

## The role of different trehalose concentrations and cooling rates in freezing of ram semen

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**Summary:** The aim of this study was to determine the influence of different cooling rates and trehalose concentrations on standard semen parameters after the freezing-thawing of ram spermatozoa. Ejaculates collected using artificial vagina from 4 Akkaraman rams were evaluated and pooled at room temperature. Semen samples diluted with a Tris-based extender containing different concentrations (50, 100 and 150 mM) of trehalose and no additives (control) were cooled to 5°C and frozen at slow (a rate of 0.5°C/min from 5°C to -25°C; -50°C/min from -25°C to -130°C), and fast (a rate of 10°C/min from 5°C to -25°C; -50°C/min from -25°C to -130°C) cooling rates and plunged into liquid nitrogen. Frozen straws were thawed individually at 37°C for 20 sec in a water bath for evaluation. In conclusion, 50 and 100 mM trehalose and slow cooling rate were found to significantly ( $p<0.05$ ) improve the freezability of ram spermatozoa which were assessed regarding post-thawing sperm motility, morphologic sperm abnormalities, viability and membrane integrity. But, 100 mM trehalose concentration and slow cooling rate were observed superior than other treatment groups.

Key words: Fast freezing, ram semen, semen parameters, slow freezing, trehalose.

### Koç spermalarının dondurulmasında farklı trehaloz konsantrasyonu ve soğutma oranlarının rolü

**Özet:** Bu çalışmanın amacı dondurma/çözdürme sonrası farklı soğutma oranları ve trehaloz konsantrasyonlarının standart spermatojik parametreler üzerine etkisini saptamaktır. Sun'î vagina kullanılarak 4 Akkaraman koçtan toplanan ejakülatlar oda sıcaklığında değerlendirildi ve karıştırıldı. Farklı konsantrasyonlarda (50, 100 ve 150 mM) trehaloz içeren ve içermeyen (control) tris bazlı solusyonla sulandırılmış sperma örnekleri 5°C'a soğutuldu ve yavaş (dakikada 0.5°C ile 5°C'dan -25°C'a; dakikada -50°C ile -25°C'dan -130°C'a) ve hızlı (dakikada 10°C ile 5°C'dan -25°C'a; dakikada -50°C ile -25°C'dan -130°C'a) soğutma oranlarında donduruldu ve sıvı azota daldırıldı. Dondurulmuş payetler değerlendirme için bireysel olarak su banyosunda 37°C'da 20 saniyede çözdürüldü. Sonuç olarak, çözdürme sonrası spermatozoa motilitesi, morfolojik spermatozoa anomalileri, canlılığı ve membrane bütünlüğü değerlendirilen koç spermatozoasının dondurulabilirliğini, 50 ve 100 mM trehaloz ile yavaş dondurma oranının önemli ölçüde ( $p<0.05$ ) geliştirdiği bulundu. Fakat, 100 mM trehaloz konsantrasyonu ve yavaş soğutma oranının diğer tedavi gruplarından daha üstün olduğu gözlemlendi.

Anahtar sözcükler: Hızlı dondurma, koç sperması, sperma parametreleri, yavaş dondurma, trehaloz..

### Introduction

The enhancement of artificial insemination is a valuable tool in genetic improvement programs and ovine breeds conservation. However, artificial insemination is not so widespread in ovine as it is in other domestic species (4). Because of ultrastructural, biochemical and functional changes, low fertility rates of cryopreserved ram sperm are undergone by a large sperm population, which leads to insufficient motility and loss of the viability of spermatozoa following the thawing (25). Because sperm membranes are exposed to ultrastructural changes during the freezing-thawing process, there is a redistribution of lipids that alters lipid-lipid and lipid-protein relations which are necessary for the normal function of sperm membranes. An intact and functional

plasmalemma is a prerequisite for maintaining membrane potential, ionic microenvironment and pH (17). Therefore, freezeability of the sperm cells can be improved by addition of some additives and using different freezing methods cryopreservation (3, 6).

