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Effect of L-(+)-Ergothioneine (EGT) on Freezability of Ram Semen

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Abstract: The aim of this study was to investigate freezability of ram semen extended with different L-(+)-Ergothioneine (EGT) doses. For this aim, semen from four ram were collected with artificial vagina (44°C) and then pooled. Pooled semen was divided five aliquots and extended with skim milk based extender containing 0 mmol/L (EGT0: Control), 1 mmol/L (EGT1), 2 mmol/L (EGT2), 5 mmol/L (EGT5) and 10 mmol/L (EGT10) EGT, respectively. After equilibration (+5°C/2 h), the extended aliquots of semen in straws were cryopreserved in Liquid Nitrogen (LN_2) vapour (-120°C/15 min) and stored in LN_2 (-196°C) until examination date. Totally two straws from 17 replications (trials) in each experimental group were thawed in water bath (37°C/1 min) and percentages of progressive motility, sperm viability, abnormality, acrosome and membrane integrity were determined and statistically assessed with SPSS. In the result, it was determined that different doses of EGT did not affect freezability of ram semen when seventeen replications considered ($p>0.05$). However, when separated trials according to good (≥20% for motility in control groups; 8 in 17 replications) or poor (<20% for motility in control groups; 9 in 17 replications) freezability were statistically analyzed, beneficial effects of 10 mmol/L concentration of EGT on progressive motility and membrane integrity were determined in poor freezability trials, compared with control ($p<0.05$). In conclusion, addition of EGT in semen extenders may be considered to improve freezability of ram semen, if there is a situation of poor-freezability.

Keywords: Cryopreservation, L-ergothionine, poor-freezability, semen, tushin ram

INTRODUCTION

Tushin (Tuj, Kars, Çıldır) sheep have been reared for its wool, meat, leather and also milk. It is important breed for Caucasian region and Tushin sheep have accommodated to this region hard climate's condition. However, number of purebred Tushin sheep in Turkey has decreased in day by day. The number of Tushin sheep (including crossbred) is estimated only 200 thousand heads (Manson, 1996). Therefore, the best way for preserve Tushin sheep breed is cryopreservation of ram semen, oocyte and embryos from Tushin breed. Extenders used for cryopreservation of Tushin ram semen determine success of cryopreservation (Ari *et al*., 2011a). Ari *et al*. (2011a) showed that skim milk (prepared from skim milk powder) and goat semi skim milk based extenders provided best freezability for Tushin ram semen. Moreover, same researchers determined that intervals of semen collection were important factor affecting freezability of Tushin ram semen (Ari *et al*., 2011b). It is known that semen from Tushin ram reared in Kars province were not affected from seasonal changes (Ari *et al*., 2011c). Nevertheless, it is clear that ram semen and also Tushin ram semen is very vulnerable for cryopreservation (cryo-sensitive).

It is well known that reactive oxygen species (ROS; i.e., super oxide, hydroxyl radical and peroxide) are produced by spermatozoa, immune cells in semen and also environmental factors (especially UV) during cryopreservation stages (Aitken and Fisher, 1994; Aitken and Baker, 2004; Alvarez and Storey, 1984). The antioxidant capacity of spermatozoa is very limited to protect itself against ROS, compared with somatic cells. Moreover, plasma and mitochondrial membrane of spermatozoa is very sensitive against oxidative stress because of high unsaturated fatty acid composition, compared with somatic cells (Alvarez and Storey, 2005; Flesch and Gadella, 2000; Holt and North, 1984; Lin *et al*., 1993). Therefore, a lot of studies were carried out about addition of different antioxidants in extenders to protect ram spermatozoa against detrimental effects of ROS (Bucak *et al*., 2007, 2008; Uysal and Bucak, 2007). However, there has not been any study about addition of defined antioxidant in extenders for cryopreservation of Tushin ram semen.

L-ergothioneine (the betaine of 2-thio-L-histidine; EGT) is a unique, naturally occurring antioxidant that is abundant in most plants and animals. High concentrations of EGT are found in a number of organ systems including liver, kidney, the eye, erythrocytes and also seminal fluid. Although various synthetic

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compounds of this chemical class exist, EGT is the only naturally occurring 2-thio-imidazole exists predominantly in the thione form (Hartman *et al*., 1990). At physiologic pH EGT does not auto-oxidize and is therefore very stable in aqueous solution, this explains why, unlike other alkylmercaptan antioxidants such as GSH (from thiol group). Moreover, EGT also differs significantly from natural thiol-containing antioxidants in that it does not stimulate lipid peroxidation in the presence of ferric ions. In physiological concentrations, EGT exhibits potent diffusion-controlled inactivation of hydroxyl radical (Heath *et al*., 1951a, b; Hans, 1992) and prevention of singlet oxygen production (Dahl *et al*., 1988; Hartman *et al*., 1990).

