

Effects of Trehalose and Catalase on the Viability and Kinetic Parameters of Cryopreserved Ram Sperm

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ABSTRACT

Background: Most part of ram spermatozoa membrane has unsaturated fatty acids (phospholipids). Membrane structure of cells is composed of double ordered phospholipid layers adorned with mosaic-like protein, glycoprotein and glycolipids. Sperm freezing protocols could be negatively affected on ram sperm motility, viability and acrosome integrity during cryopreservation. For these reasons, researchers were designed their topics has led to the search for effective antioxidant systems against peroxidative damage and spermatozoon dysfunction. There are three protective enzymatic systems against reactive oxygen species (ROS) damage in sperm. These include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase / reductase cycles. Catalase is a hemo-protein in the enzyme tetramer structure. The aim of this study was to investigate the effects of trehalose, catalase and their combinations on ram sperm parameters after the cryopreservation/thawing process.

Materials, Methods & Results: At the out of breeding season (March-May) seven rams (1-3 years of age) were used in this study. Ejaculates were collected by electro-ejaculator twice a week. Pooled ejaculates were kept at 37°C, divided into six aliquots, diluted with the Tris based extender containing Trehalose 25 mM (Group-1), Trehalose 50 mM (Group-2), Catalase 200 µg (Group-3), Catalase 400 µg (Group-4), Trehalose 50 mM + Catalase 400 µg (Group-5) and no anti-oxidant (control), respectively, were cooled to 5°C than frozen in 0.25 mL French straws on the nitrogen vapour and stored in liquid nitrogen. The extender supplemented with Group 1 (54.1 ± 1.53 ; 73.1 ± 4.37), Group 2 (58.3 ± 4.01 ; 63.1 ± 0.30) and Group 5 (56.6 ± 1.05 ; 58.3 ± 0.55) resulted in higher subjective motility in comparison to the control (40.0 ± 3.87 ; 40.5 ± 0.22) group respectively ($P < 0.05$). Besides, Group 2 (60.16 ± 4.39) and Group 5 (59.60 ± 2.21) led to higher CASA total motility when compared to control (44.40 ± 8.13) group ($P < 0.05$). Sperm progressive motility was better in Group 1 (20.57 ± 6.90) than the Group 3 (10.63 ± 3.59) [$P < 0.05$]. Casa kinetic parameters of catalase 200 µg (Group 3) was higher values than other groups in VCL, VSL, VAP, LIN parameters ($P < 0.05$). There were no statistically significant differences on the membrane integrity between the groups ($P > 0.05$).

Discussion: Freezing ram sperm is extremely difficult process when compared bull and dog semen. Previous studies showed that antioxidants which were adding into the ram freezing extender gave positive effects solely or combination. In this study similar results were taken at trehalose 25, 50 mM and trehalose 50 mM + catalase 400 µg except 200 and 400 µg catalase groups. These findings supported some researches but lots of them opposite of catalase results. Catalase is found in semen and ameliorates the sperm parameters when adding the liquid storage. Also, after diluted and equilibrated catalase groups motilities were better than the control group. During the freezing stage catalase efficiency has been restricted. On the other hand when it combined with the trehalose 50 mM, catalase activity was triggered. Trehalose acts on sperm as non-permanent had a protective action related both osmotic effect and specific interactions with membrane phospholipids. Our data suggest that solely Trehalose 50 mM or combination with Catalase 400 µg can be added to Tris based extender for improving the post-thawed sperm quality in ram semen.

Keywords: catalase, viability, freezing, ram sperm.

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INTRODUCTION

Nowadays sheep population is an important place within the livestock sector but, we cannot be say to be at the same level in terms of yield. The efficient use of rams can be achieved through the use of reproductive biotechnologies [40].

Restrictive effect is ram sperm membrane sensitivity to freezing protocols. The thermodynamic properties of these constructions and unsaturated phospholipids converted to membranes from liquid phase to the gel phase [38,39]. After the break down the stabilization of cell, cold shock damage develops in the cell, which is called cryo-injury in the terminology [18].

