Kafkas Univ Vet Fak Derg
21 (1): 81-86, 2015
DOI: 10.9775/kvfd.2014.11792

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

# The Effects of L-Ergothioneine, N-acetylcystein and Cystein on Freezing of Ram Semen [1][2]

Savaş YILDIZ [1] Yavuz ÖZTÜRKLER [1] Umut Çağın ARI [1]
Necdet Cankat LEHİMCİOĞLU [1] Emine ATAKİŞİ [2] Recai KULAKSIZ [1]

[1] *This study was supported by the TÜBİTAK-Tovag (Project No: 109O236)*
[2] *This study was presented as poster at the VII. National Reproduction and Artificial Insemination Science Congress on 1-4 July 2013, Kars, Turkey*

[1] Department of Artificial İnsemination, Faculty of Veterinary Medicine, University of Kafkas, TR36100 Kars - TURKEY
[2] Department of Biochemistry, Faculty of Veterinary Medicine, University of Kafkas, TR36100 Kars – TURKEY

**Article Code: KVFD-2014-11792 Received: 07.06.2014 Accepted: 17.09.2014 Published Online: 19.09.2014**

## Abstract

The aim of this study was to determine the influence of different doses of L-Ergothioneine (LE), cystein and N-acetylcystein (NAC) on post-thawing semen parameters in rams. Ejaculates collected from four Tushin Rams were evaluated and pooled at 35°C. Semen samples were diluted with skim-milk based extender containing LE (5, 10 mM), Cystein (5, 10 mM), NAC (5, 10 mM) or not containing any antioxidant (control) and loaded to 0.25 ml French straws. Straws were cooled to 5°C for 2 h, frozen in liquid nitrogen vapour (aprox. -120°C) for 15 min and then being stored in liquid nitrogen until thawing process. Straws were thawed in water bath (37°C for 1 min). The percentages of motility, viability, abnormal acrosome, total abnormalities, membrane integrity (hypoosmatic swelling test, HOST) were statistically assessed. Also total antioxidant capacity (TAC) and total oxidatif stres (TOS) were evaluated in samples from replications. It was seen that LE was superior to N-acetylcystein and cystein in motility, viability, defected acrosome, total morphological abnormalities, HOST and TAC, ($P<0.05$) except cystein in motility. Neverthless, there was not any statistically difference between LE and control groups ($P>0.05$). It was conlcluded that there was not any beneficial or detrimental effects of LE on post thawing semen parameters in rams while it was determined that cystein and NAC may have been some detrimental effects on post thawing semen parameters in rams. These results warrants future scientific studies on LE, NAC and cystein in ram semen cryopreservation.

***Keywords:*** *Ram semen, Cryopreservation, L-Ergothioneine, Cystein, N-acetylcystein, Tushin*

# Koç Spermasının Dondurulması Üzerine L-Ergothionin, N-asetil sistein ve Sisteinin Etkileri

## Özet

Bu çalışmanın amacı, koçlarda L-Ergothionin (LE), sistein ve N-asetil sisteinin (NAC) farklı dozlarının çözüm sonu sperma parametreleri üzerine etkisini belirlemekti. Dört Tuj ırkı koçtan alınan ejakülatlar değerlendirildi ve 35°C'de karışım yapıldı. Sperma örnekleri, LE (5, 10 mM), Sistein (5, 10 mM), NAC (5, 10 mM) içeren veya antioksidan içermeyen (kontrol), yağsız süt temelli sulandırıcıyla sulandırılarak, 0.25 ml'lik payetlere doldurulduu. Payetler 5°C'ye 2 saat süreyle soğutuldu, sıvı azot buharında (yaklaşık -120°C) 15 dak. süre ile donduruldu ve çözme işlemine kadar sıvı azot içinde saklandı. Payetler su banyosunda (37°C'de 1 dak.) çözüldü. Motilite, canlılık, anormal akrozom, toplam anormalite ve membran bütünlüğü (hipoozmotik şişme testi, HOST) yüzdeleri istatistiksel olarak değerlendirildi. Ayrıca, toplam antioksidan kapasite (TAK) ve toplam oksidatif stres (TOS), replikasyonlardan elde edilen örneklerde değerlendirildi. LE'nin motilite (sistein hariç), canlılık, akrozom hasarı, total morfolojik anormalite, HOST ve TAK oranları bakımından, NAC ve sisteine üstünlük sağladığı görüldü ($P<0.05$). Yine de, LE ve kontrol grubu arasında istatistiksel bir fark yoktu ($P>0.05$). Sistein ve NAC'ın koçlarda çözüm sonu spermatolojik parametreler üzerine bazı olumsuz etkileri belirlenirken, LE'nin olumlu ya da olumsuz etkisinin olmadığı sonucuna varıldı. Bu sonuçlar, koç spermasının dondurulmasında, LE, NAC ve sistein kullanılması üzerine yeni bilimsel çalışmaların yapılması gerektiğini göstermektedir.

