSH was originated from oxidative stress and the deterioration of the plasma membrane integrity, and accordingly this situation effects motility and viability [39]. It is proposed that cysteine (5 mM) added to sperm extender has a positive effect on endogenous antioxidant system and increases the GPx activity but does not reduce MDA levels. Increased GPx activity does not have any positive effects on sperm values [19]. Our findings indicate that changes in extender and its composition, animal species or breeds explain why antioxidant supplementation do not improve the sperm motility while some of them have effects on abnormalities and plasma membrane integrity positively.

In conclusion, although using antioxidants does not have any influence on the sperm motility after thawing, A, AS and CY cause reduction at abnormal spermatozoa; CY exhibits the greatest cryoprotective activity on plasma membrane integrity and F caused an increase at chromatin damage.

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