It is known that disaccharides protect sperm membrane structure from oxidative stress and cold shock damage during freezing-thawing process. Nonpermeant disaccharides (sucrose, trehalose) have a protective action related both to osmotic effect and specific interactions with membrane phospholipids (16). Trehalose is found in a number of plants and animals that can resist dehydration or freezing (29), and the action of trehalose appears to be connected with its ability to replace water at the membrane/solution interface (5). When trehalose

was added in hypertonic conditions, it showed a synergic effect with glycerol used as a cryoprotectant in order to avoid ice crystal formation from intracellular water on cell integrity. In addition to these functions, supplementation with trehalose of semen diluents is well known to improve the viability and motility of liquid or cryopreserved ram sperm cells (2).

Protection from dehydration and crystallization stress during semen freezing may also depend on freezing rate. With regard to the cryopreservation process, a capital objective is the achievement of the optimum cooling rate. Damage to sperm membranes primarily occurs during the freezing and thawing process over the temperature range  $-15^{\circ}\text{C}$  to  $-60^{\circ}\text{C}$  (19). Plasma membranes of ram spermatozoa are the most susceptible to damage during freezing between temperatures of  $-10^{\circ}\text{C}$  and  $-25^{\circ}\text{C}$ . While slow cooling rates result in the osmotic shrinkage of vesicles as water freezes out of the extravascular space, rapid freezing rates do not allow time for the transport of water through the lipid bilayer and increase the likelihood of intravesicular ice formation (23). Byrne et al. (8) reported that high fertility results are obtained from samples frozen with rapid cooling rates ( $-5^{\circ}\text{C}/\text{min}$ ).

This study was conducted in order to determine the influence of the addition of different concentrations of trehalose including 50, 100 and 150 mM and slow (a rate of  $0.5^{\circ}\text{C}/\text{min}$  from  $5^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ;  $-50^{\circ}\text{C}/\text{min}$  from  $-25^{\circ}\text{C}$  to  $-130^{\circ}\text{C}$ ) and fast (a rate of  $10^{\circ}\text{C}/\text{min}$  from  $5^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ;  $-50^{\circ}\text{C}/\text{min}$  from  $-25^{\circ}\text{C}$  to  $-130^{\circ}\text{C}$ ) freezing rates on standart semen parameters after freezing-thawing process.

### Material and Methods

All chemicals, reagents and antibiotics were purchased from Sigma–Aldrich chemicals. In this trial, a Tris-based extender (CTR) was used (Tris 3.63 g/100 ml, fructose 0.50 g/100 ml, citric acid 1.99 g/100 ml, egg yolk 15 ml/100 ml, glycerol 5 ml/100 ml, penicillin 100.000 IU/100 ml, streptomycin 100 mg/100 ml - pH 6.8-7, 300 mOsm) for all the ejaculates.

*Animals and Semen Collection:* The animals (3 to 4 year-old rams) were housed at Ankara University, Faculty of Veterinary Medicine, Education Research and Practice Farm under uniform nutritional conditions. Semen collected by artificial vagina from 4 mature Akkaraman rams during the breeding season was used in this study. In a total of 10 ejaculates were collected from each rams. Immediately after collection, the ejaculates were evaluated for spermatological parameters including ejaculate volume (ml) sperm motility (%), sperm concentration ( $\times 10^6/\text{ml}$ ), morphologic sperm abnormalities (%) sperm viability (%), and membrane integrity (%).

The ejaculates were pooled in the trial if the following criteria were met: volume varying between 1.0–2.0 ml, the motile sperms percentage higher than 80 %, sperm concentration of  $3 \times 10^9$  sperm/ml, less than 10 % abnormal sperm and higher than 20 % viability in a pool balancing the sperm contribution of each male to eliminate individual differences (13).