At last decade, using antioxidative substances; hypotaurin, ascorbic acid, alpha-tocopherol, butylated hydroxytoluene (BHT), catalase which were found naturally and higher concentration in the epididymis and seminal plasma [34]. Catalase has been reported to enhance the viability of bull spermatozoa in an egg yolk diluent [12]. Trehalose is known that disaccharide which protects sperm membrane structure from oxidative stress. And also trehalose may act positively spermatological parameters and fertility results to accelerate the semen antioxidant enzymes levels such as catalase, superoxide dismutase and glutathione [19].

With the light of these informations, this study was aimed to determine influence of the addition of different concentrations of trehalose including 25, 50 mM; catalase 200, 400 µg and combination of trehalose 50 mM + catalase 400 µg on standard ram semen parameters after freezing-thawing process.

MATERIALS AND METHODS

Local and animal management

This study was conducted from March to May (spring-summer northern hemisphere) at the Mehmet Akif Ersoy University Department of Artificial Insemination clinics. In this study, semen was collected from seven mature Merino-Sakiz crossbreed rams (1-3 years old), and the animals were housed at the under uniform optimal nutritional conditions. All handling process in this study was hold on general guidelines for animal welfare.

Semen collection

Ejaculates were collected twice a week from the rams with the aid of an electro-ejaculator (Electro-Ejaculator E320)¹ during the out of breeding season.

Totally 98 ejaculates were collected from seven rams. For sperm quality, collected ejaculates analyzed if normospermic properties or not. This procedure were performed in a water bath at 33°C within approximately 20 min after semen collection.

Semen analysis

Macroscopic analysis of ejaculate volume was recorded in a graduated tube, and the concentration was determined using a haemo-cytometer. Fresh sperm motility was estimated using phase-contrast microscopy (400 × magnification) at 37°C. Only ejaculates between 1 and 2 mL in volume, containing sperm with >80% initial motility and an ejaculate concentration of higher than 1×10⁹ sperm/mL were used.

Semen extending and freezing

Pooling of semen was performed to balance the sperm contribution of each male and eliminate individual ram differences. In total 98 pooled ejaculates were used in the study. A Tris-based extender (tris (Trizma® Base T1503)² 297.58 mM, citric acid (Citric acid C0759)² 96.32 mM, fructose (D-Fructose F0127)² 82.66 mM, glycerol (Glycerol G5516)² 5% (v/v), egg yolk 15% (v/v), penicillin-streptomycin (Penicillin-Streptomycin P4333)² 500 IU/mL, pH 6.8) was prepared as the base extender. Each pooled ejaculate at 37°C was split into 6 equal aliquots and diluted with the base extender containing trehalose (D-(+)-Trehalose dihydrate T0167)² 25 (Group 1) and 50 mM (Group 2), catalase (Catalase from bovine liver C40)² 200 (Group 3) and 400 µg (Group 4), combination of trehalose 50 mM +catalase 400 µg (Group 5) and no antioxidant (control, Group 6), respectively. The final semen concentration was diluted to 800×10⁶ sperm/mL. Diluted semen samples were stored into 0.25 mL French straws¹ and cooled from 37°C to 5°C in a refrigerator for equilibration at 2.5 h. The straws were placed horizontally and frozen in a vapor 5 cm above liquid nitrogen for 15 min then stored in liquid nitrogen. Post thawed sperm parameters were determined after about one month.

Assessment of sperm motility

Subjective motility was analyzed using a phase-contrast microscope (400× magnification), with a warm stage maintained at 37°C. Sperm motility estimations were performed in at least five different fields for each sample. The mean of the five successive evaluations was recorded as the motility score.