***Anahtar sözcükler:*** *Koç sperması, Dondurma, L-Ergotiyonin, Sistein, N-asetilsistein, Tuj*

**İletişim (Correspondence)**
+90 474 242 6836/5247 GSM: +90 505 2635755
sfyzyildiz@gmail.com

## INTRODUCTION

Artificial insemination with frozen semen does not yet give satisfactory results in sheep. Therefore, for increasing fertility rates are needed in the development of new semen extenders or new compositions. In semen, cooling, freezing, thawing and premature activation in genital tract cause deterioration in membrane integrity and decreases the success of artificial insemination [1,2].

It is well known that ram semen is susceptible to freezing and thawing processes. So, in recent years, various antioxidants have been used in the freezing of ram semen. In ram semen, despite the positive effects of antioxidants on the motility and membrane integrity, previous results are not satisfactory to achieve in artificial insemination. Hence, the many studies have been continuing on this subject. Although there are many studies on effect of antioxidants, the avaliable information on the antioxidant capacity and lipid peroxidation in frozen ram semen is limited [1-3]. The investigations on the selection of the best diluent, antioxidant and dose have been continued by researchers [4-8]. Some literatures documented have shown that antioxidants supplemented to semen extenders have a positive effect on the success in cryopreservation [9-11]. Even synthetic antioxidants on sperm have been investigated [2]. Not only investigation and detection of ram semen characteristics, also the measuring the activity of antioxidant enzymes in ram semen has recently gained importance in researches. The measurement of antioxidant activity for better understanding of the effects of antioxidants gives important clues in terms of the success of freezing [9,10].

Recently, a thiol compound, LE, has been used to determine the antioxidant activity in in the storage of semen and shown to have positive effects on storage of ram semen [1]. However, the studies regarding use of antioxidants in the storage of semen is very limited. Nevertheless, it has been reported that N-acetylcysteine reduce reactive oxygen species and DNA damage in human sperm [11,12]. Also, It has been shown that L-Cystein hidrochloide, reducing oxidative stress in semen, is one of major thiol compounds which is involved in glutathione structure and decreases chromatin damage and also enhances the sperm viability [13].

The oxidative damage during freezing and thawing is induced by the reactive oxygen species (ROS) produced by cellular metabolism and sperm components. Hence, ROS is one of the main causes of decrease of motility and potential fertility in spermatozoa and comprises primary factor comprising oxidative stress. For these reasons, addition of antioxidant compounds to the culture media have become widespread in recent times [14,15].

However, according to current literature knowledged, it is seen that LE which has been much more important than the other antioxidants upto now and superior features of LE have not been effectively used in culture medium of oocytes and embryos. It has been known that LE is a fungal metabolite with antioxidant function in mammalian cells [16]. The preventing of oxidative stress induced by hydrogen peroxide in the presence of catalase and glutathione (GSH) consumption stability regulated by N-ethylmaleimide is realized by intracellular LE [17]. It had been investigated the effectiveness of LE on prevention of DNA damage and revention of cell deaths caused by hydrogen peroxyde. In this study, researches had concluded that LE could act as an *in vivo* non-toxic thiol buffer antioxidant and provide the oxidative stability in pharmaceutical preparations [18]. LE is known as an unique and single antioxidant amongst antioxidants which chelates heavy metals and protects of the cells (primarily erythrocytes) from ROS damage. Further, LE is shown as a single and powerful antioxidant which induces lipid peroxidation alike thiol antioksidants in the presence of ferric acid antioxidants and neutralizes the hydroxyl radicals, peroxynitrite hypochloroik acid and peroxynitrite [19-21].