Studies (Mann and Leone, 1953; Haag and McLeod, 1959) pointed out that EGT has a critical protective role in seminal fluid approx. sixty years ago. EGT is the predominant sufhydryl in human, stallion and pig semen. Its role is evidently to protect spermatozoa from oxidative stress, given the exceptionally high metabolic rate in sperm. EGT, as consequence of its antioxidant properties, counteracts the effects of hydrogen peroxide on spermatozoa viability and survival while also enhancing the viability of sperm during storage. There are very limited studies about usage of L-ergothioneine during storage of ram semen (Yıldız and Daşkın, 2004; Çoyan *et al*., 2011) and stallion semen (Coutinho da Silva *et al*., 2008; Metcalf *et al*., 2008). In addition to semen storage, one study (Ozturkler *et al*., 2010) was carried out to test effects of L-ergothioneine on in vitro fertilization and embryonic development of ovine oocytes. There is not any study on cryopreservation of Tushin ram semen with milk based extenders containing L-ergothioneine.

The aim of this study was to test effect of different concentrations (i.e., 1, 2, 5 and 10 mmol/L) of EGT on freezability of Tushin ram semen extended with skim milk based extender.

MATERIALS AND METHODS

All chemicals, except L-(+)-Ergothioneine (EGT) (Tetrahedron®/France), were provided from Sigma-Aldrich (Germany) if not stated.

Animals: All animal procedures were approved by Local Experimental Ethical Committee at Kafkas University (KAÜ/HAYDEK; acceptance date/number: 09 March 2010/08). Prior to experiment, all rams were examined to ensure an absence of reproductive problems and all remained healthy throughout the study. Four healthy and sexually mature (2-4 years old; 50-60 kg) Tushin rams were selected and used in this study. Rams were housed at Education Research and Practice Farm, Faculty of Veterinary Medicine, Kafkas University, Turkey at 40°34’33’’N, 43°02’35’’E at an altitude of 1751 m. Rams were trained for artificial vagina and were used routinely as semen donors twice a week before the study. They were fed 0.91 kg of concentrate daily and good quality hay and water were supplied ad libitum.

Collection and evaluation of ejaculates: Ejaculates were obtained from each ram by artificial vagina according to the technique previously described by Salamon and Maxwell (1995). Ejaculates were collected twice a week during this experiment (April-May; spring). The volume and mass sperm activity were recorded before the tube was placed in a water bath at 37°C. Each ejaculate with 3+ mass activities was analyzed to determine its semen concentration, total number of spermatozoa per ml and semen motility, so that adequate semen quality was secured before ejaculates of the four rams were pooled. Only ejaculates with motility >75%, sperm concentration of $>2\times10^9$ spermatozoa/ml and semen volume of >0.5 ml were included in this study. Sperm concentration was determined using hemocytometric method, after diluting semen with Hayem solution (dilution rate 1/500). The percentage of motile spermatozoa was estimated by subjective microscopic examination using a phase contrast microscope supplied with heated stage at 37°C and magnification 400x after dilution with the extender (dilution rate 1:10).

Semen extender and semen processing: The skim milk based extender was prepared within 1 week period as following. Firstly, skim milk based solution was prepared with 10 g cow skim milk powder dissolved in 100 mL bi-distilled water and incubated at 92°C for 10 min and cooled. 0.9 % (w/v) glucose, 10 % (v/v) egg yolk and 5% (v/v) glycerol were added to skim milk based solution. After supplementation of antibiotics (500 IU of penicillin and 500 µg of streptomycin sulfate per milliliter), the Skim Milk based Extender (SME) was kept at 5°C until usage.