Add of sperm subjective motility analyze, also computer-assisted sperm motility analysis (CASA) was done with Sperm Class Analyzer (SCA® v.4.2)³ for analyze sperm motion characteristics. The spermatozoa motility properties were set as static, slow >60 µm/s, medium >90 µm/s, fast >120 µm/s [36]. The following motility values were recorded: motility (%), progressive motility (%), VCL (curvilinear velocity, µm s⁻¹), VSL (straight linear velocity, µm s⁻¹), VAP (average path velocity, µm s⁻¹), LIN (Linearity,[VSL/VCL] x100). Evaluation process was done at least 250 and most 400 spermatozoa in six microscopic fields for each parameter.

Assessment of sperm viability

The viability of spermatozoa was assessed by nigrosin–eosin staining method [11]. Sperm suspension smears were prepared by mixing a drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide; viability was assessed by counting 200 cells under phase-contrast at 400× magnification. Sperm displaying complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

Assessment of sperm membrane functional integrity

Hypo-Osmotic Swelling (HOS) test was used to calculate the ratio of intact sperm, based on curled and swollen tails. This was performed by incubating 10 µL of semen with 100 µL of 100 mOsm hypoosmotic fructose (D-Fructose F0127)² solution at 37°C for 60 min. After incubation, 10 µL of the mixed solution was spread with a cover slip on a warm slide. Totally 200 sperm cells were evaluated under 400× magnification with phase-contrast microscopy and spermatozoa with swollen or coiled tails were recorded [28].

Assessment of sperm morphology

Abnormal spermatozoon rate was determined by fixation liquid method. Spermatozoon were fixed with Hancock solution [16] and abnormalities (head, tail) detected under 1000x magnification with phase-contrast microscopy by evaluating at least 200 sperm cells [32].

Statistical Analysis

The study was replicated seven times. The results were expressed as Mean (X) ± standard deviation (SD). Means were analyzed using a one-way analysis of variance (ANOVA), followed by Duncan’s post hoc test to determine the significant differences in all parameters among all groups using the SPSS/PC computer program (version 21.0)⁴. Differences with values of *P* < 0.05 were considered statistically significant.

RESULTS

Different doses of trehalose, catalase and combination of trehalose and catalase were investigated on semen parameters through the freeze-thawing process evaluated in 7 experiments. In Table 1 and Table 3, Group 1, Group 2 and Group 5 were higher subjective and CASA motility than control group after thawed semen (*P* < 0.05). In Table 2, Group 1, Group 2 and Group 5 were higher viability compared to control (*P* < 0.05). Also, in Table 2, control group were higher abnormal spermatozoa rate compared to Group 1, Group 2 and Group 5 (*P* < 0.05). The results of CASA kinetic parameters were significant differences between the groups. Especially, catalase 200 (Group 3) was higher values than other groups in VCL, VSL, VAP, LIN parameters. Membrane integrity (HOS test) results were not statistically different compared the control group (*P* > 0.05, Tables 2 & 3).

Table 1. Effect of trehalose, catalase and combination of trehalose and catalase on subjective motility parameters (Mean±SD).

Group	After dilution %	After equilibration %	Post-thawed %
Control	80.0 ± 1.82	64.1 ± 1.53 ^b	40.0 ± 3.87 ^b
Trehalose 25 mM (Group 1)	77.5 ± 2.81	70.0 ± 1.82 ^a	54.1 ± 1.53 ^a
Trehalose 50 mM (Group 2)	78.3 ± 2.78	68.3 ± 1.66 ^{ab}	58.3 ± 4.01 ^a
Catalase 200 µg (Group 3)	76.6 ± 2.78	68.3 ± 1.66 ^{ab}	33.3 ± 2.10 ^b
Catalase 400 µg (Group 4)	76.6 ± 2.78	68.3 ± 1.05 ^{ab}	29.1 ± 6.50 ^b
Trehalose 50 mM + Catalase 400 µg (Group 5)	81.6 ± 1.66	69.1 ± 1.53 ^{ab}	56.6 ± 1.05 ^a
<i>P</i>	#	*	*

^{ab}Different superscripts within the same column means significant differences (*P* < 0.05). **P* < 0.05; #*P* > 0.05.

