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Research Article

The Effects of Different Antioxidants on Post-thaw Microscopic and Oxidative Stress Parameters in the Cryopreservation of Brown-Swiss Bull Semen [1] [2]

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Abstract

The aim of this study was to research the effects of four antioxidants that had been added to semen diluents, on spermatological parameters, anti-oxidant enzymes activities and DNA integrity after the freeze-thaw procedures in Brown-Swiss bull semen. A total of 24 ejaculates were collected from three bulls. Each ejaculate was divided into five equal parts and they diluted to the Tris + 25 mM trehalose base extender containing fetuin 1 mg/ml, dithioerithritol 1 mM, cysteamine 4 mM or linoleic acid 0.5 ml/50 ml, and no additives (control). Group dithioerithritol and linoleic acid showed the higher rates of CASA progressive motility, CASA sperm motility ($P<0.05$) compared with the other groups. Group linoleic acid provided the better protective effect for acrosome and total abnormalities ($P<0.01$, $P<0.05$; respectively). Also, group dithioerithritol showed the highest values for the HOS test ($P<0.01$). In the comet test, group dithioerithritol enhanced tail with lesser chromatin damage than the other groups ($P<0.05$). Supplementation of dithioerithritol significantly affected the GSH activity ($P<0.01$). Also groups dithioerithritol and linoleic acid gave higher CAT values than the other groups ($P<0.05$). Additionally, supplementation of fetuin and cysteamine showed the lowest total antioxidant activity value ($P<0.05$). In conclusion, we may said that, the addition of antioxidants, specially dithioerithritol and linoleic acid, to added with 25 mM trehalose Tris extender improve post-thaw sperm parameters.

Keywords: *Antioxidants, Catalase, Glutathione peroxidase, Spermatological charecteristics, Bovine*

Brown-Swiss Boğa Spermasının Dondurulmasında Çözündürme Sonrası Mikroskopik ve Oksidatif Stress Parametreleri Üzerine Farklı Antioksidanların Etkileri

Özet

Bu çalışmanın amacı Brown-Swiss boğa spermasında dondurma çözündürme işlemleri sonrasında, spermatolojik parametreleri, antioksidant enzim aktiviteleri ve DNA bütünlüğü üzerine sperma sulandırıcısına eklenen dört antioksidanın etkisini araştırmaktı. Üç boğadan toplam yirmidört ejakülat toplandı. Her bir ejakülat beş eşit kısma ayrıldı ve 25 mM trehaloz katılmış Tris temel sulandırıcısı içerisinde, fetuin 1 mg/ml, dithioerithritol 1 mM, sisteamin 4 mM veya linoleik asit 0.5 ml/50 ml, ve antioksidan içermeyen (kontrol) ile sulandırıldı. Grup dithioerithritol ve L diğer gruplarla kıyaslandığında daha yüksek CASA progressif motilite ve CASA sperma motilitesi gösterdi ($P<0.05$). Grup linoleik asit akrozom ve toplam abnormalitede daha iyi koruyucu etki sağladı (sırasıyla $P<0.01$, $P<0.05$). Keza, grup dithioerithritol, HOS test için en yüksek değeri verdi ($P<0.01$). Comet testte göre, dithioerithritol grubunda kuyruk kromatin hasarı diğer gruplara göre daha azdı ($P<0.05$). Dithioerithritol eklenmesi GSH aktivitesini önemli derecede etkiledi ($P<0.01$). Keza, dithioerithritol ve linoleik asit grupları diğer gruplara göre daha yüksek CAT değeri verdi ($P<0.05$). Buna ek olarak fetuin ve sisteamin eklenmesi endüşük total antioksidan aktivite değeri gösterdi ($P<0.05$). Sonuç olarak, 25 mM trehaloz eklenmiş Tris sulandırıcısına eklenen antioksidanların, özellikle de dithioerithritol ve linoleik asit'in, çözüm sonu sperma parametrelerini artırdığı söyleyebilir.