*Semen Extending, Freezing and Thawing:* Each pooled ejaculate was split into four equal aliquots and diluted at  $37^{\circ}\text{C}$  with the CTR extender containing different concentrations of trehalose (50 mM, 100 mM or 150 mM) and no additives (control) to a final concentration of approximately  $4 \times 10^8$  spermatozoa per ml in one step in a 10 ml-glass centrifuge tube. Diluted samples were loaded into 0.25 ml straws and sealed with polyvinyl alcohol powder. All straws were divided into two factions, consisting of three different concentrations of trehalose and no additives (control). After equilibration period at  $5^{\circ}\text{C}$  for 2.5 h, one faction was frozen at slow cooling rate (a rate of  $0.5^{\circ}\text{C}/\text{min}$  from  $5^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ;  $-50^{\circ}\text{C}/\text{min}$  from  $-25^{\circ}\text{C}$  to  $-130^{\circ}\text{C}$ ), and the other was frozen at fast cooling rate (a rate of  $10^{\circ}\text{C}/\text{min}$  from  $5^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ ;  $-50^{\circ}\text{C}/\text{min}$  from  $-25^{\circ}\text{C}$  to  $-130^{\circ}\text{C}$ ), using programmable freezer (Planer, Kryo 440-1.7). Once the straws reached  $-130^{\circ}\text{C}$ , they were plunged directly into liquid nitrogen at  $-196^{\circ}\text{C}$  (Byrne et al., 2000). After storage for one month, 10 straws were thawed in a water-bath at  $37^{\circ}\text{C}$  for 30 seconds for each of freezing rates and different trehalose concentrations. A total of 80 straws were evaluated for post-thawing sperm motility, morphologic sperm abnormalities, sperm viability and sperm membrane integrity by HOS test.

*Semen evaluation:* Analysis of standard semen parameters; The volume of ejaculates was measured in a conical tube graduated at 0.1 ml intervals. Sperm motility was assessed using a phase-contrast microscope (10x10 magnification) with a warm stage maintained at  $37^{\circ}\text{C}$ . A wet mount was made using a  $5 \mu\text{l}$  drop of semen placed directly on a microscope slide and covered by a cover slip. Sperm motility estimations were performed in 3 different microscopic fields for each semen sample. The mean of the 3 successive estimations was recorded as the final motility score.

For abnormal sperm assessment, at least three drops of each sample were added to Eppendorf tubes containing 1 ml of Hancock (26) solution [62.5 ml formalin (37%), 150 ml sodium saline solution, 150 ml buffer solution, and 500 ml of double-distilled water]. One drop of this mixture was put on a slide and covered with a cover slip. The percentage of total sperm abnormality was determined by counting a total of 400 spermatozoa under phase contrast microscope (magnification 10x100, oil immersion).

Table 1: Mean ( $\pm$ SEM) post-thawing sperm characteristics for various trehalose concentrations at rapid and slow cooling rates (n:10).

| Freezing rate and trehalose (Tr) concentrations (mM) | Sperm motility (%)<br>means $\pm$ S.E.M | Morphologic sperm abnormalities (%)<br>means $\pm$ S.E.M | Sperm viability (%)<br>means $\pm$ S.E.M | Membrane integrity (%)<br>means $\pm$ S.E.M |
|--|---|--|--|---|
| Rapid cooling Tr 0                                   | 53.5 $\pm$ 4.7 b                        | 46.2 $\pm$ 2.3 c   | 66.3 $\pm$ 7.2 a                         | 53.0 $\pm$ 5.0b                             |
| Rapid cooling Tr 50                                  | 62.5 $\pm$ 3.8 bc                       | 29.6 $\pm$ 2.9 ab  | 74.2 $\pm$ 4.5 a                         | 61.7 $\pm$ 3.5 bc                           |
| Rapid cooling Tr 100                                 | 67.5 $\pm$ 3.2 c                        | 28.1 $\pm$ 2.9 ab  | 79.5 $\pm$ 3.6 a                         | 60.9 $\pm$ 3.0bc                            |
| Rapid cooling Tr 150                                 | 26.5 $\pm$ 2.8 a                        | 34.8 $\pm$ 3.1 b   | 40.9 $\pm$ 3.8 b                         | 23.1 $\pm$ 3.3 a                            |
| Slow cooling Tr 0                                    | 62.5 $\pm$ 3.3 bc                       | 42.3 $\pm$ 2.3 c   | 75.0 $\pm$ 3.7 a                         | 61.4 $\pm$ 3.1 bc                           |
| Slow cooling Tr 50                                   | 68.0 $\pm$ 2.9 c                        | 28.7 $\pm$ 2.1 ab  | 74.5 $\pm$ 2.5 a                         | 66.1 $\pm$ 2.8 c                            |
| Slow cooling Tr 100                                  | 72.0 $\pm$ 3.7 c                        | 25.5 $\pm$ 2.5 a   | 75.9 $\pm$ 4.5 a                         | 68.2 $\pm$ 3.9 c                            |
| Slow cooling Tr 150                                  | 28.5 $\pm$ 4.2 a                        | 33.1 $\pm$ 2.2 ab  | 52.9 $\pm$ 3.6 b                         | 26.9 $\pm$ 3.8 a                            |

a, b, c, d: Different superscripts at same columns denote significant differences ( $p < 0.05$ ).