In reproductive studies, the significant protective effects of LE has been demonstrated especially on semen. LE is the predominant sulphidryl in human, equine and porcine semen. It has been reported that LE explicitly protects the semen from oxidatif stress. As a result of the antioxidant properties of LE, while it increases sperm viability during storage, eliminates the harmful effects of hydrogen peroxide on viability of spermatozoa and vital impact [1,22]. The aim of in this study was to investigate the effects of LE, NAC and Cystein on the frozen ram semen.

## MATERIAL and METHODS

The experiment was performed in accordance with guidelines for animal research from the National Instutites of Health and all procedures on animals were approved by the Kafkas University Ethics Committee on Animal Research in current study (approval date/number: 2009/02).

### *Chemicals*

All chemicals were provided from Sigma-Aldrich® (Germany) if not stated.

### *Animals*

The animals were housed at the Kafkas University, Faculty Of Veterinary Medicine, Education Research and Practice Farm and kept under uniform feeding, housing and lighting conditions.

### *Semen Extension, Freezing and Thawing*

Semen samples were obtained from four Tushin rams (2 and 4 years of age) and all of ejaculates were collected from the rams with artificial vagina twice a week during

the breeding season. The ewes in oestrus were used as phantom. Ejaculates were evaluated. Only ejaculates with in volume, spermatozoa with >80% progressive motility and concentration higher than $2\times10^9$ spermatozoa/ml were pooled, balancing the sperm output of each male to compansate individual differences [23-25]. Each pooled ejaculate was splitted into seven equal aliquots and diluted at 37°C with skimmed milk extender (prepared from 10% milk powder w/v, 0.9% glucose w/v; and added 10% egg yolk v/v, 5% glycerol v/v; pH adjusted to 6.8 with 1N NaOH) supplemented with LE (5 and 10 mM), Cystein (5 and 10 mM), N-acetylcystein (5 and 10 mM) and no-antioxidant (control), respectively, and a total of seven experimental groups were established. After dilution, final concentration was adjusted to of approximately $4\times10^8$ spermatozoa/ml. Diluted semen samples were aspirated into 0.25 ml French straws, sealed with polyvinyl alcohol powder and equilibrated at 5°C for 2 h. After equilibration, the straws were frozen in liquid nitrogen vapor, 4 cm above the liquid nitrogen (-120°C), for 15 min. Thereafter, the straws were plunged into the liquid nitrogen for storage. The frozen semen in straws were thawed at 37°C for 1 min in a water bath for post-thawing evaluation immediately after thawing.

***Analysis of Standard Semen Parameters***

***Semen Volume:*** The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals.

***Motility:*** Progressive spermozoa motility was estimated using phase-contrast microscopy (400×; Nikon Eclipes e400, Japan), with a warm stage kept at 37°C. For motility assessment, one drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm-motility estimations were performed in three different microscopic fields for each semen sample. The mean (SEM ±) of the three successive estimations was recorded as final motility percentage (%).

***The Percentage of Live and Dead Sperm:*** The percentage of live sperm in the sample was assessed by means of a nigrosin–eosin staining method [26]. In examination, the 2% solution of eosin stain powder prepared with 3% Sodium Citrate were used for staining. The stain fixation of sperm were prepared by mixing a drop of semen with two drops of the stain on a warm slide and spreading the stain immediately with a second slide. The viable sperm rate was assessed by counting 200 cells under bright field microscope (400× magnification; Olympus CX21, Japan). Spermatozoa showing partial or complete purple colorization was considered non-viable or dead and only sperm showing strict exclusion of the stain were considered to be alive.

***The Percentage of Sperm Abnormalities:*** For the assessment of sperm abnormalities, one drop of each sample were added to Eppendorf tubes containing 0.5 ml of Hancock solution (62.5 ml of 37%, formalin 150 ml sodium saline solution, 150 ml buffer solution and 500 ml double-distilled water) [27]. The mixing samples were stored at refrigerator (+4°C). After providing fixation of samples, one drop of this mixture was put on a slide and covered with a cover slip. The percentage total sperm abnormalities were determined by counting a total of 400 spermatozoa under phase-contrast microscopy (magnification 1000×/oil immersion object). Further, sperm abnormalities were classified as acrosome, head, midpiece and tail (AHMT), and each category were recorded seperately.

