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# Improvement of Incubation Resilience with Various Antioxidants in Cryopreserved Ram Semen*

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**Summary:** The aim of the current study was to evaluate different antioxidant-supplemented extenders for post-thaw semen quality and incubation resilience of ram spermatozoa. Pooled semen samples were divided into four equal volumes and each volume were diluted with two-step dilution method in control and antoxidant supplemented groups (5mM methionine, 5mM cysteamine and 1mM cysteine). Semen samples were assessed for the sperm motility, plasma membrane integrity using hypoosmotic swelling test (HOST) and Hoechst 33258 test, damaged acrosome using FITC-Pisum sativum agglutinin (PSA-FITC) and DNA integrity using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Membrane lipid peroxidation was also analyzed using the malondialdehyde (MDA) concentration assay. This study showed that antioxidant supplemented extenders had beneficial effect on motility, plasma membrane integrity and acrosome integrity of ram semen compared to control group (P<0.05). The motility (60.40%) and acrosome integrity (30%) values of cysteine group was significantly higher than those of the groups (P<0.05). However, there were no differences about DNA fragmentation rates at 0 h of incubation. In addition, we achieved a higher motility (35%), HOST (50%), Hoechst (56.50%) and lower defected acrosome (35%) and DNA fragmentation (8.80%) rates in post thawed ram semen even after 6 h incubation when the extender was supplemented with 1 mM cysteine.

**Key words:** Antioxidants, cryopreservation, incubation resilience, ram semen

**Koç Spermasının Dondurulmasında, İnkubasyon Direncinin Çeşitli Antioksidanlar ile Artırılması**

**Özet:** Çalışmamızda farklı antioksidanların dondurma çözdürme sonrası sperma parametreleri ve spermanın inkubasyon direncinin artırılması üzerine etkilerinin belirlenmesi amaçlanmıştır. Birleştirilien sperma örnekleri dört eşit hacme bölünerek, antioksidan ilave edilmiş (5mM methiyonin, 5mM sisteamin, 1mM sistein) ve edilmemiş (kontrol) sulandırıcılarla iki aşamalı sulandırma yöntemi ile sulandırılmıştır. Sperma örneklerinin değerlendirilmesi amacıyla motilite, plazma membran bütünlüğünün değerlendirilmesi amacıyla hipoozmotik şişme testi (HOST) ve Hoechst 33258 testi, akrozom ve DNA bozukluklarının belirlenmesi amacıyla ise sırasıyla FITC-Pisum sativum agglutinin (PSA-FITC) ve terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) boyama değerlerine bakılmıştır. Ayrıca membran lipid peroksidasyonun değerlendirilmesi için malondialdehyde (MDA) konsantrasyon ölçümü yapılmıştır. Değerlendirmeler doğrultusunda koç spermasının dondrulmasında antioksidan ilave edilen grupların motilite, plazma mebran bütünlüğü ve akrozom bütünlü değerlerinin kontrol grubundan daha yüksek olduğu sonucuna varılmıştır (P<0.05). Sistein ilave edilen grubun motilite (%60.40) ve akrozomal bütünlük (%70) oranları diğer gruplardan daha yüksek buunmuştur.Ancak inkubasyonun 0. Saatinde grupların DNA bütünlük oranları arasında bir fark bulunmamaktadır (P>0.05). Dondurma çözdürme işlemleri ve 6 saat inkubasyon sonrası en yüksek motilite (%35), HOST (%50), Hoechst (%56.50) ve en düşük akrozomal bozukluk (%35) ve DNA bozukluk (%8.80) oranları 1mM sistein içeren sulandırıcı ile dondurulan grupta elde edilmiştir.

**Anahtar kelimeler:** Antioksidan, inkubasyon direnci, koç sperması, sperma dondurma

## Introduction

Cryopreservation of semen is one of the most important techniques in order to improve animal reproduction (4). However, post thaw semen quality has to be improved by semen extenders to achieve a better reproductive efficiency. The succession of the preservation of ram semen is related to modification of extenders (21). Due to this relation, various components have been tested for the maintenance of sperm motility, fertilizing capacity and preserving sperm membrane integrity (34).

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Freeze-thawing processes cause many defects on spermatozoon such as functional, biochemical and morphological features (13). Cold shock and the formation-dissolution of ice during the freeze-thawing process affect the integrity and the functions of the acrosome, nucleus, mitochondria and plasma membrane that impairs fertilizing ability of spermatozoa (28,29).