The viability of spermatozoa in samples was assessed by means of the nigrosin–eosin stain method (12). The final composition of the stain was: eosin-Y 1.67 g, nigrosin 10 g, and sodium citrate 2.9 g, dissolved in 100 ml distilled water. 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 400 cells under phase-contrast at 1000x magnification. Sperm displaying partial or complete purple staining were considered nonviable, only sperm showing strict exclusion of stain were counted as viable.

The hypoosmotic swelling test (HOST) was used to evaluate the integrity of the sperm membrane, based on curled and swollen tails, and was performed by incubating 30  $\mu$ l of semen with 300  $\mu$ l of a 100 mOsm hypoosmotic solution (9 g fructose plus 4.9 g sodium citrate per liter of distilled water) at 37°C for 60 min. After incubation, 0.2 ml of the mixture was spread with a cover slip on a warm slide. Four hundred sperm were evaluated under bright-field microscopy. Sperm with swollen or coiled tails were recorded (7).

The sperm evaluations were repeated 10 times and the results were expressed as the mean  $\pm$  S.E.M. Means were analyzed by analysis of variance (ANOVA), followed by the Duncan test to determine significant differences between the 6 experimental groups and 2 control groups- with additives or no additive after the freezing-thawing process for sperm characteristics using the SPSS/PC version 12.0 software (SPSS, Chicago). Differences with values of  $p < 0.05$  were considered to be statistically significant (11).

## Results

The influence of trehalose additions and two different cooling rates on the standard semen parameters of frozen ram semen were evaluated in 8 independent

experiments. Different concentrations of trehalose exhibited a dose-related response on the sperm characteristics.

As shown in Table 1, the best post-thawing results (72.0 $\pm$ 3.7 % for sperm motility, 25.5 $\pm$ 2.5 % for morphologic sperm abnormalities, 75.9 $\pm$ 4.5 % for sperm viability, 68.2 $\pm$ 3.9 % for sperm membrane integrity) were obtained from 100 mM concentration of trehalose, compared to the others at slow cooling rate.

In this study, differences among trehalose concentrations and between cooling rates were found significant for post-thawing sperm characteristics statistically ( $p < 0.05$ ).

## Discussion and Conclusion

The sperm plasma membrane is rich in polyunsaturated fatty acids (PUFA). Therefore, it is susceptible to oxidative damage and lipid peroxidation due to attacks from ROS generated during freezing-thawing process. These effects cause consequent loss of membrane integrity, decreased sperm motility, and eventually loss in fertility (22). Improving the sperm characteristics and decreasing oxidative damage following freezing-thawing requires the use of additives, such as antioxidants and disaccharides in the sperm processing techniques. Disaccharides have several functions including energy providing, antioxidative properties and membrane stabilizing effect on the sperm. The beneficial effects of the trehalose on the post-thawing viability of mammalian sperm cells have been reported in many studies (6). In some reports, trehalose conferred better cryoprotection and post-thawing fertilizing ability of ram, bull and mouse sperm due to diminished death and damage of sperm (9). Molinia et al. (20) demonstrated that the motility of frozen-thawed ram spermatozoa was higher in the presence of trehalose than that of glucose when glycerol is not incorporated in the

diluent. Aboagla and Terada (1) reported that substitution of Tris-citric acid diluent with trehalose was found to significantly improved the freezability of goat spermatozoa which were assessed for motility, acrosome integrity, and membrane fluidity. Matsuoka et al. (18) demonstrated that the addition of trehalose enhanced motility and viability of ram spermatozoa after freezing-thawing.