***Sperm Concentration:*** The sperm concentration was measured by using a haemocytometer [28]. The obtained results were recorded as $10^9$/ml.

***The Hypo-osmotic Swelling Test (HOST):*** The hypo-osmotic swelling test (HOST) was used to evaluate the functional integrity of the sperm membrane, basedon curled and swollen tails. This procedure was carried out by incubating 30 µl of semen with 300 µl of a 100 mOsm hypo-osmotic solution (9.0 g fructose + 4.9 g sodium citrate in 1.000 ml of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spreaded with a cover slip on a warm slide. Four hundred sperms were evaluated (magnification 400×) with bright-field microscopy (Olympus CX21, Japan). Sperm with coiled tails were recorded as membrane intact [29,30].

***Biochemical Assays***

Biochemical assays were performed on the sperm samples immediately after thawing and without washing by using spesific kits. Total antioxidant capacity (TAC) in samples was assayed by following the procedures as described in recent years [31]. After semen samples were centrifuged in rapid of 3.000 rpm/5 min, seminal plasma were obtained. TAC and TOC levels were measured by using kit (Rel Assay Diagnostic, Turkey). The test named as blue-green ABTS radical cation which is based on the principle of color loss. In this method, 2,2'-azino-di (3-sulfonate to etlylbenzthiozol=ABTS) reacts with peroxidase and hydrogen peroxide. At the end of this reaction, radical cation ABTS positive (+) is formed and a blue-green color is obtained spectrophotometrically. This color was measured by spectrophotometry at 600 nm [32].

***Statistical Analysis***

The study was replicated seven times. The means of results obtained from seven different experimental groups were analyzed. Results were expressed as the mean±S.E.M mean were analyzed using a one-way analysis of variance (ANOVA), followed by the Tukey's post hoc test to determine significant differences in all the parameters between groups using the SPSS/PC computer program (version 14.0, SPSS, Chicago, IL). Differences with values of $P<0.01$ were considered to be statistically significant [33].

## RESULTS

The mean±S.E.M average of motility, viability, total sperm abnormalities, acrosomal anormalities and the values of HOST, TAC and TOS values in frozen ram semen dilueted skimmed milk powder with supplemented of different antioxidants in different doses are shown in *Table 1*. The results of spermatological parameters, the values of TAC and TOS in all experimental groups were not superior with the addition of the anti-oxidants, when compared to the control. However, LE supplemented groups were superior to the other antioxidant supplemented groups (P<0.01) *(Table 1)*.

## DISCUSSION

The evaluation of the data obtained from control, N-acetyl cystein, cystein and LE groups pointed out that motility rates of LE groups were statistically superior to the other experimental groups except control in current study. Viability rate in control and LE groups was found as numerically superior to other groups. Acrosome and total morphological defects rates in the control group was statistically significant lower than other groups, except LE group. On the other hand, the acrosomal defects in LE group were lower than the other groups, this lowerness was found as statically signficant compared to NAC5 and NAC10 groups, except control. From these results, acrosome defect rate and total morphological defect rates were understood to be parallel with each other.

In present study, the best membrane integrity (HOS Test) results were obtained from groups of LE5 and LE10, similar to rates in other groups. These findings demonstrated that there was consistency among sperm parameters evaluated in current study. In the examinig of TAC, although the LE group was relatively more successful, there were no any differences between groups in TOS values. Considering all the assessments mentioned above, it might be suggested that LE tried for the first time, Cystein and N-acetylcystein in the freezing of semen did not give succesful results compared to control. Unlike, regarding with spermatological parameters (motility, viability, total morphological abnormalities, intact acrosome and HOST) LE group have been successfully compared to other anti-oxidant groups. According to the literatures documented, as it has not been found any literature except for two studies (Çoyan et al.[34] and Ari et al.[35]) regarding with LE and the other antioxidants investigated in the present study, the main theme of the experiment could not be done subject-specific discussion in detail. However, in different number of mammalian species, analyzing the results of different or similar antioxidants with some studies [36,37] especially, motility results obtained in the present study is lower and in those of some others is similar [38,39]. In the same study, in the group added 2 mM LE subjective, CASA and progressive motility values were 81.3%, 57.9% and 31.0%, while in the group added 4 mM, these scores were 80.6%, 65.2% and 32.4% respectively. It was observed that the percentage of motility in LE group was significantly higher cystein group and the more addition dose was increased the success of study increased in LE group. The motility percentages were significantly lower than those of the results in the study of Çoyan et al.[34] similarly, in the present study, LE group was higher than cystein group. Moreover, Ari et al.[35] determined that 10 mM LE improved motility and membrane integrity in replication with poor freezability compared with 0, 1, 2, 5 mM LE. However, they could not determine any beneficial effects of LE in replication with good freezabillity.

