

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

Umut TAŞDEMİR ¹ 
Taner ÖZGÜRTAŞ ³

Pürhan Barbaros TUNCER ¹ Serhat BÜYÜKLEBLEBİCİ ²
Emre DURMAZ ⁴ Olga BÜYÜKLEBLEBİCİ ⁵

^[1] This study was financed under a project supported by the Scientific and Technological Research Council of Turkey (TUBITAK) (Project No: 106G005, TURKHAYGEN-1)

^[2] This study had been presented 8th Congress of the Turkish Society of Toxicology with Intenational Participation, 15-18 November 2012, Antalya - Turkey

¹ Ministry of Food Agriculture and Livestock, General Director of Food and Control, TR-06060 Ankara - TURKEY

² Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine, Aksaray University, TR-68100 Aksaray - TURKEY

³ Biochemistry and Clinical Biochemistry Laboratory, Gulhane Military Medical Academy, TR-06010 Ankara - TURKEY

⁴ Department of Toxicology, Faculty of Pharmacy, Gazi University, TR-06330 Ankara - TURKEY

⁵ Department of Biochemistry, Faculty of Veterinary Medicine, Aksaray University, TR-68100 Aksaray - TURKEY

Makale Kodu (Article Code): KVFD-2013-9964

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 15×10^6 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 equilibrated slowly to 4°C during 4 h and then frozen using a digital freezing machine.. Frozen straws were thawed individually in water bath 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 ($17.5 \pm 0.57\%$; $15.5 \pm 1.98\%$, 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 Spermaları Kalitesi Üzerine Değişik Antioksidanların Etkileri

Özet

Bu çalışmanın amacı, sperma sulandırıcısına ilave edilen antioksidan supplementi (A), fetuin (F), aminoasit (AS) ve sisteinin (CY) dondurma çözündürme sonrası spermatolojik parametreler, plazma membran bütünlüğü, kromatin hasarı ve antioksidan aktivite üzerine etkilerini değerlendirmektir. Ejakülatlar 5 eşit parçaya ayrıldı ve mililitrede 15×10^6 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 ($17.5 \pm 0.57\%$; $15.5 \pm 1.98\%$, 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 spermaları, DNA bütünlüğü, Oksidatif stres, Sperma dondurma



İletişim (Correspondence)



+90 535 6654375



tasdemiru@gmail.com

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 occurrence 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 1.0×10^9 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 15×10^6 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, $\mu\text{m/s}$), straight linear velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), amplitude of lateral head displacement (ALH, $\mu\text{m/s}$), 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 Chromatin Damage

Sperm chromatin 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; Ca²⁺ and Mg²⁺ free) twice to yield a concentration of 1x10⁵ spermatozoa/cm³. 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 Na²-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 chromatin 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 (\pm SE) sperm values in frozen thawed bull semen**Tablo 1.** Boğa spermasının dondurma çözündürme sonrası ortalama spermatojik değerleri

