RESEARCH ARTICLE

F.U. Vet. J. Health Sci.
2018; 32 (2): 135 - 142
http://www.fusabil.org

Effects of Some Antioxidant Additives on Spermatological Parameters, Oxidative Stress and DNA Damage After Freezing-Thawing Process in Ram Semen *, **

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In this study, determing the effects of antioxidants on the computer assisted sperm analysis (CASA), oxidative stress parameters and the DNA damage of the thawed sperm was aimed. Semen collected from 10 Pirlak rams was divided into five equal aliquots and diluted with the tris base extender containing taurine, trehalose, trolox, lipoic acid and no antioxidant (control), cooled to 5 ^{0} C and frozen in 0,25 mL French straws in liquid nitrogen vapour and stored in liquid nitrogen. In the thawed semen, the highest values were detected in taurine group with regard to subjective motility and CASA motility, average path velocity µm/s (VAP) and straight linear velocity µm/s (VSL). The lowest values in spermatozoa with abnormal head, middle piece and acrosome were found in all antioxidant groups when compared with the control group. The increases in hypoosmotic eosin staining test (H+/E- type) spermatozoa were significant (P<0.05) in taurine, trehalose and lipoic acid groups compared with the control group. The lowest value in DNA damage was observed in trehalose and lipoic acid groups. In terms of malondialdehyde (MDA), lipoic acid and trolox, which had the lowest value, indicating that it provided preservation. In conclusion, the efficacies of taurine, trehalose, lipoic acid and trolox that were used as antioxidants for freezing ram semen show differences, and lipoic acid is more effective on reducing oxidative stress and DNA damage.

Key Words: *Antioxidant, DNA damage, H-E test, ram semen, oxidative stress*

Koç Spermasına Katılan Bazı Antioksidanların Dondurma ve Çözdürme Sonrası Spermatolojik Parametreler, Oksidatif Stres ve DNA Hasarı Üzerine Etkileri

Bu çalışmada, spermaya katılan antioksidanların dondurma çözdürme sonrası spermatolojik, bigisayar destekli sperma analiz cihazı (CASA), oksidatif stres parametreleri ve DNA hasarları üzerine etkilerinin belirlenmesi amaçlandı. Afyonkarahisar koşullarında yetiştirilen 2-3 yaşlı Pırlak ırkına ait toplam 10 koçtan alınan spermalar birleştirilip 5 eşit hacme bölünerek kontrol başta olmak üzere taurin, trehaloz, trolox ve lipoik asit içeren Tris bazlı sulandırıcılar ile sulandırıldı. Sulandırılan örnekler 0.25 mL'lik payetlerde 5 ^{0}C'de 3 saat ekilibrasyona tabi tutulduktan sonra sıvı azot buharında dondurularak sıvı azot içerisinde (-196 ^{0} C) saklandı. Çözdürülen spermalarda subjektif motilite, CASA motilitesi, ortalama rota hızı µm/s (VAP) ve düz çizgi hızı µm/s (VSL) açısından taurin grubunda en yüksek değerler tespit edildi. Anormal spermatozoon baş, orta kısım ve akrozom oranı açısından kontrol grubuna göre tüm gruplarda en düşük değerler elde edildi. Spermatozoon hipo-ozmotik eosin boyama testi (HE-test) parametrelerinden H+/E- tip spermatozoonlarda kontrol grubuna göre taurin, trehaloz ve lipoik asit gruplarındaki artışlar önemli (P<0.05) bulunmuştur. DNA hasarında en düşük değer yine kontrol grubuna kıyasla trehaloz ve lipoik asit gruplarında gözlendi. Oksidatif stres parametrelerinden malondialdehit (MDA) yönünden lipoik asit ve trolox en düşük değeri göstererek koruma sağlamıştır. Sonuç olarak koç spermasının dondurulmasında antioksidan olarak kullanılan taurin, trehaloz, lipoik asit ve trolox'un etkinliklerinin farklılık gösterdiği bunun yanında lipoik asitin gerek oksidatif stres ve gerekse DNA hasarını azaltıcı etkisinden dolayı diğerlerine nazaran üstünlük sağladığı belirlendi.