Anahtar sözcükler: *Antioksidanlar, Katalaz, Glutatyon peroksidaz, Spermatolojik özellikler, Boğa*

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INTRODUCTION

Animal breeding industry has been extensively used frozen bull semen in artificial insemination (AI). Achievement of AI is contingent upon the quality and quantity of fresh semen and its capacity for dilution and storage with minimum deprivation of fertilizing capacity [1]. Freezing-thawing processes conduct to the production of reactive oxygen substances (ROS) that weaken post-thaw motility, viability, intracellular enzymatic activity, fertility and sperm functions [2,3]. Spermatozoa consist of high concentrations of polyunsaturated fatty acids (PUFA) in the sperm plasma membrane exposed to peroxidation, and consequently are extremely responsive to lipid peroxidation (LPO), which leads to a following loss of motility, membrane integrity, fertilizing capability and metabolic changes of sperm cells [4,5]. When frozen semen is used for AI, spermatozoa are exposed to oxygen and light radiation which could irreversibly influence sperm functions. Under these conditions, it is declared that addition of antioxidants increased the post-thaw motility, viability, membrane integrity and fertility of boar [6], bull [1], ram [7] and goat [8] sperm cells. Oxidative stress is the result of an extreme production of ROS and/or diminishes in the antioxidant defence system, and these oxidative stress targets lipids, proteins and DNA [9]. Fetuin, which is a micro heterogeneous protein, appears in fetal calf serum and a protease inhibitor, and has been shown to inhibit zona pellucida hardening during the *in vitro* maturation of equine oocytes [10]. In additionally, fetuin is a commercially available protein, which improves sperm motility [11]. Dithiothreitol is known as an antioxidant and it decreases protamine disulfide bond [12]. It avoids the oxidation of sulfhydryl groups, and it has a mucolytic effect on mucoprotein disulfide bonds, which may likely damage the frozen membranes [13]. Deshpande and Kehrer [14] revealed that dithiothreitol provides a defensive effect against apoptosis and oxidative damage. It is declared that cysteamine stimulates glutathione (GSH) synthesis during the *in vitro* maturation of ovine oocytes, promoting embryonic growth [8]. Additionally, it improves post-thaw motility, reduces total abnormality rates and advances the antioxidant capacity of goat sperm [15]. Takahashi et al.[16] revealed that low molecular weight thiol compounds such as mercaptoethanol (P3-ME) and cysteamine supported to cell viability. Limited number of studies on the supplementation of unsaturated fatty acids for sperm production or cryopreservation provided positive findings. Fair et al.[17] stated that dietary addition of wide range of PUFA complements has shown to modify the sperm fatty acid profile. There are contradictory results about the effects of using PUFA on fresh and frozen-thawed sperm. Some authors suggest that supplementation of fatty acids on the preservation of sperm function after long-term liquid storage or freezing needs to be addressed [18].

The composition of the extender is highly important for semen cryopreservation. Sugar is utilized by spermatozoa as an energy source to support sperm motility and movement [19]. Trehalose is a non-reducing disaccharide which is able to protect the integrity of cells against a variety of environmental stresses such as dehydration, heat, cold and oxidation [20]. The extender containing trehalose improved antioxidant action and reduced the oxidative stress induced by cryopreservation [21]. So, we have added in 25 mM trehalose to tris extender. The novelty of this study was to evaluate the effects of four antioxidants added to this modified tris extender prior to cryopreservation on post-thawing microscopic sperm parameters (motility, acrosome and total abnormalities, HOS test), antioxidant activities (GPx, LPO, GSH, CAT and Total antioxidant activity) and DNA integrity of frozen-thawed Brown-Swiss bull semen.

MATERIAL and METHODS

Chemicals

All chemicals used in this study were obtained from Sigma-Aldrich Chemical Co. (Interlab Ltd., Ankara, Turkey).

Animals, Semen Collection and Semen Processing

The research materials belong to the Lalahan Livestock Central Research Institute (Ankara, Turkey) and they were fed under the same conditions. A total of 24 ejaculates were obtained from the three Brown-Swiss bulls (between 3-4 years of age) with the aid of an artificial vagina twice a week, according to AI standard procedures. Immediately after collection, the initial semen characteristics (ejaculate volume, sperm motility and concentration) were microscopically evaluated using routine laboratory procedures and methods described by Tuncer et al.[1]. The ejaculates comprising sperm cells which had more than 80% forward progressive motility and concentrations higher than 1.0 x 10^9 spermatozoa/ml were used in the study. The ejaculates were pooled in a warm water bath at 35°C until they were evaluated in the laboratory. A modified Tris-based extender (T) (189.5 mM of Tris, 63.2 mM of citric acid, 55.5 mM of fructose, 25 mM trehalose, 20% egg yolk (v/v) and 1000 ml of distilled water at a pH of 6.8) was used as diluents. Each ejaculate was divided into five equal parts. After that, they diluted to a final concentration of 60 x 10^6 ml^{-1} spermatozoa with the Tris + 25 mM trehalose base extender containing fetuin 1 mg/ml, dithioerithritol 1 mM, cysteamine 4 mM or linoleic acid 0.5 ml/50 ml, and no additives (control). Diluted semen samples cooled down to 4°C in 4 h and afterwards they were inserted in a digital freezing machine (Digitcool 5300 ZB 250; IMV) and were 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. Subsequently the straws were put into liquid nitrogen. The straws were stored in liquid nitrogen at least 24 h and thawed in a water bath (37°C) during 20 s.