In the present study, the viability percentages in the groups added LE and cystein were similar to the results obtained from some studies carried out in various animal species [39,40], while the results of the group supplemented with N-acetylcystein were lower than the results of some investigators [40,41]. On the other hand, the percentages

***Table 1.*** *The mean±S.E.M average of motility, viability, total sperm abnormalities, acrosomal anormalities and the values of HOST, TAC and TOS values in frozen ram semen dilueted skimmed milk powder with supplemented of different antioxidants*

***Tablo 1.*** *Farklı antioksidanlar içeren yağsız süt tozu sulandırıcısında dondurulmuş koç spermasında ortalama motilite, canlılık, toplam anormalite, akrozom bozuklukları, HOST, TAK ve TOS değerleri*

| Parameters | Control | LE5 | LE10 | NAC5 | NAC10 | C5 | C10 |
|---|---|---|---|---|---|---|---|
| Motility (%) | 26.28±3.16$^{a}$ | 24.71±2.85$^{a}$ | 26.14±4.13$^{a}$ | 4.00±2.69$^{b}$ | 2.85±2.42$^{b}$ | 15.14±3.28$^{c}$ | 16.00±4.38$^{c}$ |
| Viability (%) | 32.28±4.10$^{a}$ | 28.71±3.12$^{a}$ | 26.85±4.21$^{ab}$ | 17.57±3.56$^{bc}$ | 14.00±3.92$^{d}$ | 24.71±2.40$^{ab}$ | 25.00±3.40$^{ab}$ |
| Total Morphological Abnormalities (%) | 46.14±3.48$^{a}$ | 49.42±2.56$^{ab}$ | 51.71±3.80$^{ab}$ | 78.42±2.92$^{c}$ | 82.57±3.24$^{c}$ | 57.00±3.47$^{b}$ | 59.28±3.65$^{b}$ |
| Acrosome anormalities (%) | 42.00±3.92$^{a}$ | 45.85±2.74$^{ab}$ | 49.00±3.60$^{ab}$ | 73.14±3.45$^{c}$ | 77.57±4.08$^{c}$ | 53.71±4.50$^{b}$ | 55.42±3.62$^{b}$ |
| HOST (%) | 30.28±3.25$^{a}$ | 29.57±2.62$^{a}$ | 28.57±2.66$^{a}$ | 15.50±3.80$^{b}$ | 14.80±4.31$^{b}$ | 24.14±3.13$^{a}$ | 23.00±3.75$^{a}$ |
| TAC (mM/lt) | 155.14±2.72$^{a}$ | 173.89±5.09$^{a}$ | 250.00±28.0$^{ab}$ | 328.01±17.2$^{b}$ | 357.08±17.8$^{b}$ | 356.69±23.5$^{b}$ | 359.82±19.2$^{b}$ |
| TOS (mM/lt) | 66.61±18.5 | 61.93±12.7 | 61.83±20.5 | 45.57±23.6 | 49.91±21.4 | 73.12±10.9 | 70.00±11.9 |

$^{a, b, c}$ *Different letters within the same column are significantly different (P<0.01)*

***LE5:*** *5 mM L-Ergothionin supplementation,* ***LE10:*** *10 mM L-Ergothionin supplementation,* ***NAC5:*** *5 mM N-Acetyl Cystein supplementation,* ***NAC10:*** *10 mM -Acetyl Cystein supplementation,* ***C5:*** *5 mM Cystein supplementation,* ***C10:*** 10 mM Cystein supplementation

of viability in a study which has been added 6 mM N-acetylcystein to dog semen [37] had been higher than those of the present study's results.