Egg yolk is being widely used in mammalian semen extenders to protect spermatozoon against the cold shock and the lipid-phase transition effect. The most effective ingredient of the egg yolk is the low density protein, which prevents spermatozoa from cold shock during cryopreservation (17). Furthermore, phospholipids in egg yolk such as phosphatidylcholine are crucial for the maintenance of sperm membrane integrity in freeze-thawing process (5).

During freeze-thawing process, ice crystallization causes functional and biochemical changes in spermatozoon that induce increments in reactive oxygen species (ROS) and lipid peroxidation which leads to the oxidative stress. Overproduction of ROS leads to decrease in sperm motility, viability and fertilizing ability. In semen, there is a natural antioxidant system, but this system is partly removed and severely altered during cryopreservation. Therefore, the addition of antioxidants to the extender may have positive effects on semen cryopreservation in various species (8,9,27). Among various antioxidant species methionine cysteamine and cysteine play a vital role in detoxification (7,10,11,32,33).

Effects of different antioxidants have been examined; however, there are few reports about the effect of antioxidant additives on incubation resilience of ram spermatozoa. In this experiment, it was designed to evaluate methionine, cysteamine and cysteine on post-thaw quality and incubation resilience of cryopreserved ram spermatozoa.

## Materials and Methods

All issues concerning the experimental methods and evaluation techniques were approved by the Scientific Ethical Committee, Uludag University, Bursa, Turkey (No: 2015-07/03).

### *Semen extender preparation*

Two-step dilution method was used in this study. Extender A consisted of 223.7 mmol/L Tris (Sigma, St. Louis, MO, USA), 55.5 mmol/L fructose (Sigma), 66.6 mmol/L citric acid (Merck, Darmstadt, Germany), 4 g/L penicillin G, 3g/L dihydrostreptomycin, 20% egg yolk (v/v) and antioxidants (5 mM methionine, 5 mM cysteamine, 1mM cysteine or no antioxidant [control]) in distilled water. Extenders B were prepared by adding 100.4 mmol/L trehalose, 4.03 mmol/L EDTA and 6% glycerol (v/v) to extender A (control and 3 different antioxidant groups) (2).

### *Semen collection, evaluation and dilution*

Five rams aged 3–5 years old which maintained at Uludag University, Faculty of Veterinary Medicine in Bursa, Turkey, were used as a material during breeding season. Rams were maintained under uniform feeding and housing conditions; water was administered ad libitum. Ram semen was collected five times every other day by electrically stimulated ejaculation (Ruakura Goat Probe Plastic Products, Hamilton, New Zealand). Collected semen was placed in a warm water bath (28-32°C) and immediately evaluated for consistency, wave motion (0–5 scale), and the percentage of motile spermatozoa. Ejaculates with a thick consistency, rapid wave motion (3–5 on a 0–5 scale) and >75% initial motility were pooled (28).

Briefly, with the concentration of at least $1x10^9$ spermatozoa/mL pooled ejaculates were split into four equal aliquots. Each aliquot was diluted to a ratio of 1/2 (semen/extender) with extender A, then cooled to 5°C within 60 min. Cooled sperm aliquots were then diluted to a ratio of 1/1 (semen/extender) with relevant extender B (previously cooled at 5°C), respectively, at five steps with 10 min intervals. The diluted samples were equilibrated at 5°C for 120 min.

### *Semen freezing and thawing*

Equilibrated semen was placed into 0.25 mL straws and frozen at 3°C/min from +5°C to -8°C and at 15°C/min from -8°C to -120°C in liquid nitrogen vapor using the Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France). The straws were then plunged into liquid nitrogen at -196°C where they were stored for at least one month. Three straws from each group were thawed at 37°C for 30 s in a water bath and incubated for 6 h in 5% $CO_2$ in humidified air at 39°C to evaluate post-thaw semen characteristics.

### *Semen evaluation*

All semen parameters were assessed at post-thaw 0 h and 6 h. semen samples were frozen by the same person, and each of the studied semen parameters was measured by the same

person on each occasion throughout the study. Sperm motility was assessed subjectively using a phase-contrast microscope [Olympus BX51-TF (Olympus Optical Co., Ltd., Japan)] (400x) with a warm slide (38°C) (2).

***Fluorescein lectin staining assay (FITC conjugated Pisumsativum agglutinin [PSA-FITC])***

PSA-FITC staining performed for assessment of acrosome integrity. The staining was performed according to description of Nur et al. (28) with a fluorescence microscope (Olympus BX51, Olympus Optical Co., Tokyo, Japan). At least 200 spermatozoa per smear were evaluated for acrosome integrity.