The experimental procedures were approved by the Animal Care Committee of Istanbul University, Faculty of Veterinary Medicine (number and date: 2006/172 and September 27, 2006).

Assessment of Sperm Quality

A computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, Beverly, MA, USA) was also used to analyse sperm motion characteristics. The method was described by Büyükleblebici et al.[22]. We used accurate identification of sperm is easy with use of the IDENT Stain - a specialized, DNA-specific, fluorescent dye that it is CASA analysis to avoid the effects of egg-yolk particles on sperm discernibility. This method was described in Hamilton Thorne website [23]. For the evaluation of sperm abnormalities, at least three drops of each sample were placed into Eppendorf tubes containing 1 ml Hancock solution [24]. One drop of this mixing was placed on a slide and covered with a coverslip. The percentages of total sperm abnormalities (acrosome and other abnormalities) were ascertained by counting a total of 200 spermatozoa under phase-contrast microscope (x1000, oil immersion, Olympus BX43, Tokyo, Japan). The integrity of the sperm membrane tail was evaluated using the hypo-osmotic swelling test (HOS test). A hypotonic solution containing fructose and sodium citrate was prepared with an osmolality of 100 mOsm/kg. A 30 µl of prewarmed (37°C) semen was mixed with 300 µl of the hypotonic solution and incubated at 37°C for 60 min. Smears were prepared from the incubated semen sample; a minimum of two hundred cells were counted under phase-contrast microscope (x400, Olympus BX43) [25]. The swelling is characterized by a coiled tail, indicating that the plasma membrane is intact. Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which was performed at high alkaline conditions. The method described by Tuncer et al.[26]. The images of 100 randomly chosen nuclei were examined using a fluorescent microscope at a magnification of 400x (Zeiss, Germany). The percentage of the total DNA in the comet tail was taken as a measure of DNA break frequency. Tail DNA (%) was assessed in 100 cells by using Comet Assay III image analysis system (Perceptive Instruments, UK). Analysis was done blindly by one slide reader.

Biochemical Assays

Semen samples were thawed in 37°C water for 20 s and they were centrifuged at 4°C at 1.000 x g for 15 min in order to separate spermatozoa. Pellet was washed 3 times with a 0.5 ml of PBS. This final solution was homogenized 5 times by sonication in cold for 15 s for the Lipid Peroxidation Analysis (LPO), 120 µl of homogenate was mixed with 10 µl 0.5 mM butyl hydroxyl toluene (BHT) and kept in –80°C until the analysis. The rest of the homogenate was centrifuged at 8.000 x g for 15 min and the supernatant was separated and kept in –80°C for the other enzyme analysis. The levels of lipid peroxidase (LPO) were measured with the commercial LPO-586TM Oxis research kit, glutathione peroxidase (GPx) levels with GPx-340TM Oxis research kit, superoxide dismutase with Sigma–Aldrich Fluka FL 19160 kit, catalase (CAT) with Oxisresearch TM Catalase-520TM kit, GSH (glutathione) with Oxisresearch-420TM kit and antioxidant capacity with Sigma-Aldrich Antioxidant assay CS 0790 kit with spectrophotometric analysis. The assessment of sperm biochemical assays was investigated using spectrophotometric analysis by the method of previously described by Taşdemir et al.[27].

Statistical Analysis

The study was replicated eight times. The results were expressed as mean ± SEM. Data set is normally distributed using the Shapiro Wilk normality test. Homogeneity of variances with Levene's test groups was compared. The test revealed that the variances were homogeneous. After that, comparisons between the groups were made variance with Duncan post hoc test using the SPSS/PC computer programme (version 14.1, Chicago, IL). The $P<0.05$ value was considered as significant.