The percentages of acrosomal defects in the present study were lower than some studies's results [24] while were higher than those of some studies [37,38,40,41]. However, the percentages of acrosomal defects in a study [38] which was subjected to addition of cystein in bull semen were highly lower than those of the present study. It also suggests that bull semen is more resistance than ram semen to thawing procedure. On the other hand, the acrosomal defects rates of the groups supplemented with N-acetyl cystein and cystein in the present study were higher than those of the other experimental groups (LE and control groups) and some other studies [24,40,41].

In present study, it was seen that total morphological abnormalities showed similar pattern with the percentage of acrosome disorders, so in the same way, these rations has shown similar differences with other studies [24,37,38,40,41].

Compared with Çoyan et al.[34] study in ram semen, the findings obtained from control and LE groups of the present study were higher and similar to those of N-acetylcysteine and cysteine added.

Our findings about LE doses was similar with findings from Ari et al.[35] when all replications considered. However, Ari et al.[35] pointed out that higher doses of LE (i.e. 10 mM) improved post-thaw motility and membrane integrity in poor freezability replications, while lower doses of LE (1, 2, 5 mM) did not show any beneficial effects on post thawing sperm parameters neither in poor nor in good freezability replications.

Ari et al.[42] determined that higher doses of NAC (i.e. 0.75 mM) had some detrimental effect on post-thawing motility and other semen parameters on ram semen diluted with milk based extender. Similarly, we found that 5 mM and 10 mM doses of NAC detrimentally affected post thawing parameters in ram semen.

Compared HOST results obtained from present study in all groups with other studies [35-37,39] are seen to below.

TAC and TOS relevant results, obtained from the present study, have not been able to discuss in detail with the results from other studies because limited literatures found. However, Çoyan et al.[34] reported that LE and cystein did not affect significantly the superoxide dismutase (SOD) and gluthation peroxidase (GPx) activity. In TOS terms, similar situation was observed in the present study. On the other hand, in the same study, it was shown that the motility rates were positively affected in the groups of the addition of LE compared with control.

Differences between results in our study and the other studies might have been orginated from different methods and sheep breeds or species. Because it has been pointed out that the different methods and breeds or species used may cause variations in sensitivity to oxidative stres in sperm [39]. In addition, it is also known that individual characteristics of rams, season, native quality of semen, extenders/diluents, cryoprotectants, procedure of cryopreservation and also thawing procedure can directly affect results of post-thawing semen quality in rams [40].

In conclusion, in this study, while the 5 and 10 mM doses of LE were superior to the 5 and 10 mM doses of NAC in the view of the percentages of motility, viability, acrosomal defects, total morphological abnormalities, membrane integrity (HOST) and TAC values, except motilty rates in the 5 and 10 mM doses of cystein. However, 5 mM dose of LE were more succesful than all doses of NAC and cystein, considered TAC values. Neverthless there were not statistically significant differences between LE and control groups. The results of current study and limited literature knowledge about LE, described as unique and strong antioxidant, warrants future studies on ram semen cryopreservation.

## REFERENCES

**1. Yıldız S, Daşkın A:** Koç spermasının farklı antioksidanlar içeren sulandırıcılarla kısa süreli saklanması. *Kafkas Univ Vet fak Derg,* 10 (2): 155-159, 2004.

**2. Marti E, Mara L, Marti JI, Muiño-Blanco T, Cebrián-Pérez JA:** Seasonal variations in antioxidant enzyme activity in ram seminal plasma. *Theriogenology,* 67 (9): 1446-1454, 2007.

**3. Pagl R, Aurich JE, Müller-Schlösser F, Kankofer M, Aurich C:** Comparison of an extender containing defined milk protein fractions with a skim milk-based extender for storage of equine semen at 5-8°C. *Theriogenology*, 66, 1115-1122, 2006.

**4. Erokhin AS, Nikifirov GA, Epishina TM:** Protective effect of new synthetic antioxidants in the cryopreservation of ram semen. *Sel'skokhozyaistvennaya Biologiya*, 6, 62-67, 1996.

**5. Maxwell WMC, Stojanov T:** Liquid storage of ram semen in the absence or presence of some antioxidants. *Reprod Fertil Dev*, 8 (6): 1013-1020, 1996.