Immediately after collection and evaluation, pooled semen was divided five aliquots and each aliquot were extended with SME containing 0 mmol (EGT0; Control), 1 mmol/L (EGT1), 2 mmol/L (EGT2), 5 mmol/L (EGT5) and 10 mmol/L (EGT10) doses of L-ergothioneine (Tetrahedron®; room temperature stable form), respectively. Diluted semen was loaded into 0.25 mL French straws constituting doses of 100×10^6 spermatozoa per straw. French straws were sealed with polyvinyl alcohol powder. The straws were placed in cassettes and then in refrigerator at 5°C. The semen was allowed to equilibrate for 2 h before freezing.

After equilibration, the straws were frozen horizontally on a rack about 4 cm above Liquid Nitrogen (LN_2) held in an insulated container. The LN_2 vapor reduced the temperature within the straws to approximately -120°C in 15 min. Then the straws were transferred rapidly to LN_2 containers at -196°C. The straws were stored in LN_2 until evaluation time.

After storage, two straws from each doses of L-ergothioneine (i.e., EGT0, EGT1, EGT2, EGT5 and EGT10) were thawed in a warm bath at 37°C. After 1 min, the contents of the straw were examined microscopically as described at below.

Semen examination:

Progressive motility: After thawing of straws, a 3 µL aliquot of each sample was placed on a warmed (37°C) slide and covered with a cover slip before examination under a phase-contrast microscope (Nikon Eclipse E400, Nikon Corp., Japan) at 400x magnification. After observing four or five different fields, the percentage of progressively motile spermatozoa was recorded for each concentration of EGT. Throughout the experiment, two technicians (UÇA and RK) evaluated all the samples without knowing experimental groups and their mean values were recorded as a percentage.

Morphology: For evaluation of acrosomal integrity and morphological abnormalities, one drop sample from groups was diluted in 1 ml Hancock's solution (prepared with 62.5 mL formalin, 150 mL sodium saline solution, 150 mL buffer solution and 500 mL distilled water) (Shafer and Holzmann, 2000), placed under a cover slip and evaluated by differential interference phase-contrast microscopy (Nikon Eclipse E400, Nikon Corp., Japan) under immersion. The morphological alternations (including acrosomal integrity) were classified as described by Ax *et al*. (2000). Throughout the experiment, same technician (RK) evaluated all samples without knowing experimental groups.

Membrane integrity: To evaluate membrane integrity, 50 µL of semen sample was diluted with 450 µL of 100 mmol/L hypotonic solution (composed of 9 g fructose plus 4.9 g sodium citrate per liter of distilled water). After 45 min, smear was prepared and evaluated considering sperm tail curling (%) using bright-field microscopy (Olympus CX21, Olympos Optical Co. Ltd., Japan) (Hypo-osmotic swelling Test/HOST) (Azeredo *et al*., 2001). Throughout the experiment, same technician (UÇA) evaluated all samples without knowing experimental groups.

Viability: Percentage of viability in the extenders after thawing was evaluated with eosin staining (eosin-Y 1.67 g and sodium citrate 2.9 g dissolved in 100 mL distilled water) as described by Ax *et al*. (2000). The sperm smears were prepared by mixing a drop of semen with two drops of stain on a warm slide and spreading the stain immediately with the aid of a second slide. The viability was assessed by counting 200 sperm cells with bright-field microscopy (400x). Sperm showing partial or complete colorization were considered non-viable or dead. Only sperm showing strict exclusion of the stain were considered to be alive (Ax *et al*., 2000). Throughout the experiment, same technician (UÇA) evaluated all samples without knowing experimental groups.

Statistical analysis: The mean (± standard error of the mean) post-thaw semen parameters of sperm motility, viability, abnormality, acrosomal integrity and hypo-osmotic swelling test for the 17 trials (totally 85 samples for each semen parameters) carried out during this study were analyzed with ANOVA with subsequent Tukey's test to compare the mean values resulting from the various treatments at a significance level of $p<0.05$. Moreover, the totally 17 trials were divided in two groups (i.e., poor and good freezability), according to after thawing motility of control (EGT0 with lower than 20% motility were considered as a poor freezability trial). Totally eight good freezability trials and nine poor freezability trials were determined and separately analyzed. In this way, the effect of different concentrations of L-ergothionine was evaluated in total (seventeen), the good (eight) and the poor (nine) freezability trials. All analyses were carried out using the SPSS 11 for Windows statistical software package.