Table 2. Effect of trehalose, catalase and combination of trehalose and catalase on viability, HOS-test and normal spermatozoa rate after freeze thawed ram semen (Mean±SD).

Group	Viability %	Membrane integrity %	Morphology %
Control	40.5 ± 0.22 ^{de}	38.0 ± 3.64	73.0 ± 4.99 ^b
Trehalose 25 mM (Group 1)	73.1 ± 4.37 ^a	38.0 ± 6.13	84.0 ± 2.11 ^a
Trehalose 50 mM (Group 2)	63.1 ± 0.30 ^b	34.8 ± 5.50	82.1 ± 0.65 ^a
Catalase 200 µg (Group 3)	48.3 ± 3.46 ^{cd}	30.8 ± 4.92	64.3 ± 0.91 ^c
Catalase 400 µg (Group 4)	38.8 ± 4.48 ^e	24.6 ± 1.22	67.0 ± 0.85 ^{bc}
Trehalose 50 mM + Catalase 400 µg (Group 5)	51.3 ± 0.55 ^c	35.5 ± 5.15	81.8 ± 0.83 ^a
<i>P</i>	*	#	*

^{a,b,c,d,e}Different superscripts within the same column means significant differences ($P < 0.05$); * $P < 0.05$; # $P > 0.05$

Table 3. Effect of trehalose, catalase and combination of trehalose and catalase on CASA motility and kinetic parameters after freeze thawed semen (Mean±SD).

Group	Casa Total Motility (%)	Progressive Motility (%)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)
Control	44.40 ± 8.13 ^{bc}	15.82 ± 5.13 ^{ab}	85.15 ± 2.54 ^{ab}	44.88 ± 5.84 ^b	57.88 ± 5.19 ^{abc}	52.61 ± 5.68 ^{ab}
Trehalose 25 mM (Group 1)	50.02 ± 4.85 ^{cd}	20.57 ± 6.90 ^b	84.71 ± 2.83 ^{ab}	45.21 ± 8.85 ^b	59.30 ± 8.42 ^{abc}	53.22 ± 9.26 ^{ab}
Trehalose 50 mM (Group 2)	60.16 ± 4.39 ^d	18.65 ± 4.47 ^{ab}	84.89 ± 3.29 ^{ab}	46.09 ± 7.67 ^b	57.37 ± 6.20 ^{ab}	51.84 ± 7.41 ^{ab}
Catalase 200 µg (Group 3)	26.81 ± 7.25 ^a	10.63 ± 3.59 ^a	86.88 ± 2.93 ^b	56.85 ± 6.28 ^c	66.76 ± 4.42 ^c	60.56 ± 9.38 ^b
Catalase 400 µg (Group 4)	32.69 ± 8.11 ^{ab}	12.06 ± 4.00 ^{ab}	83.59 ± 4.39 ^{ab}	50.90 ± 5.59 ^{bc}	63.62 ± 4.71 ^{bc}	57.17 ± 7.90 ^b
Trehalose 50mM + Catalase 400 µg (Group 5)	59.60 ± 2.21 ^d	12.23 ± 2.78 ^{ab}	79.82 ± 6.89 ^a	34.38 ± 1.51 ^a	51.03 ± 2.99 ^a	43.21 ± 2.46 ^a
<i>P</i>	*	*	*	*	*	*

^{a,b,c,d}Different superscripts within the same column demonstrate significant differences ($P < 0.05$); * $P < 0.05$.

DISCUSSION

The present study aimed restoring the balance of oxidant-antioxidants in the system by addition of trehalose and catalase in extender together or solely. For this aim, evaluated on spermatological parameters during cryopreservation (after dilution, before freezing, and after thawing), *in vitro* quality (after thawing) of Merino-Sakiz crossbreed ram spermatozoa.

The spermatozoon plasma membrane is extremely sensitive to lipid peroxidation as it is rich in unsaturated fatty acids. Cells may be exposed to lipid peroxidation at long term storage of the sperm. As a result, there are losses in spermatozoon motility, membrane integrity and fertility rates [30].