RESULTS

The effects on the spermatological parameters of different antioxidant additives in the cryopreservation of Brown-Swiss bull semen are presented in *Table 1*, group dithioerithritol and linoleic acid resulted the higher rates of CASA progressive motility (21.88±1.29% and 22.75±1.35%; $P<0.05$), CASA sperm motility (47.25±2.58% and 49.75±2.80%; $P<0.05$), respectively compared to the other groups. Group linoleic acid provided the better protective effect for acrosome ($P<0.01$) and total abnormalities ($P<0.05$). Also, group dithioerithritol showed the highest values for the HOST (49.88±0.35%; $P<0.01$). In the comet test, group dithioerithritol enhanced tail length; group fetuin, dithioerithritol and linoleic acid also enhanced tail movement intensity and tail moment with lesser chromatin damage than the other groups ($P<0.05$).

As set out in *Table 2*, when fetuin or cysteamine was added to the extender, sperm motion characteristics such as VAP ($P<0.05$), VSL ($P<0.05$) and VCL ($P<0.01$) had significantly decreased. No significant differences were observed among the groups for ALH and STR values ($P>0.05$).

As shown in *Table 3*, there is no significant differences observed in the level of GPx and LPO activities among the groups ($P>0.05$). Supplementation of dithioerithritol significantly affected the GSH activity ($P<0.01$). Also dithioerithritol and linoleic acid groups gave higher CAT values than other groups ($P<0.05$). Additionally, supplementation of fetuin and Cys showed the lowest total antioxidant activity value ($P<0.05$).

Table 1. *Mean (±SE) sperm values in frozen thawed bull semen*
Tablo 1. *Dondurulmuş çözdürülmüş boğa spermasında ortalama spermatolojik değerler*

Groups	C	F (1 mg/ml)	D (1 mM)	Cys (4 mM)	L (0.5 ml/50) ml	P
Progressive Motility (%)	18.88±1.04^{b}	8.00±0.70^{c}	21.88±1.29^{a}	13.00±3.26^{c}	22.75±1.35^{a}	*
Motility (%)	40.00±2.28^{b}	34.50±4.52^{c}	47.25±2.58^{a}	29.38±4.92^{c}	49.75±2.80^{a}	*
Acrosome (%)	5.38±0.32^{c}	6.50±0.54^{d}	4.25±0.25^{b}	4.00±0.10^{b}	2.63±0.18^{a}	**
Total Abnormality (%)	16.50±0.33^{c}	15.13±0.97^{c}	14.38±1.07^{b}	15.50±0.82^{c}	12.63±0.37^{a}	*
HOST (%)	40.25±0.37^{c}	32.75±0.68^{d}	49.88±0.35^{a}	38.88±0.35^{c}	45.63±0.60^{b}	**
Tail length	74.93±7.28^{c}	55.31±6.28^{b}	48.07±9.24^{a}	73.73±8.85^{c}	62.33±5.90^{b}	*
Tail intensity (%)	19.06±2.24^{b}	14.86±2.76^{a}	13.27±0.66^{a}	18.22±2.34^{b}	14.56±0.65^{a}	*
Tail moment (μm/s)	8.29±2.14^{b}	4.77±2.00^{a}	3.70±0.57^{a}	8.37±1.99^{b}	4.86±0.77^{a}	*

*$^{a, b, c, d}$ Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05)*
***C:** Control, **F:** Fetuin, **D:** Dithioerithritol, **Cys:** Cysteamine, **L:** Linoleic acid*

Table 2. *Mean (±SEM) CASA sperm motion charecteristics in frozen–thawed bull semen*
Tablo 2. *Dondurulmuş çözdürülmüş boğa spermasında ortalama CASA sperm hareket özellikleri*

Groups	C	F (1 mg/ml)	D (1 mM)	Cys (4 mM)	L (0.5 ml/50) ml	P
VAP (μm/sec)	99.63±2.34^{a}	85.83±5.15^{b}	99.00±2.20^{a}	77.00±4.81^{c}	98.31±3.14^{a}	*
VSL (μm/sec)	77.50±1.65^{a}	60.25±1.42^{b}	77.88±1.42^{a}	63.25±3.14^{b}	75.91±1.96^{a}	*
VCL (μm/sec)	155.63±4.49^{a}	143.75±8.82^{b}	158.25±3.53^{a}	108.25±8.10^{c}	157.71±6.07^{a}	**
ALH (μm)	6.50±0.27	8.88±6.31	6.63±0.18	5.75±0.25	6.86±0.18	-
BCF (Hz)	10.00±0.46^{c}	13.25±1.11^{a}	12.25±0.31^{abc}	12.63±1.69^{abc}	11.64±0.96^{abc}	*
STR	67.63±1.02	68.38±1.96	69.63±8.82	76.50±2.69	76.88±1.01	-
LIN (%)	51.63±1.19^{a}	42.75±1.07^{b}	51.00±0.54^{a}	49.75±2.45^{a}	50.00±1.09^{a}	*
Elongation	39.00±0.71^{b}	45.13±2.51^{a}	38.38±0.94^{b}	38.38±0.65^{b}	38.38±0.75^{b}	*