**6. Marti JI, Marti E, Muino-Blanco T, Cebrian-Perez CA:** Study on the freezing of ram semen. Variations in the antioxidant capacity during the process. *ITEA,* Extra, 22 (2): 853-855, 2001.

**7. Neild DM, Brouwers JFHM, Colenbrander B, Agüero A, Gadella BM:** Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. *Mol Reprod Dev*, 72 (2): 230-238, 2005.

**8. Kheradmand A, Babaei H, Abshenas J:** Comparative evaluation of the effect of antioxidants on the chilled-stored ram semen. *Iranian J Vet Res*, 7 (4): 40-45, 2006.

**9. Oeda T, Henkel R, Ohmori H, Schill WB:** Scavenging effect of n-2007 acetyl-l-cysteine against reactive oxygen species in human semen: A possible therapeutic modality for male factor infertility? *Andrologia*, 29 (3): 125-231, 1997.

**10. Nichi M, Goovaerts IGF, Cortada CNM, Barnabe VH, De Clercq JBP, Bols PEJ:** Roles of lipid peroxidation and cytoplasmic droplets on ın vitro fertilization capacity of sperm collected from bovine epididymides stored at 4 and 34°C. *Theriogenology*, 67 (2): 334-340, 2007.

**11. Hughes LCM, Lewis SEM, McKelvey-Martin VJ, Thompson W:** The effects of antioxidant supplementation during percoll preparation on human sperm DNA integrity. *Human Reprod*, 13, 1240-1247, 1998.

**12. Szczesnıak-Fabıanczy B, Bochenek M, Smorąg Z, Ryszka F:** Effect of antioxidants added to boar semen extender on the semen survival time and sperm chromatin structure. *Biol Reprod*, 3 (1): 81, 2003.

**13. Bucak MN, Ateşşahin A, Yüce A:** Effect of anti-oxidants and oxidative stress parameters on ram semen after the freze-thawing process. *Small Rum Res*, 75 (2-3): 128-134, 2008.

**14. Mitsuyama H, May JM:** Uptake and antioxidant effects of ergothioneine in human erythrocytes. *Clin Sci Lond*, 97 (4): 407-411, 1999.

**15. Guerin P, El Mouatassim S, Menezo Y:** Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and ıts surroundings. *Human Reprod Update,* 7, 175-189, 2001.

**16. Öztürkler Y, Yıldız S, Güngör Ö, Pancarcı ŞM, Kaçar C, Arı UÇ:** The effects of L-ergothioneine and L-ascorbic acid on *in vitro* maturation and embryonic development in sheep oocytes. *Kafkas Univ Vet Fak Derg,* 16, 757-763, 2010.

**17. Aruoma OI, Spencer JP, Mahmood R:** Protection against oxidative damage and cell death by the natural antioxidant ergothioneine. *Food Chem Toxicol*, 37 (11): 1043-1053, 1999.

**18. Akanmu D, Cecchini R, Aruoma OI, Halliwell B:** The antioxidant action of ergothioneine. *Arch Biochem Biophys*, 288, 10-16, 1991.

**19. Heath H, Larson A, Remington CJ, Searle C:** Some effects of administrating ergothioneine to rats. *Chem Soc*, 50, 530-533, 1952.

**20. Mann T, Leone E:** Studies on the metabolism of semen, L-ergothioneine as a normal constituent of boar seminal plasma. *Biochem J*, 53, 140-148, 1953.

**21. Aruoma OI, Whiteman M, England TG, Halliwell B:** Antioxidant action of ergothioneine assessment of its ability to scavenge peroxynitrite. *BBRC*, 231, 389-391, 1997.

**22. Haag FM, Mc Leod J:** Relationship between nonprotein sulfhydryl concentration of seminal fluid and motility of spermatozoa in man. *J Appl Physiol*, 14, 27-30, 1959.

**23. Aisen EG, Medina VH, Venturino A:** Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. *Theriogenology,* 57, 1801-1808, 2002.

**24. Gil J, Lundeheim N, Söderquist L, Rodriguez-Martinez H:** Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. *Theriogenology*, 59 (5-6): 1241-1255, 2003.