In the present study, supplementation with 50 and 100 mM concentrations of trehalose improved the post-thawing results of ram spermatozoa, and 100 trehalose exerted more cryoprotective effect on sperm characteristics compared to the other and control groups significantly ( $p < 0.05$ ).

On the other hand, the worse post-thawing results were obtained from higher trehalose concentration than 100 mM. It can be explained that 150 mM concentration of trehalose exhibited toxic effect on spermatological parameters during freezing-thawing process. This study demonstrated that cryoprotective effect of 50 and 100 mM trehalose was significantly higher than other doses of trehalose and control group, when frozen at rapid and slow cooling rates.

Frozen-thawed spermatozoa have been shown to exhibit the inverted “u” shaped curve with cooling rate (14). If cooling rate is increased, cell survival also increases up to a maximum. This curve has been interpreted as resulting from two opposing factors affecting cell survival. Increasing cell death at low cooling rates has been attributed to so-called effects while at high cooling rates, there is an increasing tendency for lethal intracellular ice formation (10).

Seigneurin and Blesbois (27) cryopreserved rooster spermatozoa from  $+5^{\circ}\text{C}$  to  $-35^{\circ}\text{C}$  at rates of  $-1$ ,  $-5$ ,  $-7$ ,  $-10$  and  $-15^{\circ}\text{C}/\text{min}$ . The highest fertilization rates following artificial insemination were obtained from semen frozen at  $-5^{\circ}\text{C}$  or  $-7^{\circ}\text{C}$ . This result would agree with Mazur (19), who suggested that spermatozoa frozen at sub-optimal temperatures are exposed too long to “solution effects”. According to the results of Byrne et al. (8), semen analysis showed that there were significant differences between the different cooling rates in terms of post-thawing results, but freezing at the slow rate appears to induce a semi-lethal change in the sperm.

With regard to the cryopreservation process, a capital objective is the achievement of the optimum cooling rate. Acceptable fertility resulted from rapid cooling rates ( $-5^{\circ}\text{C}/\text{min}$ ) observed both *in vivo* and *in vitro* in ram by Byrne et al. (8). Kumar et al. (15) also observed that optimum cooling rates are between  $-20$  and  $-30^{\circ}\text{C}/\text{min}$ . Generally, in the frozen process of ram semen, the majority of cell damage occurs from  $-10$  to  $-25^{\circ}\text{C}$  (24).

We observed that slow cooling rate improved sperm motility ( $p < 0.05$ ), viability ( $p < 0.05$ ) and membrane integrity ( $p < 0.05$ ) and decreased morphologic sperm abnormalities ( $p < 0.05$ ) of frozen-thawed ram semen significantly. According to Watson (28), slow dehydration of spermatozoa is associated with cell survival, whereas rapid cooling rate are considered more likely to cause cell death. But according to O’Neill (21), semen frozen rapidly (from  $5^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$  at  $5^{\circ}\text{C}/\text{min}$ ) have significantly better viability, mitokondrial activity and acrosome integrity than a slow ( $0.5^{\circ}\text{C}/\text{min}$ ) cooling rate over the same interval. Some researchers have attained the best post-thawing results at rate of  $-5^{\circ}\text{C}/\text{min}$  (8). But, we used  $-10^{\circ}\text{C}/\text{min}$  as fast cooling rate that means probably due to it is over optimal cooling rate, therefore post-thawing results from slow cooling rate may have reflected better than those of rapid cooling rate in this study. Related to trehalose, it may protect sperm cells against solution effects at slow freezing rate ( $-0.5^{\circ}\text{C}/\text{min}$ ) through the critical temperature zone of  $-15^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ , where cell damage is most likely to occur differ from findings of other researchers .

Our results demonstrated that supplementation with various concentration of trehalose of semen diluents and different cooling rates used in semen freezing have influenced the sperm characteristics following the freezing-thawing process. Additionally, this study has shown that many aspects of sperm protection e.g. sperm motility, viability and membrane stabilization of sperm cells have primer importance. Trehalose 50 or 100 mM provided a near-optimal concentration for improved sperm survival during freezing-thawing process. But 100 mM concentration of trehalose and slow cooling rate were found to significantly improve the freezability of ram spermatozoa and slow cooling rate were observed superior than other treatment groups.

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