Anahtar Kelimeler*: Antioksidan, DNA hasarı, H-E testi, koç sperması, oksidatif stres*

Received : 22.06.2018
Accepted : 18.07.2018

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Introduction

Nowadays, freezing spermatozoa, the male gamete cells that constitute a significant part of the genetic material, has become an extremely important tool for the genetic protection of endangered, valuable and unique animals (1)

Cryoprotectants are used as preservatives against freezing, to decrease the damage caused by cold shock, osmotic change, intracellular crystal formation, and free oxygen radicals that occur as a result of physical, chemical and oxidative stress during the cooling, freezing and thawing of the cell (2). Reactive oxygen species (ROS) directly lead to lipid peroxidation (LPO) and also cause the apoptosis and death of the cell by leading to the irreversible stoppage of spermatozoa motility and protein damage with nucleic acid (3). The membrane lipid phase change occurring during the freezing-thawing of sperm, the LPO that develops due to osmotic-mechanical stress and free

* 7. National Reproduction and Artificial Insemination Congress, 1-4 July 2013, Kars/TURKEY.

** The project numbered 10.VF.15 was supported by Scientific Research Project Coordination Unit of Afyon Kocatepe University. This article was summarized from Phb thesis entitled "The effects of some antioxidant additives on spermatological parameters, oxidative stress and DNA damage after frozen-thawed process in ram".

oxygen in the medium, denaturation in membrane proteins, structural deformation in cell organelles, fragmentations in DNA, the addition of cellular lysis antioxidants, and improve spermatozoon functions after thawing (4).

The cells use antioxidants as a defence mechanism to resist the negative effects of ROS, and there needs to be a balance between these two events. Structural and functional disorder occurs in the cell, along with an increase in the amount of ROS due to the disruption of balance between ROS and antioxidants, which is called oxidative stress or damage (5). It is reported that some antioxidants added to sperm diluents minimize the oxidative damage caused by LPO occurring in the medium during short- and long-term preservation, protect viability, and improve the quality and function of spermatozoa (4).

When taurine, which is a sulphonic amino acid, is exposed to the aerobic conditions and the freezing-thawing of spermatozoa, it plays an important role in preservation by acting as a non-enzymatic scavenger against ROS (6). Taurine exhibits its effects by being involved in the motility, capacitation and osmoregulation of spermatozoa, and the antioxidant mechanism (7). It is known that trehalose reduces the release of ROS, which can penetrate into the spermatozoon plasma membrane and provide the surface width of membrane phospholipids by forming hydrogen bonds with polar head groups during freezing and thawing, and the consumption of reduced glutathione (GSH) in spermatozoon and sperm plasma. Also, trehalose has a scavenging effect via trapping free radicals and alleviating GSH consumption by the enzymatic antioxidant defences. In this way, a decrease of GSH oxidation and LPO might be a consequence of reduced ROS levels. It also ensures protection against cold shock by acting as an osmotic buffer between the cell and the medium (8). α-tocopherol, which is fat-soluble and the most important chain-breaking antioxidant in the cell membrane, decrease LPO by increasing the motility and viability of spermatozoa. Vitamin E has a protective characteristic against oxidative stress and delays the cell death. Vitamin E, as a reducing agent, has the effect of inhibiting the functions of ROS in the cell (9). The most important duty of α-tocopherol, which is a very potent antioxidant, is to protect the unsaturated fatty acids in the membrane lipids against the attacks of free oxygen species (Rice 10). Alpha lipoic acid (ALA) is a disulphide compound and is present in mitochondria as a coenzyme for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. The addition of this agent exogenously increases the level of free ALA, which ensures a decrease in oxidative stress, both in vivo and in vitro, and enables it to function as a potent antioxidant. ALA is an antioxidant that is both water- and fat-soluble, that decreases free radicals, including LPO, in the cell membrane and is as effective as mitochondria-derived free radical scavengers (11).

This study aims to contribute to the development of the existing freezing method by determining the effects of different antioxidants added to sperm diluents on subjective spermatological as well as objective spermatological by assessing Computer Assisted Semen Analysis (CASA) parameters, oxidative stress and DNA damage after freezing-thawing, along with the effectiveness of antioxidants in minimizing the damages that may occur after thawing.