Process of cryopreservation has a detrimental effect on spermatological parameters so that researchers investigated various methods for protect to ram semen [4,25,40]. In last decade, trehalose was added to the extenders of bull [10,37], buffalo bulls [27,33], ram [6,14,15,26], goat [5], rabbit [41] to improve the semen

quality. A sugar such as trehalose is feasible, because decreased the negative effects of water flow through the membrane during freezing, such as formation of ice crystals [3]. Trehalose, also performing as a cryoprotectant [3,24,31] interacts with modifies sperm membrane phospholipids and proteins resulting in improved membrane flexibility that with stand cryo-injuries [6,25]. On the other hand, Trehalose stabilizes cellular membrane by acting on the polar heads of phospholipids and avoiding dehydration and rehydration during phase transitions due to temperature changes [8].

When evaluating the different concentrations of Trehalose (50 and 100 mM), it was observed that a Trehalose concentration of 50 mM had positively effect on the total and progressive sperm motility and morphological integrity in ram semen [9]. These findings were basis for the use of Trehalose 25 and 50 mM in the present study. Also we get similar results with addition of trehalose 50 mM and combination of Trehalose 50 mM with Catalase 400 µg.

This presented study demonstrated that supplementation of Trehalose 25, 50 mM and combination of Trehalose 50 mM with Catalase 400 µg were gave higher viability and lower abnormal spermatozoa ratio compared the control. These results were in agreement to bull [8], ram [6,24] goat [1], mouse [35] sperm cryopreservation studies.

Trehalose 50 mM has ameliorating effect on ram semen during short term storage [6]. In the present study this findings observed similar at the end of equilibration time. Trehalose not only reacts at energy balance it also effects on heat stress. Jafaroghli *et al.* [20] were noticed that best concentration of trehalose was 100 mM, in the present study we found 50 mM dose of trehalose could act more positively on freeze-thawed ram sperm motility and viability against the 25 mM Trehalose.

In the present study addition of Trehalose 25, 50 mM and combination group gave lower morphological defects compare the control. However, catalase 200 and 400 µg/mL were not protect the spermatozoa and have increased the morphological defects. This was because catalase could not protect against cold shock itself. This had to be because combination group were better normality rates against catalase 200 and 400 µg/mL.

ROS levels have an important effect on sperm physiological processes such as capacitation, hyperactivation, signaling processes and acrosome reaction to ensure fertilization [2]. In this study, it observed that adding catalase 200 and 400 µg/mL were decreased the post thawed motility, viability but also increased the abnormalities compared the other groups. These two concentration it seemed, increasing the amount of available antioxidants per cell, thereby higher concentrations of the antioxidants per cell may be lethal to sperm during the freeze-thawing procedure. Also catalase 100 IU/mL had improved post-thawed quality of rooster semen this finding could support lower dose of catalase could act positively on post-thawed sperm parameters [23]. Similar results were observed in liquid-stored ram semen, where the sperm motility

increased catalase 100 U/mL and 200 U/mL groups while the functionality of the sperm membrane was not affected after catalase addition [7]. Nevertheless, the beneficial effect of catalase on both the viability and motility was demonstrated for boar [29] and canine [22] sperm after freeze thawing.

In kinetic parameters there were significant differences between the groups. Especially, group 3 was higher values than other groups in VCL, VSL, VAP, LIN parameters. VCL and VAP are evaluate of sperm velocity over specifical paths, so these values indicate that sperm classified as high mobility swim faster than those classified as lower mobility [21]. Thus, VSL's role in sperm transport may be during the passage through the female reproductive tract and penetration of the oocyte vestments [13,17].

CONCLUSION

Based on the results of the present study, the addition of catalase (200 and 400 µg/mL) cold not reduced the negative effect of freezing on the sperm parameters of ram sperm. However, affect on the sperm membrane functionality. The addition of 50 mM trehalose solely or combination with catalase 400 µg/mL had showed a positive effects on the sperm parameters. Also these findings could be support with the in-vivo fertility trials.

MANUFACTURERS

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Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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