Analysis	Control	Antioxidant (0.5 ml)	Fetuin (2 mg/ml)	Aminoacids (13%)	Cysteine (5 mM)	P
Subjective motility (%)	51.9 \pm 3.89	58.8 \pm 4.51	58.1 \pm 3.53	57.5 \pm 4.43	52.5 \pm 5.00	-
CASA motility (%)	48.3 \pm 4.34	53.1 \pm 5.56	50.8 \pm 6.59	49.4 \pm 4.52	53.3 \pm 5.03	-
Progressive motility (%)	20.8 \pm 2.40	22.4 \pm 3.79	21.4 \pm 3.55	20.5 \pm 2.43	18.1 \pm 3.73	-
VAP (μ m/s)	100.8 \pm 1.19 ^a	102.6 \pm 1.78 ^a	100.6 \pm 2.05 ^a	102.0 \pm 1.86 ^a	120.5 \pm 1.97 ^b	*
VSL (μ m/s)	77.1 \pm 1.16	78.6 \pm 1.16	79.5 \pm 1.30	79.5 \pm 1.19	81.5 \pm 1.87	-
VCL (μ m/s)	176.0 \pm 1.79 ^a	178.5 \pm 4.70 ^a	169.5 \pm 5.23 ^a	175.6 \pm 3.81 ^a	207.4 \pm 3.32 ^b	*
ALH (μ m/s)	7.9 \pm 0.12	7.9 \pm 0.24	7.6 \pm 0.31	7.7 \pm 0.51	8.5 \pm 0.24	-
BCF (Hz)	16.3 \pm 0.35 ^a	16.0 \pm 0.62 ^a	17.2 \pm 1.07 ^a	17.3 \pm 0.55 ^a	13.7 \pm 0.50 ^b	*
HOS T (%)	41.1 \pm 0.40 ^a	44.5 \pm 1.04 ^b	42.5 \pm 0.80 ^{ab}	41.6 \pm 0.53 ^a	48.1 \pm 0.79 ^c	*
Acrosome abnormalities (%)	5.8 \pm 0.45	4.3 \pm 0.75	4.3 \pm 0.59	3.9 \pm 0.61	3.4 \pm 0.53	-
Total abnormalities (%)	17.5 \pm 0.57 ^a	12.3 \pm 1.69 ^{bc}	15.5 \pm 1.98 ^{ab}	13.3 \pm 0.88 ^{bc}	9.8 \pm 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 (\pm 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 (2 mg/ml)	Aminoacids (13%)	Cysteine (5 mM)	P
Tail intensity (%)	10.7 \pm 0.49 ^a	9.7 \pm 0.66 ^a	14.6 \pm 1.34 ^b	10.6 \pm 0.83 ^a	9.6 \pm 0.41 ^a	*
Tail moment (μ m/s)	3.9 \pm 0.25 ^a	4.1 \pm 0.18 ^a	7.4 \pm 1.21 ^b	4.0 \pm 0.16 ^a	2.42 \pm 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 (\pm 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 (2 mg/ml)	Aminoacids (13%)	Cysteine (5 mM)	P
GPx (mU/ml-10 ⁹ cell/ml)	14.9 \pm 0.64 ^c	14.8 \pm 0.18 ^c	15.4 \pm 0.53 ^c	12.8 \pm 0.83 ^b	10.8 \pm 0.62 ^a	*
LPO (μ m/ml-10 ⁹ cell/ml)	0.5 \pm 0.27	0.7 \pm 0.23	0.5 \pm 0.29	0.6 \pm 0.28	0.44 \pm 0.28	-
GSH (μ m/ml-10 ⁹ cell/ml)	37.7 \pm 10.86	37.5 \pm 10.16	27.1 \pm 9.23	27.2 \pm 7.50	20.7 \pm 1.46	-
CAT (μ m/ml-10 ⁹ cell/ml)	18.9 \pm 4.94	17.5 \pm 4.91	7.7 \pm 1.35	13.6 \pm 2.49	15.7 \pm 4.73	-
Total antioxidant activities (mmol/trolox/ml-10 ⁹ cell/ml)	15.2 \pm 3.30	14.2 \pm 3.27	7.7 \pm 0.90	11.6 \pm 1.66	13.0 \pm 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 not have any 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 potential 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.