Material and Method

Semen Collection, Processing, Extending, Freezing and Thawing

Ten 2-3-year-old Pirlak (Daglic x Kivircik) rams raised at Afyon Kocatepe University Animal Husbandry Application and Research Center were used in this study. The study's experimental design was approved by the Animal Care and Ethics Committee Afyon Kocatepe University Veterinary Medicine Faculty in terms of ethics, with the authorisation number B.30.2.AKÜ.0.9Z. 00.00/10. The semen was collected using an artificial vagina from ten rams. Semen was collected from each ram regularly twice a week during the breeding season (autumn to early winter) and this process was repeated twenty four times (24 replication). Ejaculates which met the following criteria were evaluated: volume of 0.5–2 mL; minimum sperm concentration of $3x10^9$ sperm/mL; motility of 80%. In the study, Tris (Tris 3.63 g/100 mL, citric acid 1.99 g/100 mL, fructose 0.5 g/100 mL, egg yolk 15% (v/v), glycerol 5% (v/v), pH 6.8, osmolality 307 mOsm/kg) was used as a diluents at 37 °C, and then 5 different groups were formed by adding 100 mM Trehalose, 50 mM Taurine, 1 mM Trolox and 1 mM Lipoic acid and without adding any antioxidant (control). The semen samples that were taken into separate tubes were pooled in a tube and divided into 5 equal aliquots. The semen samples were diluted with the pre-prepared diluents containing different antioxidants with 5% glycerol by being dosed as $5x10^8$ sperm/mL. Following the dilution process, the samples were loaded into 0.25 ml straws and equilibrated at 5 °C for 3 hours, and then they were frozen in nitrogen vapour 4 cm above the liquid nitrogen, for 15 minutes and plunged into liquid nitrogen (-196 °C) until in vitro evaluation. After the straws were stored for one year, they were separately thawed in a water bath at 37 °C for 25 seconds for a microscopic evaluation.

Semen Evaluation

Evaluation of Subjective and CASA Motilities: For the subjective motility determination, 5 µL drop of semen was placed on the heated stage of a phase-contrast microscope set at 37 °C with a 2.9% solution of sodium citrate buffer ($NaH_2PO_4.H_2O$) on a microscope slide, and the coverslip was covered with 45^0 angle. The subjective motility assessments were performed in at least five microscopic fields (400 magnification) for the determination of total motility and was recorded as a percentage (%). CASA (Version 12 IVOS, Hamilton-Thorne Biosciences, Beverly, MA, USA) was used in the evaluation of the objective motility and movements of spermatozoa. Before starting the evaluation, CASA phase contrast was set at 60 Hz frame rate, minimum contrast -70, small and large static size entries -0.64.32, small and large density entries -0.20–1.92, small and

large extension entries 7–91, default cell size -10 pixels and default cell density-80. The thawed semen was immediately evaluated by being diluted with glycerol and egg yolk-free buffer tris diluent (5 µL semen + 95 µL diluent). 4 µL of the diluted semen sample was put on a preheated compartment microscope slide (Leja 4, Leja Products, Luzernestraat B.V., Holland), and the motilities and movement characteristics of spermatozoon were determined at 37 °C under 200 magnification. The values of total motility (%), progressive motility (%), VAP (average path velocity, µm/s), VSL (straight linear velocity, µm/s), VCL (curvilinear velocity, µm/s) and ALH (lateral head displacement, µm amplitude), BCF [beat-cross frequency rhythm Hertz (Hz)] were recorded as motility and movement characteristics. For each evaluation, at least 300 cells were examined in 10 microscopic fields.

Assessment of Sperm Abnormality and Acrosome Integrity: Abnormal spermatozoon and acrosome ratios in sperm samples were determined by the liquid fixation method (12).

Assessment of Sperm Plasma Membrane Integrity: The HE-test, which is called the Hypo-osmotic Eosin staining test in which the dead-living spermatozoon ratio and hypo-osmotic swelling test are applied together, was used in sperm samples (13).

Biochemical Assay: For the purpose of evaluating the oxidative stress parameters at the end of thawing, the spermatozoa were washed 3 times with phosphate buffered saline (PBS) by being centrifuged at 800 g for 20 minutes with refrigerated centrifuge to separate them from the diluent, and then the supernatant was made up to 0.5 mL with PBS. For homogenization, the samples were taken to falcon tubes in ice, and the sonication process was repeated 6 times by keeping them in the ice for 30 seconds after a 10 second-sonication process. For the lipid peroxidation analysis, 120 µL homogenate was taken and 10 µL 0.5 mM BHT (butyl hydroxy toluene) (B1378 BHT, Sigma-Aldrich Co., St. Louis, USA) was added and mixed. All homogenates were stored at -20 °C until GSH, glutathione (GSH), glutathione peroxidase (GSH-Px**)**, catalase (CAT) and superoxide dismutase (SOD) analyses were performed.