***The hypoosmotic swelling test (HOST)***

HOST was used to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 10µL of semen with 100µL of 100 mOsm hypoosmotic solution (9g fructose + 4.9g sodium citrate per liter of distilled water) at 37ºC for 60 min. After incubation, 20µL of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm cells were evaluated under 1000x magnification with phase-contrast microscope. Sperms with swollen or coiled tails were recorded (12).

***Hoechst 33258***

The percentage of spermatozoa with intact membranes was measured according to Perez et al. (31). Briefly, samples ($4\times10^6$ spermatozoa/mL) were added to an equal volume of Hoechst 33258 solution (10µL/mL in PBS) and incubated for 3 min at 37°C. Spermatozoa were immotilized by adding 10µL of formaldehyde (50 mg/L in distilled water). Then, 20µL aliquots were placed on glass slides and covered with cover slips. The samples were viewed with an Olympus BX51-TF epifluorescence microscope, using a UV- 2A filter. Under these conditions, the membrane-damaged spermatozoa were stained light-blue and the membrane intact cells remained unstained. At least 200 cells were evaluated for unstained spermatozoa in duplicates for each sample.

***TUNEL assay***

For the TUNEL technique, In Situ Cell Death Detection Kit with fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) was used according to the manufacturer's protocol with slight modifications. At least 200 sperm cells were evaluated to determine the percentage of TUNEL positive sperm cells. Each microscopic field was evaluated first under fluorescence microscopy (400x magnification) to determine the number of reactive sperm and the total number of sperm per field under phase-contrast microscope (2).

***Malondialdehyde (MDA) concentrations***

The samples were thawed before the lipid peroxidation (LPO) analyses. After thawing semen sample immediately was centrifuged at 800 g for 10 min and supernatant was separated. Malondialdehyde (MDA) levels were measured spectrophotometrically (Mannheim Boehringer Photometer 4010, Germany) by using the thiobarbituric acid method (30). One ml of 0.67% thiobarbituric acid solution were added to 0.2 ml of the supernatant in a glass tube and kept in 100°C for 60 min. After cooling the tubes, absorbance of the supernatant was read at 546 nm. 1, 1, 3, 3,-Tetramethoxypropane was used as MDA standard and the results were expressed as pmol/mg protein.

***Statistical analysis***

Statistical analysis was performed using IBM SPSS version 20. Five replications were performed for all the parameters measured. Shapiro Wilk test was used as normality test. Means of obtained semen parameters were analyzed using one-way ANOVA test followed by Tukey test. Pearson correlation coefficient was used to assess the relationships among the values of motility, plasma membrane and functional integrity, defected acrosome and DNA fragmentation.

**Results**

Table 1 shows the percentages of sperm motility, plasma membrane functional integrity (HOST), plasma membrane integrity (Hoechst 33258, Germany), defected acrosomes and DNA fragmentation after thawing (0 h) and incubation (6 h) in cryopreserved ram semen from control and antioxidant groups.

***Motility***

Sperm motility was progressively reduced after 6 h incubation ($P<0.001$). Post-thaw motility values at 0 h in antioxidant groups were higher than those of control group ($P<0.01$). At the end of incubation time, cysteine group had the highest motility rate than the other antioxidants and control groups ($P<0.05$).