REFERENCES

- Polge C, Smith AU, Parkes AS:** Revival of spermatozoa after vitrification and dehydration at low temperatures. *Nature*, 164, 666, 1949.
- Polge C, Rowson LEA:** Results with bull semen stored at -79°C. *Vet Rec*, 64, 851-853, 1952.
- Watson PF:** The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci*, 60, 481-492, 2000.
- Alvarez JG, Storey BT:** Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res*, 23, 77-99, 1989.
- Drevet JR:** The antioxidant glutathione peroxidase family and spermatozoa: A complex story. *Mol Cell Endocr*, 250, 70-79, 2006.
- Bilodeau JF, Chatterjee S, Sirart MA, Gagnon C:** Levels of antioxidant defences are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev*, 55, 282-288, 2000.
- Kao SH, Chao HT, Chen HW, Hwang TI, Liao TL, Wei YH:** Increase in oxidative stress in human sperm with lower motility. *Fertil Steril*, 89, 1183-1190, 2008.
- Alvarez JG, Storey BT:** Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a model of sublethal cryodamage to human sperm during cryopreservation. *J Androl*, 13, 232-241, 1992.
- Kadirvel G, Kumara S, Kumaresan A:** Lipid peroxidation, mitochondrial membrane potential and DNA integrity of spermatozoa in relation to intracellular reactive oxygen species in liquid and frozen-thawed buffalo semen. *Anim Reprod Sci*, 114, 125-134, 2009.
- Kasimanickam R, Kasimanickam V, Thatcher CD, Nebel RL, Cassell BG:** Relationships among lipid peroxidation, glutathione peroxidase, superoxide dismutase, sperm parameters, and competitive index in dairy bulls. *Theriogenology*, 67, 1004-1012, 2007.
- Ross C, Morriss A, Khairy M, Khalaf Y, Braude P, Coomarasamy A, El-Toukhy TA:** Systematic review of the effect of oral antioxidants on male infertility. *Reprod BioMed Online*, 20, 711-723, 2010.
- Kang JH, Hakimov H, Ruiz A, Friendship RM, Buhr M, Golovan SP:** The negative effects of exogenous DNA binding on porcine spermatozoa are caused by removal of seminal fluid. *Theriogenology*, 70, 1288-1296, 2008.
- Khlifaoui M, Battut I, Bruyas JF, Chatagnon G, Trimeche A, Tainturier D:** Effects of glutamine on post-thaw motility of stallion spermatozoa: An approach of the mechanism of action at spermatozoa level. *Theriogenology*, 63, 138-149, 2005.
- Li Y, Si W, Zhang X, Dinnyes A, Ji W:** Effect of amino acids on cryopreservation of cynomolgus monkey (*Macaca fascicularis*) sperm. *Am J Prim*, 59, 159-165, 2003.
- Berger T, Bierle JW:** Inhibition of sperm motility by bovine serum components. *Biol Reprod*, 42, 545-551, 1990.
- Bucak MN, Tuncer PB, Sariözkan S, Başpınar N, Taşpınar M,**