**25. Bucak MN, Atessahin A, Varisli Ö, Yüce A, Tekin N, Akcay A:** The influence of trehalose, taurine, cysteamine and hyaluronan on ram semen microscopic and oxidative stress parameters after freeze-thawing process. *Theriogenology*, 67 (5): 1060-1067, 2007.

**26. Evans G, Maxwell WMC:** Handling and Examination Semen. **In,** Maxwell WMC (Ed): Salamon's Artificial Insemination of Sheep and Goat. 93-106, Butterworths, Sydney, 1987.

**27. Schafer S, Holzmann A:** The use of transmigration and spermac stain to evaluate epididymal cat spermatozoa. *Anim Reprod Sci,* 59, 201-211, 2000.

**28. Smith JT, Mayer DT:** Evaluation of sperm concentration by the hemocytometer method. *Fertil Steril,* 6, 271-275, (1955).

**29. Revell SG, Mrode RA:** An osmotic resistance test for bovine semen. *Anim Reprod Sci,* 36, 77-86, 1994.

**30. Buckett WM, Luckas MJ, Aird IA, Farquharson RG, Kingsland CR, Lewis-Jones DI:** The hypo-osmotic swelling test in recurrent miscarriage. *Fertil Steril,* 68, 506-509, 1997.

**31. Badarinath AV, RAo Mallikarjuna K, Madhu Sudhana Chetty C, Ramkanth S, Rajan TVS, Gnanaprakash K:** A review on *in-vitro* antioxidant methods: comparisions, correlations and considerations. *Int J Pharm Tech Res,* 2 (2): 1276-1285, 2010.

**32. Erel O:** A New automated colorimetric method for measuring total antioxidant status. *Clin Biochem*, 38 (12): 1103-1111, 2005.

**33. Daniel WW:** Analysis of variance. **In,** Daniel WW (Ed): Biostatistic: A Foundation for Analysis in the Health Sciences. 274-320, John Wiley & Sons, Hoboken, 1991.

**34. Çoyan K, Baspınar N, Bucak MN, Akalın PP:** Effects of cysteine and ergothioneine on post-thawed merino ram sperm and biochemical parameters. *Cryobiology*, 63, 1-6, 2011.

**35. Ari UÇ, Kulaksiz R, Ozturkler Y, Yildiz S, Lehimcioglu NC:** Effect of L-(+)-Ergothioneine (EGT) on freezability of ram semen. *Int J Anim Vet Adv,* 4 (6): 378-383, 2012.

**36. Bucak MN, Tuncer PB, Sarıozkan S, Ulutaş PA:** Comparison of the effects of glutamine and an amino acid solution on post-thawed ram sperm parameters, lipid peroxidation and anti-oxidant activities. *Small Rumin Res,* 81, 13-17, 2009.

**37. Michael A, Alexopoulos C, Pontiki E, Hadjipavlou-Litina D, Saratsis P, Boscos C:** Effect of antioxidant supplementation on semen quality and reactive oxygen species of frozen-thawed canine spermatozoa. *Theriogenology*, 68, 204-212, 2007.

**38. Fraser L, A. Dziekońska A, Strzeżek R, Strzeżek J:** Dialysis of boar semen prior to freezing-thawing: Its effects on post-thaw sperm characteristics. *Theriogenology*, 67, 994-1003, 2007.

**39. Ciftci H, Verit A, Savas M, Yeni E, Erel O:** Effects of *N*-acetylcysteine on semen parameters and oxidative/antioxidant status. *Urology*, 74 (1): 205-207, 2009.

**40. Marco-Jimenez F, Puchades S, Gadea J, Vicente JS, Viudes-de-Castro MP:** Effect of semen collection method on pre- and post-thaw guirra ram spermatozoa. *Theriogenology*, 64, 1756-1765, 2005.

**41. D'Alessandro AG, Martemucci G:** Evaluation of seasonal variations of semen freezability in leccese ram. *Anim Reprod Sci,* 79, 93-102, 2003.

**42. Arı UÇ, Kulaksız R, Yıldız S, Öztürkler Y, Lehimcioğlu NC:** Effect of N-acetylcysteine (NAC) on post-thaw semen quality of Tushin rams: Higher doses of NAC may be toxic. (Abstract) *VII. National Reproduction and Artificial Insemination Science Congress,* Book of Congress, p.148-149, Kars/TURKEY, 2013.