RESULTS

In current study, when all trials (seventeen; Table 1) and only good freezability trials (eight in seventeen; Table 2) were considered, any statistically significant differences among experimental groups was not determined for all parameters after thawing ($p>0.05$). Nevertheless, different concentration of EGT numerically improved sperm parameters post-thawing ($p>0.05$; Table 1 and 2). Moreover, whilst only poor freezability trials (nine in seventeen) were considered, 10 mmol/L EGT beneficially affected post-thawing

Table 1: The mean (±SE) percentage of motility, viability, membrane integrity, abnormal spermatozoa and acrosomal integrity in different doses of L-(+)-Ergothioneine, considered all trials (n = 17)

Concentration of ergothioneine	Number of trials (n)	Progressive Motility (%)	Viability (%)	Membrane integrity (%)	Abnormal spermatozoa (%)	Acrosomal integrity (%)
0 mmol/L	17	14.1±3.4[a*]	19.7±2.7[a]	27.9±3.2[a]	72.8±2.6[a]	30.0±2.7[a]
1 mmol/L		16.7±4.0[a]	23.0±2.8[a]	33.9±3.1[a]	70.8±2.3[a]	30.3±2.4[a]
2 mmol/L		16.1±3.9[a]	22.4±2.6[a]	34.2±3.8[a]	72.6±1.9[a]	29.4±1.8[a]
5 mmol/L		17.3±4.2[a]	21.8±2.2[a]	32.8±3.0[a]	73.7±1.7[a]	29.5±1.6[a]
10 mmol/L		22.9±5.5[a#]	27.1±2.2[a]	37.1±2.6[a]	72.3±2.3[a]	30.0±2.4[a]
p-value		>0.05				

[ab]: Different letters within columns indicate differences ($p<0.05$); [*#]: Different symbols within columns indicate tendency for motility ($p<0.06$)

Table 2: The mean (±SE) percentage of motility, viability, membrane integrity, abnormal spermatozoa and acrosomal integrity in different doses of L-(+)-Ergothioneine, considered good freezability trials (n = 8)

Concentration of ergothioneine	Number of trials (n/n)	Progressive motility (%)	Viability (%)	Membrane integrity (%)	Abnormal spermatozoa (%)	Acrosomal integrity (%)
0 mmol/L	8/17	22.5±1.8[a]	26.5±3.5[a]	29.2±2.7[a]	71.1±3.9[a]	32.1±3.8[a]
1 mmol/L		23.1±3.6[a]	28.8±4.1[a]	34.5±3.8[a]	67.3±3.1[a]	33.6±3.5[a]
2 mmol/L		18.7±3.7[a]	25.3±4.1[a]	35.1±4.8[a]	71.3±3.5[a]	30.9±4.2[a]
5 mmol/L		23.1±3.1[a]	25.8±3.3[a]	32.9±3.6[a]	71.8±3.3[a]	31.7±3.6[a]
10 mmol/L		26.8±2.8[a]	29.9±2.5[a]	33.3±2.2[a]	71.5±2.7[a]	30.0±4.0[a]
p-value		>0.05				

[ab]: Different letters within columns indicate differences (p<0.05)

Table 3: The mean (±SE) percentage of motility, viability, membrane integrity, abnormal spermatozoa and acrosomal integrity in different doses of L-(+)-Ergothioneine, considered poor freezability trials (n = 9)

Concentration of ergothioneine	Number of trials (n/n)	Progressive motility (%)	Viability (%)	Membrane integrity (%)	Abnormal spermatozoa (%)	Acrosomal integrity (%)
0 mmol/L	9/17	6.6±1.4[a]	13.7±3.1[a*]	22.7±3.7[a]	74.6±3.7[a]	27.5±3.2[a]
1 mmol/L		11.1±1.6[ab]	17.9±3.0[a]	30.6±4.0[ab]	74.7±3.1[a]	26.5±4.3[a]
2 mmol/L		13.8±3.0[ab]	19.7±3.3[a]	30.9±4.4[ab]	74.2±1.9[a]	27.7±2.0[a]
5 mmol/L		12.2±2.0[ab]	18.2±2.5[a]	30.6±3.9[ab]	75.9±1.0[a]	26.9±1.4[a]
10 mmol/L		19.4±2.5[b]	24.6±2.5[a#]	39.5±4.2[b]	73.2±3.2[a]	30.0±2.7[a]
p-value		<0.05	>0.05	<0.05	>0.05	>0.05

[ab]: Different letters within columns indicate differences (p<0.05); [*#]: Different symbols within columns indicate tendency for viability (p<0.06)

motility and membrane integrity of spermatozoa, compared with control (p<0.05; Table 3).