Determination of MDA, GSH, SOD, CAT and GSH-Px Levels: The circulating MDA concentration an index of lipid peroxidation was measured by the double heating method of Draper and Hardley (14). The GSH concentration was measured using the method described by Sedlak and Lindsay (15). SOD activity was determined using commercial kit by Sigma-Aldrich Fluka FL19160 (Chemical Co. USA) by spectrophotometry. CAT activity was determined using commercial kit by Sigma–Aldrich CAT 100 (Chemical Co. USA) by colorimetric. GSH-Px activity was determined using commercial kit by Sigma-Aldrich CGP1 (Chemical Co. USA) by spectrophotometry.

Assessment of Sperm DNA Damage: Sperm DNA damage was investigated using the single cell gel electrophoresis (comet) assay, which is generally performed under high alkaline conditions (pH≥13). The evaluation was performed by the visual scoring method. DNAs with no damage were scored as 0, and the damaged DNAs were scored from 1 to 4 according to the degree of damage. The results were evaluated as arbitrary unit (AU) (13).

Statistical Analysis: The study was repeated twenty four times. Parameters were normally distributed according to the Shapiro–Wilk normality test. Homogeneities of variance with Levene's test groups were compared using the SPSS/PC computer programme (Version 13.0; SPSS/PC, Chicago, IL). The results were expressed as mean±SEM. Means were analyzed using a one-way analysis of variance, followed by Duncan's post hoc test to determine significant differences in all the parameters between the groups. Differences with values of $P<0.05$ were considered to be statistically significant.

Results

Spermatological Parameters After Freezing-Thawing: The findings related to subjective motility and CASA parameters that were obtained after thawing are presented in Figure 1. Accordingly, it was observed that the group containing taurine achieved a significant advantage ($P<0.05$) in terms of subjective motility compared to the control and other groups. In terms of the CASA motility, a decrease in the group containing Trolox was found to be significant ($P<0.05$) compared to the control group. In terms of BCF, a decrease in the group containing lipoic acid was found to be significant ($P<0.05$) compared to the control group.

Abnormal Spermatozoon Rates After Freezing-Thawing: The findings related to abnormal spermatozoon and acrosome obtained in this study are presented in Table 1. Accordingly, it was determined that the decrease in the groups containing trehalose and Trolox was significant ($P<0.05$) in terms of head abnormalities compared to the control group. Moreover, in terms of the mid-piece part the decrease in all groups containing antioxidants was significant ($P<0.05$) compared to the control group. Nevertheless, the decrease in all groups containing antioxidants was found to be significant ($P<0.05$) in terms of the abnormal acrosome ratio compared to the control group.

HE-Test Parameters After Freezing-Thawing: The findings obtained as a result of the HE-test are presented in Figure 2. Upon assessing the modified HOS Test, it was determined that the increase in the groups containing taurine, trehalose and lipoic acid was significant ($P<0.05$) in terms of the H+/E- ratio compared to the control group, the increase in the taurine group was significant ($P<0.05$) in terms of the H-/E- ratio compared to the control group, and the decrease in all groups containing antioxidants was significant ($P<0.05$) in terms of the H-/E+ ratio compared to the control group.

DNA Damage After Freezing-Thawing: The findings obtained in relation to DNA damage in this study and the variations of the groups are presented in Figure 3. Accordingly, the decreases in the groups containing trehalose and lipoic acid were found to be statistically significant ($P<0.05$) compared to the control group.

Oxidative Stress Parameters After Freezing-Thawing: The findings related to oxidative stress parameters are presented in Table 2. In comparison with the control group, the decreases in the group containing lipoic acid and Trolox in terms of spermatozoon MDA levels, in the group containing taurine, trehalose and Trolox in terms of CAT activity, and in the group containing trehalose, lipoic acid and Trolox in terms of GSH values were found to be statistically significant (P<0.05).