- Çoyan K, Bilgili A, Peker-Akalın P, Büyükleblebici S, Aydos S, Ilgaz S, Sunguroğlu S, Öztuna D:** Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: Antioxidants protect DNA integrity against cryodamage. *Cryobiology*, 61, 248-253, 2010.
- 17. Funahashi H, Sano T:** Select antioxidants improve the function of extender boar semen at 10°C. *Theriogenology*, 63, 1605-1616, 2005.
- 18. Bucak MN, Tuncer PB, Sariözkan S, Alkım-Ulutaş P, Çoyan K, Başpınar N, Özkalp B:** Effects of hypotaurine, cysteamine and aminoacids solution on post-thaw microscopic and oxidative stress parameters of Angora goat semen. *Res Vet Sci*, 87, 468-472, 2009.
- 19. Tuncer PB, Bucak MN, Büyükleblebici S, Sariözkan S, Yeni D, Eken A, Peker Akalın P, Kinet H, Avdatek F, Fidan AF, Gündoğan M:** The effect of cysteine and glutathione on sperm and oxidative stress parameters of post-thawed bull semen. *Cryobiology*, 61, 30307, 2010.
- 20. Revel SG, Mrode RA:** An osmotic resistance test for bovine semen. *Anim Reprod Sci*, 36, 77-86, 1994.
- 21. Schafer S, Holzman A:** The use of transmigration and spermac stain to evaluate epididymal cat spermatozoa. *Anim Reprod Sci*, 59, 201-211, 2000.
- 22. Taşdemir U, Büyükleblebici S, Tuncer PB, Coşkun E, Özgürtaş T, Aydın FV, Büyükleblebici O, Gürçan İS:** Effect of various cryoprotectants on bull sperm quality and oxidative stress parameters. *Cryobiology*, 66, 38-42, 2013.
- 23. Guthrie HD, Welch GR:** Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in viable boar sperm using fluorescence activated flow cytometry. *J Anim Sci*, 84, 2089-2100, 2006.
- 24. Martin G, Sabido O, Durand P, Levy R:** Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol Reprod*, 71, 28-37, 2004.
- 25. Garner DL, Hafez ESE:** Spermatozoa and seminal plasma. In: Hafez ESE (Ed): *Reproduction in Farm Animals*. 167-182, Lea&Febier, Philadelphia, 1993.
- 26. Tuncer PB, Bucak MN, Sariözkan S, Sakin F, Yeni D, Çiğerci IH, Ateşşahin A, Avdatek F, Gündoğan M, Büyükleblebici O:** The effect of raffinose and methionine on frozen/thawed Angora buck (*Capra hircus ancyrensis*) semen quality, lipid peroxidation and antioxidant enzyme activities. *Cryobiology*, 61, 89-93, 2010.
- 27. Cerolini S, Maldjian A, Surai P, Noble R:** Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. *Anim Reprod Sci*, 58, 99-111, 2000.
- 28. Michael AJ, Alexopoulos C, Pontiki EA, Hadjipavlou-Litina DJ, Saratsis P, Ververidis HN, Boscos CM:** Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Anim Reprod Sci*, 112, 119-135, 2009.
- 29. Ma H, Quana F, Chena D, Zhenga Y, Zhanga B, Wang Y, Zhanga Y:** Protective function of alpha-lipoic acid on sperm motility and mitochondrial function during goat sperm-mediated gene transfer. *Small Rum Res*, 96, 178-184, 2011.
- 30. Lahnsteiner F, Mansour N, Kunz FA:** The effect of antioxidants on the quality of cryopreserved semen in two salmonid fish, the brook trout (*Salvelinus fontinalis*) and the rainbow trout (*Oncorhynchus mykiss*). *Theriogenology*, 76 (5): 882-890, 2011.
- 31. Sariözkan S, Bucak MN, Tuncer PB, Ulutaş PA, Bilgen A:** The influence of cysteine and taurine on microscopic-oxidative stress parameters and fertilizing ability of bull semen following cryopreservation. *Cryobiology*, 58, 134-138, 2009.
- 32. Aitken RJ, Gordon E, Harkiss D, Twigg JP, Milne P, Jennings Z, Irvine DS:** Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod*, 59, 1037-1046, 1998.
- 33. Twigg J, Fulton N, Gomez E, Irvine DS, Aitken RJ:** Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: Lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Human Reprod*, 13, 1429-1436, 1998.
- 34. Larson-Cook KL, Brannian JD, Hansen KA, Kasperon KM, Aamold ET, Evenson DP:** Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. *Fertil Steril*, 80, 895-902, 2003.
- 35. Evenson DP, Wixon R:** Comparison of the Halosperm® test kit with the Sperm Chromatin Structure Assay (SCSA®) infertility test in relation to patient diagnosis and prognosis. *Fertil Steril*, 8, 846-849, 2004.
- 36. Szczesniak-Fabianzyk B, Bochenek M, Smorag Z, Ryszka F:** Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. *Reprod Biol*, 3, 83-89, 2003.
- 37. Holland MK, Alvarez JG, Storey BT:** Production of superoxide and activity of superoxide dismutase in rabbit epididymal spermatozoa. *Biol Reprod*, 27, 1109-1118, 1982.
- 38. Çoyan K, Başpınar N, Bucak MN, Peker-Akalın P, Ataman MB, Doğan AO, Güngör S, Küçükğünay S, Özkalp B, Sariözkan S:** Influence of methionine and dithioerythritol on sperm motility, lipid peroxidation and antioxidant capacities during liquid storage of ram semen. *Res Vet Sci*, 89, 426-431, 2010.
- 39. Stradaoli G, Noro T, Sylla L, Monaci M:** Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: Comparison between two extenders. *Theriogenology*, 67, 1249-1255, 2007.