DISCUSSION

This study was carried out to investigate effect of different doses of EGT on freezability of ram semen. In current study, beneficial effects of 10 mmol/L EGT on post-thawing progressive motility and membrane integrity of ram semen were determined in poor freezability trials. Moreover, other concentrations of EGT did beneficially affect post-thawing sperm parameters although statistical differences were not found in total and good freezability trials. A lot of studies have been carried out to determine effects of antioxidant on freezability of ram semen (Bucak *et al*., 2007, 2008; Uysal and Bucak, 2007; Camara *et al*., 2011; Çoyan *et al*., 2011). While Bucak *et al*. (2008) determined beneficial effects of thiols on freezability of ram semen, Camara *et al*. (2011) did not find any effect of antioxidants from thiols on freezability of ram semen.

Only one study about EGT on ram semen cryopreservation has been carried out and current study is the first study to be carried out in Tushin ram semen cryopreservation. Çoyan *et al*. (2011) tried three (1, 2 and 4 mmol/L) different doses of EGT on freezability of merinos ram semen and determined any beneficial effect of EGT on freezability of ram semen, except CASA motility for 4 mmol/L EGT. In current study, similar results were obtained for 1, 2 and 5 mmol/L EGT, but 10 mmol/L EGT statistically improved post thawing progressive motility and membrane integrity in poor freezability trials. Nevertheless, all EGT concentrations numerically improved post-thawing sperm parameters in our study for all trials and any detrimental effect of EGT observed on semen parameters.

Another important finding of current study was that protective effect of EGT is clearer in poor freezability trials compared with good or all trials. All concentration of EGT numerically improved all post-thawing sperm parameters, but only 10 mmol/L EGT statistically improved motility and membrane integrity of post thawing ram semen especially in poor freezability trials. Lower doses of EGT (<10 mmol/L) may have not affected freezability of ram semen. However, Çoyan *et al*. (2011) determined that lower doses of EGT improved some spermatological parameters after thawing with TRIS extender. This difference with our findings may be rooted from breeding differences and also extenders used during experiments. In this study, skim milk based extender was used and it is well known that skim milk based extenders including goat milk provide more protective effect to ram spermatozoa compared with TRIS based extenders during cryopreservation and thawing process (Ari *et al*., 2011a). Therefore, EGT showed protective effect only in poor freezability trials when skim milk based extenders were used. And, usage of EGT may be more efficient with poor extenders or trials with poor freezability risks.

In this study, although any biochemically test were not carried out, 5 and 10 mmol/L concentrations of EGT did not affect total antioxidant and oxidant capacity in frozen thawed Tushin ram semen in our preliminary study (unpublished data). Therefore, beneficial effect of 10 mmol/L concentration of EGT on frozen-thawed ram semen may have been originated from other characteristic of EGT, in addition to as an antioxidant. Akanmu *et al*. (1991) stated that EGT is a powerful scavenger of hydroxyl radicals (.OH) and an inhibitor of iron or copper ion-dependent generation of

.OH from hydrogen peroxide (H_2O_2). By contrast it does not react rapidly with superoxide (O_2^-) or hydrogen peroxide (H_2O_2). Moreover, Çoyan *et al*. (2011) showed that EGT had not affected LPO, SOD activity, GPx Activity and CAT activity, but improving post-thawed sperm motility and also motility characteristics.

Higher concentration of EGT (i.e., 5 and 10 mmol/L) did not detrimentally affect post-thawing semen parameters in all trials while 10 mmol/L EGT improving motility and membrane integrity in current study in poor freezability trials. Similarly, Aruoma *et al*. (1999) determined that concentration of the natural antioxidant ergothioneine in mammalian tissue is 1-2 mmol/L, which suggests that EGT may serve as a non-toxic thiol buffering antioxidant in vivo. And, the researchers suggested that EGT may find applications in pharmaceutical preparations where oxidative stability is desired.

CONCLUSION

In conclusion, high concentrations of L-(+)-ergothioneine may tolerably be used to improve post-thawing semen parameters in species with higher cryo-sensivity of its spermatozoa. Moreover, it is clear that higher concentration of EGT (>10 mmol/L) have to be tried to determine real effects of EGT on cryopreservation of ram semen and also fertilization trials must be carried out in future studies in ram semen frozen with extender containing EGT.

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