*$^{a, b, c}$ Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05)*
***C:** Control, **F:** Fetuin, **D:** Dithioerithritol, **Cys:** Cysteamine, **L:** Linoleic acid*

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. *Dondurulmuş çözdürülmüş boğa spermasında ortalama glutatyon peroksidaz (GPx), lipit peroksidaz (LPO), redükte glutatyon (GSH), katalaz (CAT) ve total antioksidan değerleri*

Analysis	C	F (1 mg/ml)	D (1 mM)	Cys (4 mM)	L (0.5 ml/50) ml	P
GPx (mU/ml-10^{9} cell/ml)	9.99±0.09^{a}	10.00±0.10^{a}	13.00±0.19^{b}	12.00±0.60^{b}	13.00±0.65^{b}	*
LPO (μm/ml-10^{9} cell/ml)	0.50±0.27	0.75±0.37	0.63±0.32	0.13±0.13	0.48±0.22	-
GSH (μm/ml-10^{9} cell/ml)	15.75±0.88^{d}	20.13±2.84^{bc}	36.63±11.87^{a}	19.25±1.33^{bc}	24.33±4.97^{b}	**
CAT (μm/ml-10^{9} cell/ml)	8.88±2.82^{b}	2.13±0.61^{c}	11.50±5.14^{a}	2.50±0.65^{c}	11.92±3.05^{a}	*
Total antioxidant activities (mmol/ trilox/ml-10^{9} cell/ ml)	10.25±1.74^{bc}	6.63±1.41^{bc}	18.50±3.52^{a}	8.13±1.54^{bc}	13.88±2.75^{ab}	*

*$^{a, b, c, d}$ Different superscripts within the same row demonstrate significant differences (** P<0.01, * P<0.05*
***C:** Control, **F:** Fetuin, **D:** Dithioerithritol, **Cys:** Cysteamine, **L:** Linoleic acid*

DISCUSSION

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 acrosome and plasma membrane integrity and structural damage to DNA [2,28]. The sperm cryopreservation procedure, which involves the decrement in temperature, cause oxidative stress and impress the sperm surface. This results in irreversible damage to the sperm organelles and modifies in enzymatic activity, related with a decrease in sperm motility, membrane integrity and fertilizing ability [29]. In the current study, addition of different doses of antioxidants significantly increased the percentages of post-thaw sperm progressive and CASA motilities, except for fetuin and cysteamine. Contradict with our results; Başpınar et