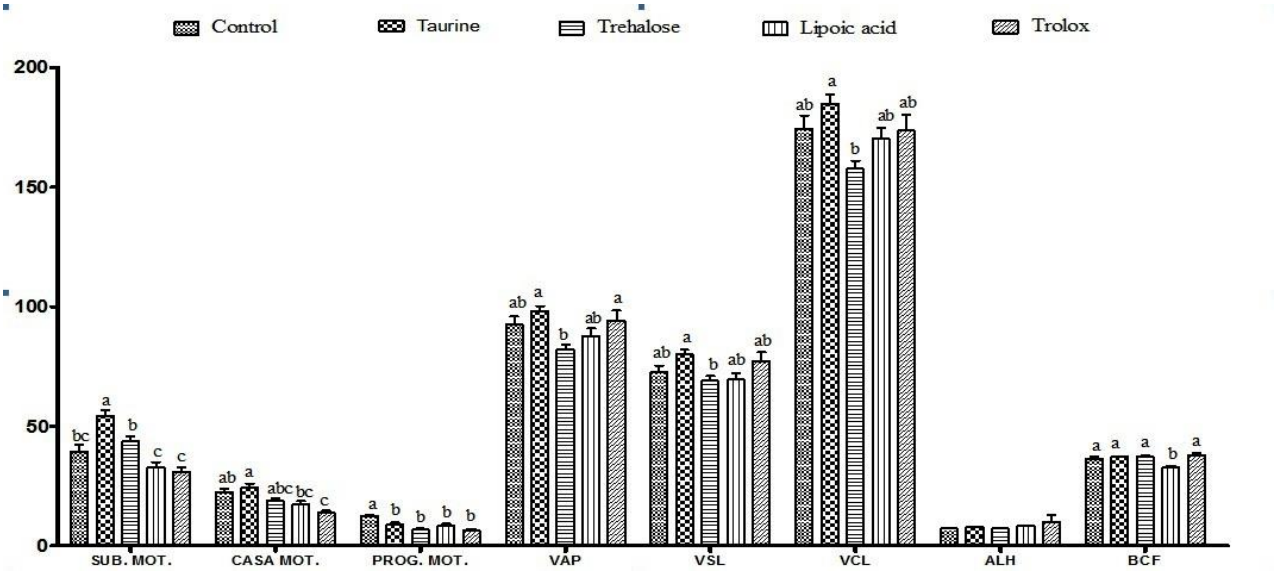

Figure 1. Effect of antioxidants on subjective motility and CASA parameters ram semen following freze – thawing ($\overline{X}$ ±SEM, n:24). a-c: Different letters are significant between control and antioxidant groups (P<0.05). SUB MOT.: Subjective motility, CASA MOT.: Casa total motility, PROG. MOT.: CASA progressive motility, VAP: Average path velocity, µm/s, VSL: Straight linear velocity, µm/s, VCL: Curvilinear velocity, µm/s, ALH: Lateral head displacement, µm amplitude, BCF: Beat-cross frequency rhythm Hertz (Hz).

Table 1. Abnormal spermatozoon rates after freezing-thawing ($\overline{X}$ ± SEM, n: 24)

Groups	Head (%)	Mid-Piece (%)	Tail (%)	Acrosome (%)
Control	1.2 ± 0.21^{a}	1.2 ± 0.21^{a}	8.7 ± 0.54^{b}	13.3 ± 0.70^{a}
Taurine	0.8 ± 0.15^{ab}	0.2 ± 0.08^{b}	9.2 ± 0.40^{b}	10.7 ± 0.51^{b}
Trehalose	0.5 ± 0.07^{b}	0.1 ± 0.04^{b}	13.1 ± 0.48^{a}	5.2 ± 0.26^{d}
Lipoic Acid	1.0 ± 0.12^{ab}	0.2 ± 0.07^{b}	9.7 ± 0.38^{b}	7.1 ± 0.41^{c}
Trolox	0.4 ± 0.08^{b}	0.1 ± 0.04^{b}	10.1 ± 0.39^{b}	3.6 ± 0.21^{d}
P	0.006	0.002	0.000	0.000

Values (Mean ± S.E.M.) with different superscripts (a and d) within the same column showed significant differences (P<0.05).