**Table 1.** The mean (x̄±Sx̄) of studied sperm resilience parameters on different extender groups.

| Incubation period (h) | Group | Motility (%) | Plasma membrane integrity | | Defected Acrosome (%) | DNA fragmentation (%) |
|---|---|---|---|---|---|---|
| | | | HOST (%) | Hoechst 33258 (%) | | |
| 0 h | Control | 50.00±0.82$^{a}$ | 54.25±1.11$^{a}$ | 58.75±0.85$^{a}$ | 40.25±0.63$^{a}$ | 7.50±0.87 |
| | Methionine | 56.60±0.68$^{b}$ | 62.00±1.17$^{b}$ | 66.40±2.16$^{ab}$ | 35.00±1.30$^{bc}$ | 5.80±0.37 |
| | Cysteamine | 58.00±0.55$^{bc}$ | 59.40±1.66$^{b}$ | 60.40±2.62$^{a}$ | 36.40±1.36$^{ab}$ | 7.40±0.51 |
| | Cysteine | 60.40±1.29$^{c}$ | 65.20±1.77$^{b}$ | 68.20±1.16$^{b}$ | 30.00±0.71$^{c}$ | 5.80±0.37 |
| 6 h | Control | 10.00±2.04$^{a}$ | 36.75±1.49$^{a}$ | 47.67±1.84$^{a}$ | 43.75±1.38$^{a}$ | 13.00±1.00$^{a}$ |
| | Methionine | 20.00±1.58$^{b}$ | 43.40±1.29$^{a}$ | 46.60±1.54$^{a}$ | 39.20±0.20$^{ab}$ | 9.80±1.39$^{ab}$ |
| | Cysteamine | 11.00±1.00$^{a}$ | 39.60±1.69$^{a}$ | 47.75±2.78$^{a}$ | 39.40±1.03$^{ab}$ | 10.00±0.55$^{ab}$ |
| | Cysteine | 35.00±2.24$^{c}$ | 50.00±0.89$^{b}$ | 56.50±2.10$^{b}$ | 35.00±2.35$^{b}$ | 8.80±0.58$^{b}$ |

a,b and c: Values with different superscripts in the same column for each of incubation time are significantly different (P<0.05).

***Plasma Membrane Integrity***

Plasma membrane functional integrity decreased with the freeze-thawing and incubation processes (P<0.001). Membrane functional integrity was better preserved in antioxidant groups than that of the control group (P<0.05). After incubation, cysteine group had the highest HOST and Hoechst 33258 unstained spermatozoa values than those of the other groups (P<0.05).

***Acrosomal Status***

Acrosome integrity deteriorated during the freeze-thawing process (P<0.05). After incubation, the acrosomal integrities were successfully protected by group supplemented with cysteine when compared to the integrities of control group (P<0.05).

***DNA Fragmentation***

The results of the TUNEL assay demonstrated that the post-thaw percentages of DNA damaged spermatozoa in all groups were not significant. After 6 h incubation, cysteine group had better DNA integrity than the control group (P<0.05).

***MDA concentrations***

Table 2 shows the effect of antioxidants on the MDA level after freeze-thawing process. As shown in table, it was found that MDA levels in the antioxidant groups were lower than control group (P<0.05). In addition, there were no significantly differences among antioxidant groups.

The results of the Pearson correlation tests are shown in Table 3. Although a significant negative correlation was found between sperm motility with DNA fragmentation and defected acrosomes, a positive correlation was found between motility with HOST and Hoechst 33258. In addition, there were negative correlations between HOST and DNA fragmentation and between HOST and defected acrosome rates (P<0.01). There were positive correlation between HOST and Hoechst 33258 rates and between defected acrosome and DNA fragmentation rates (P<0.01).

**Table 2.** Malondialdehiyde (MDA) levels in frozen-thawed ram sperm

| Groups | Control | Methionine | Cysteamine | Cysteine | |
|---|---|---|---|---|---|
| MDA (nmol/ml) | 5.76±0.61$^{a}$ | 3.38±0.31$^{b}$ | 2.67±0.37$^{b}$ | 3.89±0.24$^{b}$ | P<0.05 |

Different superscripts (a, b) in the same line indicate significant differences (P<0.05)

**Table 3.** Correlation coefficient (r) between the results of studied ram semen parameters

| | Plasma membrane integrity | | | |
|---|---|---|---|---|
| | **Host (%)** | **Hoechst 33258 (%)** | **Defected Acrosome (%)** | **DNA fragmentation (%)** |
| Motility | 0.884** | 0.835** | -0.644** | -0.779** |
| Host (%) | | 0.776** | -0.577** | -0.722** |
| Hoechst 33258 (unstained) (%) | | | -0.679** | -0.606** |
| Defected Acrosome PSA (%) | | | | 0.528** |

**Correlation is significant at the P<0.01.

## Discussion

Oxidative stress, which is resulted from adverse effect of cryopreservation, damages to the sperm structure via irreversible changing of membrane fluidity and enzymatic activity (27). As a consequence of these undesired changes deteriorate the sperm parameters and reduce the fertilizing ability of spermatozoa (2,28,39). In comparison with other mammalians, ram spermatoza are more sensitive to cryopreservation process because of their membrane structure which is formed from higher molar rate of unsaturated phospholipids (25). In the present study, the effect of various antioxidants (methionine, cysteamine and cysteine) supplemented egg yolk based extenders were compared for post thaw quality and incubation resilience of ram semen by conducting quality tests in breeding season.