al.[30] reported that when dithioerithritol was suplemented to semen extender, it did not produce a beneficial effect on sperm motility. Moreover, according to Çoyan et al.[31], the supplementation of dithioerithritol did not improve bull sperm motility during liquid storage. Its declared that, dithioerithritol had no significant improvement in motility characteristics, and CASA and progressive motilities of bovine sperm [32]. After freeze-thawing process, motility analysis did not give enough data for the evaluation of sperm. Sperm morphology has major importance for fertilizing capacity and the assessment of membrane functions. Non-motile sperm would have an intact plasmalemma and acrosome and morphology integrity [7]. In our study, acrosome abnormalities decreased in the presence of antioxidants in the extender, except for fetuin. Total abnormalities also decreased in the presence of dithioerithritol and linoleic acid. These results were in agreement with those reported by some researchers, suggesting that antioxidants could decrease the sperm acrosome and total abnormalities for bovine [27,32,33] and for goat [26]. The sperm plasma membrane is sensible to peroxidative damage with accompanying loss of membrane integrity, reduced sperm motility, and finally loss in fertility [34]. According to Büyükleblebici et al.[35], addition of 25 mM trehalose beneficially effected acrosome morphology and total abnormalities in Tris extenders. This study was assumed to determine which antioxidants, would afford the most effective protection against membrane damage during the freeze–thawing process. The results presented clearly showed that dithioerithritol and linoleic acid provided the strongest protective effect against cryodamage. Bucak et al.[36] showed that supplementing antioxidant to the diluent had positive effect on plasma membrane integrity. In contrast with our study, addition of 5 mM dithioerithritol did not have any positive effect on acrosome and total abnormality and plasma membrane integrity [22]. The COMET assay is a widely applied technique for measuring and analysing DNA breakage in individual cells [37]. In this study, by the addition of the antioxidants fetuin, dithioerithritol and linoleic acid, DNA integrity was well-kept, compared to the control group. These results were consistent to those reported in a previous research on bovine sperm [32]. On the other hand, the antioxidants which were used had no effects on upgrading at DNA integrity [27]. Adding cysteamine to the diluent had cryo-protective effect on goat [15] and ram [38] semen collected by artificial vagina. However, according to another study, there were no favorable effects of cysteamine on ram semen quality before or after freezing in which sperm was collected by electro-ejaculator [39]. On the basis of our results, we may hypothesize that the differences among our results may be associated with the types and amounts of antioxidants that were used. Several studies have been reported contradict with our findings. In terms of post-thaw sperm motion kinematic characteristics (VAP, VSL, VCL, ALH and BCF), our results were in accord with those reported by Taşdemir et al.[37] who demonstrated that using ethylene glycol and dimethyl sulfoxide as cryoprotectant instead of glycerol did not give marked effect on sperm motion kinematic characteristics. In contrast with our study, Tris-based extender containing sucrose reduced sperm motion kinematic parameters [40]. Based on our findings, we may hypothesize that these differences among the studies may be due to the turbidity of other substances in Tris based extender.

Oxidative stress commonly causes deprivation of motility, enlargement and the blebbing of the acrosomal membrane and disruption or gained permeability of the plasma membrane of spermatozoa [41]. Morphologic damage of the plasma membrane increases the susceptibility to LPO when high ROS was produced during the freeze-thawing process. This was declared for boar [6], buck [26], ram [30] and bull [37] sperm. When cells are frozen, they are subjected to various stresses such as cold shock and oxidative stress that arise through ice crystallization and LPO due to membrane changes [26]. Sperm cells and seminal plasma contain ROS scavengers, including the enzymes SOD, GSH-PX and catalase, which convert superoxide (O_2^-) and peroxide (H_2O_2) radicals into O_2 and H_2O. SOD and catalase also transfer (O_2^-) generated by NADPH-oxidase in living cells [42]. However, GSH-Px removes peroxyl radicals from different peroxides, including H_2O_2, to better sperm motility [43]. GPx is a selenocysteine containing antioxidant enzyme that participates in a role in the elimination of hydrogen peroxide and is also recognized to be involved in the detoxification of reactive lipids [8]. In the current study, supplementation of antioxidants had better GPx activity except for fetuin. According to Sarıözkan et al.[44], addition of 2.5 and 7.5 mM of cysteamine to the sperm diluent procured a higher rise in GPx antioxidant enzyme activities. It's declared that, addition of 2 mM dithioerithritol improved GSH and GPx activity [31]. In the present study, the supplementation of antioxidants elevated GSH levels after thawing. These results were consistent with the study of Bucak et al.[45], which they researched ram sperm in which an increase of total GSH level was reported in the presence of GSH for the frozen state or during storage. They were also discrepant with the studies in goat [8] and ram [38] sperm, in which total GSH levels were not elevated when, had been cryopreserved or stored with various antioxidants. Additionally, De Matos et al.[46], revealed that the supplementation of cysteamine in the maturation media concluded with elevate in GSH activity.

In conclusion, Group dithioerithritol and linoleic acid have result the higher rates of CASA progressive motility and CASA sperm motility. Group linoleic acid provided the better protective effect for acrosome and total abnormalities. Also, group dithioerithritol showed the highest values for the HOST. In the comet test, group dithio-erithritol enhanced tail length; group fetuin, dithioerithritol and linoleic acid also enhanced tail movement intensity and tail moment with lesser chromatin damage than

the other groups. Supplementation of dithioerithritol significantly affected the GSH activity. Also dithioerithritol and linoleic acid groups gave higher CAT values than the other groups. We may said that, the addition of antioxidants, specially dithioerithritol and linoleic acid, to added with 25 mM trehalose Tris extender improve post-thaw sperm parameters.

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