Table 2. Oxidative stress parameters after freezing-thawing ($\overline{X}$ ± SEM, n: 24)

Groups	MDA (nmol/mL)	SOD (10^{9}cells/mL)	CAT (µmol/min/mL)	GSH (mg/dL)	GSH-Px (nmol/min/mL)
Control	5.4 ± 0.33^{a}	30.3 ± 2.29	17.4 ± 0.80^{a}	6.1 ± 0.41^{a}	0.9 ± 0.08
Taurine	4.9 ± 0.41^{ab}	33.7 ± 1.78	14.4 ± 0.52^{b}	6.2 ± 0.68^{a}	0.9 ± 0.06
Trehalose	6.0 ± 0.71^{a}	38.9 ± 3.00	9.3 ± 0.58^{c}	4.1 ± 0.32^{b}	0.8 ± 0.07
Lipoic Acid	3.7 ± 0.21^{b}	31.1 ± 2.40	18.1 ± 1.02^{a}	3.5 ± 0.24^{b}	0.8 ± 0.01
Trolox	3.6 ± 0.24^{b}	29.9 ± 1.91	9.2 ± 0.39^{c}	3.5 ± 0.22^{b}	0.8 ± 0.03
P	0.000	0.040	0.000	0.000	0.851

Values (Mean ± S.E.M.) with different superscripts (a and c) within the same column showed significant differences (P<0.05).

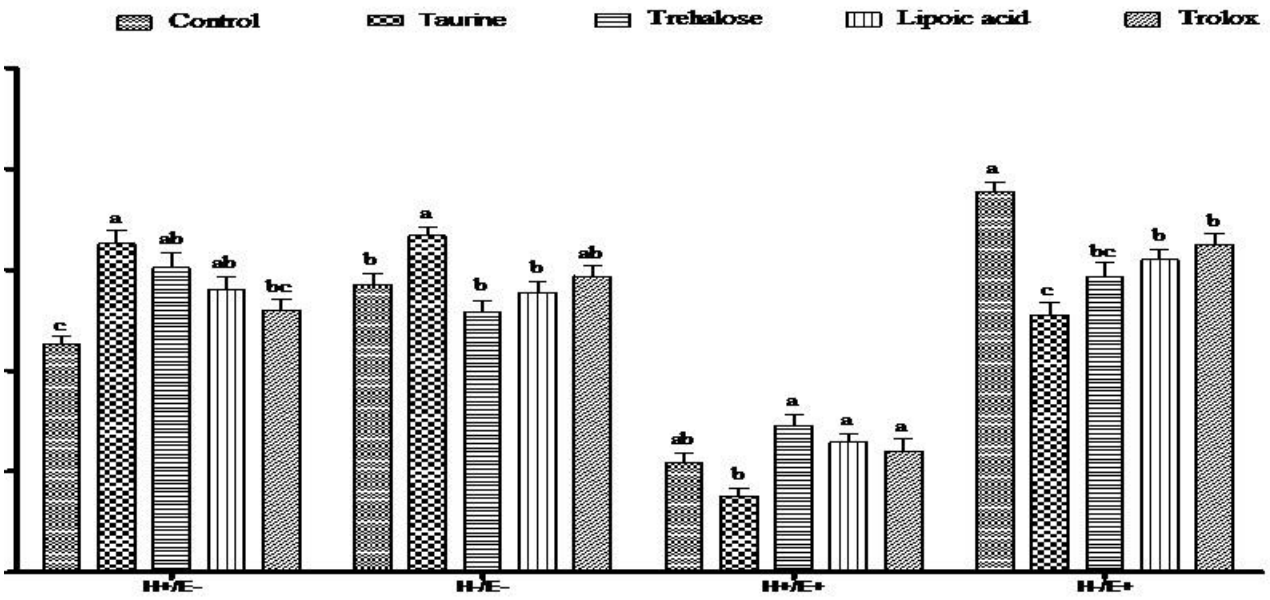

Figure 2. Effect of antioxidants on viability and hypoosmotic swelling test response ram semen following freze-thawing ($\overline{X}$ ± SEM, n:24). Values (Mean ± S.E.M.) with different superscripts (a and c) within the same column showed significant differences (P<0.05). H+/E-: tail swollen and head white; H-/E-: tail nonswollen and head white; H+/E+: tail swollen and head red; H-/E+: tail nonswollen and head red.