Only motile spermatozoa can reach to the region of fertilization, pass through cumulus layer of oocyte and finally penetrate the zona pellucida. Therefore, sperm motility is one of the indicators of fertilization ability (16). Our study showed that antioxidant supplementation had a positive effect on sperm motility at 0 and 6 h of incubation. Although post-thaw motility values of all antioxidant groups were significantly higher than control group, at the end of the incubation only cysteine and methionine maintained their beneficial effects on sperm motility (P<0.05). It is known that, cysteamine induces the uptake of cysteine by cells thereby enhancing the GSH synthesis (24) and mammalian cells can only utilize cysteine (23,35). Thus, cysteamine and cysteine enhanced motility and elevated the antioxidant capacity of post-thawed ram sperm (7). Many researchers investigated the efficiency of egg yolk based extenders for ram semen cryopreservation. Motility values of these researches are ranged between 45.7-68.0% (2,15,17,28,36,39). In this study, the motility values of control group at 0 h showed similarity with the findings of these studies.

Plasma membrane integrity is crucial for sperm metabolism; it also plays essential role in capacitation and sperm-oocyte fusion (17). One of the survival skill of sperm cells is to protect the plasma membrane (2,20,40). Therefore, plasma membrane and plasma membrane functional integrities are considered to be important sperm quality parameters. Hoechst 33258 is used for detection of structural integrity of the spermatozoon membrane (1,3). However, hypoosmotic swelling test has recently been used for specify both structural and functional integrity of sperm membranes (12,40). In our study, in terms of HOST (+) values, cysteine included extender provided the best production on sperm plasma membrane when compared with other antioxidant and control groups (P<0.05). Also our results showed that the percentages of unstained spermatozoa obtained by Hoechst 33258 assay are in agreement with the HOST results. Acrosomal integrity is the indicator of penetration, digestion of zona pellucida and fusion ability of spermatozoa so it has been related to fertility (41). Plasma and also acrosome membrane of ram spermatozoa contain high rate of unsaturated phospholipids. Therefore, ram sperm is more sensitive to cryopreservation process than the sperm of other mammalian (18). In our study, acrosome integrity values of cysteine group were significantly higher than control group at 0 h and 6 h of incubation. Protective effect of cysteine on plasma membrane and acrosome integrity of spermatozoa is originated from its chemical structure. Cysteine consists of thiol groups, which individually act as a non-enzymatic antioxidant and easily penetrates into the sperm (14). Cysteine has been shown to prevent the loss of motility, viability and mem-

brane integrity in the frozen state (11). The post thaw acrosome integrity values of previous studies ranged between 41.3-70.3% (2,28,36,39,40). Similar results were obtained from our groups.

Contrary to other sperm quality parameters, DNA integrity rates are not directly related to fertilizing ability. This parameter is related to identification of seriously damaged sperm concentration (19) and maintainability of early embryo development. After freeze-thawing process, spermatozoa are particularly susceptible to DNA damage (6). It was observed in the present study that cysteine group protected DNA integrity of spermatozoa compared to the control group at the end of incubation ($P<0.05$). Beneficial effects of cysteine for protection of sperm chromatin have been proved for chilled (37) and frozen (38) semen.

Malondialdehyde is a final product of lipid peroxidation in the cells (26). Therefore, it is often used as an indicator of lipid peroxidation. In this study, MDA levels of control group were significantly higher than those of antioxidant groups. Our results are in agreement with those of previously published studies (22,26,40).

Superior semen quality is related to higher motile spermatozoon rates (2). In our study, there was a positive correlation between motility and membrane integrity (HOST and Hoechst 33258) values ($P<0.01$). This is not an unexpected relation because input-output of metabolites and other compound across the plasma membrane with active or passive transportation are necessary for motility (2). Similar findings have been reported for sperm motility and membrane integrity values earlier (40). In contrast to plasma membrane, acrosome and DNA fragmentation rate negatively correlated with motility ($P<0.01$). These findings were in agreement with the results of studies previously reported by Alcay et al. (2).

In conclusion, the present results seem to confirm that antioxidant supplemented extenders have beneficial effect on post-thaw ram sperm motility, acrosome and plasma membrane integrity. In addition, in ram semen, we achieved best result after thawing and 6 h incubation when the extender was supplemented by 1mM cysteine. Further studies should aim at confirming the usefulness of the supplementation with these antioxidants regarding field fertility.

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