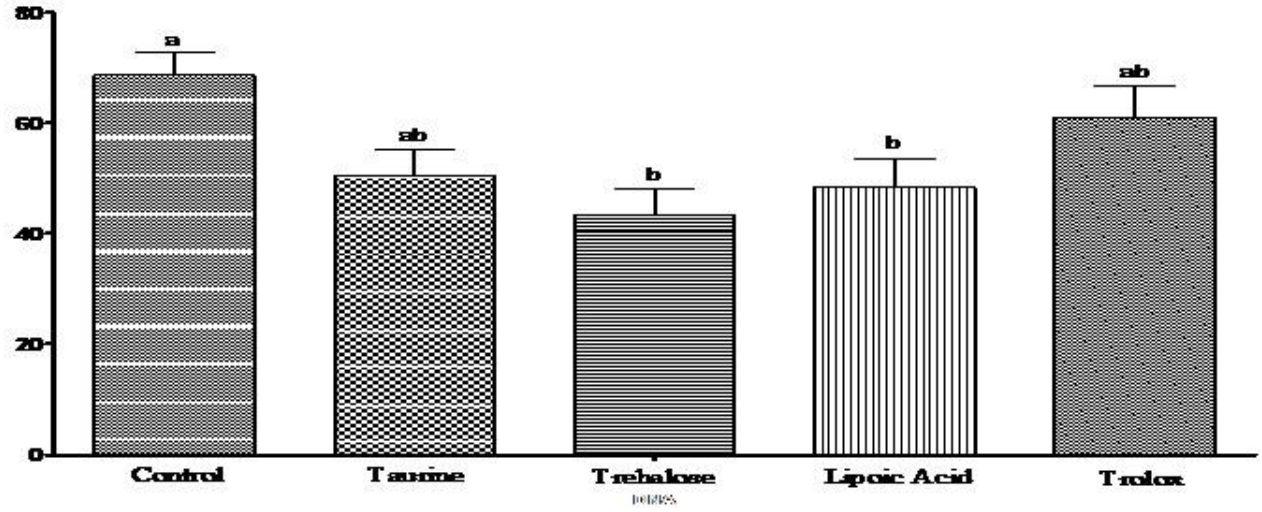

Figure 3. Effect of antioxidants on DNA damageram semen following freze-thawing ($\overline{X}$ ± SEM, n:24). Values (Mean ± S.E.M.) with different superscripts (a and c) within the same column showed significant differences (P<0.05).

Discussion

The most important factor that leads to infertility during the preservation of sperm is the peroxidation of spermatozoon membrane lipids. Free radicals such as superoxide radical, hydrogen peroxide and hydroxyl radical react with cell membranes and cause oxidative damage (16). The disruption of membrane integrity also leads to the permeability of the cell to electrolytes. The inclusion of calcium and partial sodium ions into the cell destroys the energy generation mechanism of the cell and causes adenosine triphosphate (ATP) to decrease. An intracellular calcium increase causes proteins and fats to be damaged by activating proteases and lipases. Furthermore, ROS also cause damage to DNA, which results in cell death (17). For this reason, during the freezing of ram semen, antioxidant substances are added to semen diluents because it is thought that it may have positive effects on spermatological parameters after thawing.

The number of motile spermatozoa is accepted to be an important parameter for fertility (18). Another factor ensuring that the motility in spermatozoa occurs properly is cyclic adenosine monophosphate (cAMP). The increase in cAMP concentration increases the protein kinase activation, so the axoneme protein becomes phosphorylated and causes an increase in the motility of spermatozoon and hyper activation (19). In this study, it was observed that the group containing taurine achieved a significant advantage ($P<0.05$) in terms of subjective motility after thawing compared to both the control and the other groups. The following research studies have different motility results than those obtained from the antioxidant groups in this study: the motility ratio that Başpınar et al. (20) obtained from the group containing alpha lipoic acid 1 mM of which was added to a tris-based Merino ram semen diluent; the motility ratio that Bucak et al. (21) obtained from 100 mM trehalose added to a ram semen diluent after thawing; the motility ratio that Anghel et al. (22) obtained from the group containing 1 mM vitamin E which was added to a ram semen diluent. The CASA that resulted from the need for an objective, accurate, reliable and repeatable spermatozoon analysis is commonly used for this purpose. The CASA system was developed to monitor the movements of spermatozoa under a photographic microscope many times (23). The fact that some of the values reported in relation to CASA parameters in this study paralleled those from other studies (24, 25) indicates that results across studies may be consistent. Furthermore, they are higher than the findings of some researchers (26, 27). It is thought that the differences observed in subjective motility and CASA parameters after thawing may be caused by the diluents used in the freezing process, the amounts and ratios of the components contained in the diluents, the changes in thawing time and temperature and the installation and software settings of the device on which the analysis was performed.

The morphological examination of the spermatozoon is important in terms of the abnormal spermatozoa having a low fertilization capacity and some hereditary disorders. There is a close relationship between the localization, variety and quantity of morphological disorders and fertility. In morphological examinations, the acrosome has a particular importance since it plays a critical role in fertilization (18). In the present study, while the findings obtained with regard to the abnormal spermatozoon ratio were found to be lower than the values of some researchers (21, 22), it was determined that the values obtained with regard to the acrosome ratio were close to the values reported by Uysal et al. (28) The differences observed in the abnormal spermatozoon and acrosome ratios obtained after the thawing process could be due to genotypic changes in rams, as well as factors such as breed, season and semen collection method.

Membrane integrity is of vital importance not only for spermatozoa metabolism but also for capacitation, acrosome reaction and the realization of the adsorption of sperm to the oocyte surface. For this reason, the HE-test, in which the osmotic tolerance and dead-living examination are combined, is one of the potential sperm function tests used in estimating fertility in humans and other mammals (29). According to the findings of the HOS test and the modified HE-test in which the dead-living spermatozoon ratio was determined in the study, the increases in the taurine, trehalose and lipoic acid groups were found to be statistically significant ($P<0.05$) in terms of the H+/E- value compared to the control group. This is the first study that evaluates the combination of osmotic tolerance and dead-living examination in cryopreserved ram semen.

Nowadays, the determination of spermatological characteristics, as well as spermatozoon DNA damage, is important for the estimation of male fertility and the sustainability of herd fertility. The comet assay has become a simple, versatile, fast, visual, sensitive and widespread test in which the cells can be evaluated individually (30). Since increased DNA damage is associated with infertility, defective embryonic development, implantation defects, and recurrent abortions, the determination of DNA damage at different levels between the spermatozoa of fertile and infertile males brings the usability of spermatozoon DNA damage as a marker in the evaluation of male fertility potential to the forefront. In particular, the high level of DNA damage in human spermatozoa is associated with infertility (31). The fact that DNA damage obtained from the control and antioxidant groups after thawing parallels the values of other researchers (32, 33) indicates that this study is consistent with similar studies. Furthermore, DNA damage was found to be lower when compared to the studies of some researchers (34, 35) and higher when compared to others (36, 37). Factors, such as breed, semen collection method, the diluents used in the freezing process and the amounts and ratios of the components contained in the diluents, can be considered among the reasons for differences observed in DNA damage after thawing. Factors, such as the difference of antioxidants added to semen diluents and the difference of techniques used in the dilution and freezing of semen may also play a role.

SOD, CAT, and GSH-Px are biological enzymatic antioxidants found in large quantities in seminal plasma. These enzymatic antioxidants protect the motility against LPO by converting superoxide and peroxide radicals into oxygen and water. Moreover, MDA formed as a result of LPO shows the degree of the membrane damage of the spermatozoon (5). According to the findings obtained with regard to oxidative stress parameters in the study, differences were observed between the groups in terms of the relevant parameters. In particular, the decrease in the groups containing lipoic acid and trolox was found to be significant ($P<0.05$) in terms of MDA compared to the control group. The MDA data obtained in this study are consistent with some studies (20, 21). The highest value in terms of GSH was obtained in the control group and in the group containing taurine, and the differences between them and the other trehalose, lipoic acid and trolox groups were found to be statistically significant ($P<0.05$). The findings of the present study in terms of

GSH is parallel the findings of some researchers (20, 21). These differences in oxidative stress parameters may have resulted from the factors, such as the differences in the techniques used in the dilution and freezing of semen, and the method used for the analysis of the antioxidant and doses used.

As a result, it was concluded that taurine, trehalose, lipoic acid and trolox used as antioxidants in the freezing of ram semen had different effectiveness; moreover, the lipoic acid achieved an advantage compared to the others because of the oxidative stress and its DNA damage-reducing effect.

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