



## Effects of Sucrose and Trehalose on the Freezability of Markhoz Goat Spermatozoa

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**ABSTRACT :** The present study was conducted to observe the effect of increased osmolality of basic tris extender supplemented with trehalose and sucrose on post-thawing quality (motility, progressive motility, viability, the rate of acrosome abnormality, total abnormality and membrane integrity) of Markhoz goat spermatozoa. Fresh semen samples were evaluated for motility and sperm concentration. Only semen samples with motility more than 70% and sperm concentration higher than  $3 \times 10^9$  sperm/ml were used for cryopreservation. In Exp. 1, trehalose (50, 75 or 100 mM) and sucrose (40, 60 or 80 mM) were added to a basic tris diluent. Based on the results of experiment 1, the goal of Exp. 2 was to investigate the combinational effects of the highest and lowest concentrations ( $T_{100}+S_{80}$  or  $T_{50}+S_{40}$ ) of trehalose and sucrose. As the control, semen was diluted and frozen in the tris diluent without trehalose or sucrose. The results in Exp. 1 showed that all evaluated spermatozoa characteristics improved significantly after freezing and thawing ( $p < 0.05$ ) and at the same time the increase of trehalose and sucrose concentrations in basic extenders was seen, with the best results obtained for extenders containing 70 and 100 mM trehalose and 80 mM sucrose. Comparing these results with those of control diluents, the effects of supplementation were significantly ( $p < 0.05$ ) better. In Exp. 2, the results showed no significant differences ( $p > 0.05$ ) between  $T_{100}+S_{80}$  and  $T_{50}+S_{40}$  extenders, but the results of  $T_{50}+S_{40}$  were slightly better than obtained with  $T_{100}+S_{80}$  diluents. Furthermore, the results of this experiment indicated that the sperm characteristics in the isotonic control extender were significantly ( $p < 0.05$ ) lower than examined extenders. In conclusion, the results of this study indicated that goat sperm can tolerate hypertonic trehalose and sucrose solutions better than isotonic control diluents in the freezing period. In particular, these positive effects have been shown for acrosome integrity, which is very important for the fertilization capacity of sperm. The data indicated that addition of trehalose plus sucrose to the freezing extender can be recommended for cryopreservation of goat spermatozoa, but more data is needed on pregnancy rate, acrosome reaction and IVF to ascertain the real effect. (**Key Words :** Markhoz Goat, Cryopreservation, Semen, Trehalose, Sucrose)

### INTRODUCTION

The freezing and thawing of spermatozoa is a complex process that induces several forms of cellular lesions (Amann and Pickett, 1987; Purdy, 2006). These lesions have been attributed to cold shock, intercellular ice crystals, membrane alteration, and osmotic changes (Watson and Martin, 1975; Isachenko, 2003), which may decrease motility, viability and the fertilizing ability of sperm after artificial insemination (Matsuoka et al., 2006). Regarding the latter, intracellular ice formation is one of the main damaging factors that reduces the viability of frozen-thawed sperm (De Leeuw et al., 1993) and the degree of damage

also depends on the composition of the semen extender and the nature of cryoprotectant (Hammersted et al., 1990; Curry et al., 1994). Therefore, cryoprotectants are included in cryopreservation extender to reduce the damaging effects of the freezing process (Purdy, 2006).

Furthermore, one other important factor in the efficacy of an extender is its supplementation with disaccharides such as trehalose and sucrose, whose beneficial effects have been reported in many studies (De Leeuw et al., 1993; Molinia et al., 1994a; Woelders et al., 1997; Yildiz et al., 2000; Sztejn et al., 2001; Aisen et al., 2002; Aboalga and Teranda, 2003; Farshad and Akhondzadeh, 2008). These sugars create an osmotic pressure that results in cell dehydration and lowers the incidence of intracellular ice formation (Aisen et al., 2002; Purdy, 2006).

It is important to note, that during the dehydration and rehydration of cryopreservation, trehalose and sucrose interact with the plasma membrane phospholipids, re-

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organize the sperm membrane, increase its fluidity and induce a depression in the membrane phase transition temperature of dry lipids and form a glass drying (Molinia et al., 1994a; Aisen et al., 2002; Aboagla and Terada, 2003; Fernandes-Santos, 2007). It has been demonstrated with artificial membranes that damage measured by intermixing and fusion can be reduced by a series of cryoprotectants such as trehalose and sucrose. Thus, these sugars probably play a key role in preventing deleterious alteration to membranes during reduced-water states (see for review, Fernandez et al., 2007; Aisen et al., 2002).

However, despite years of research, the cryopreservation of goat sperm still cannot be carried out efficiently (Watson, 2000; Purdy, 2006). During this time, several iso-osmotic extenders have been commonly used as a semen extender for the freezing of goat spermatozoa, but hyperosmotic diluents over a wide range of sugar concentration have shown an improvement of sperm integrity after freezing and thawing (Aisen et al., 2002; Farshad and Akhonzadeh, 2008). There are few available reports in which the effects of trehalose and sucrose, as disaccharides, on the post-thaw viability of goat spermatozoa were studied. Therefore, within this study, an attempt was made to partially dehydrate Markhoz goat spermatozoa before freezing by means of concentrated trehalose and sucrose solutions since these may reduce the damaging effects of intracellular formation of ice crystals.

## MATERIALS AND METHODS

### Animal, location and basic semen extender

This experiment was performed at the testing station located in Sanandaj, 35° 20' N latitude and 47°E longitude, and lasted from September to November. 4 mature Markhoz goat bucks, 2 to 4 years of age and 45-60 kg were used in the study. The animals were kept under natural photoperiod and nutritional levels which were adjusted to meet maintenance requirements. Goats were fed twice a day with a diet of 530 g alfalfa hay, 190 g barley straw and 300 g concentrates. They had free access to salt lick and fresh water.

The base extender consisted of 3.786 g Tris (hydroxymethyl-aminoethane, Merck 64271, Germany), 2.172 g citric acid (BHD 1081, England), and 1 g fructose (BDH 28433, England) in 100 ml distilled water, 5.0% (v/v) glycerol (Merck, 2400 Germany) and 2.5% (v/v) egg yolk (Evans and Maxwell, 1987).

### Semen collection and evaluation

Semen was collected twice a week (11 weeks) by artificial vagina (42-43°C) using an estrous female as a mount. Within a maximum 10 minutes after collection, the

semen was taken to the laboratory and kept in a water bath at 37°C. Ejaculates were evaluated for volume (ml), sperm concentration ( $3 \times 10^9$  sperm/ml, sperm cells were counted in four squares of a hemocytometer after 1:200 dilution of semen with 0.5% eosin solution), mass activity (% undiluted semen), motility (% diluted with normal saline), progressive motility using an arbitrary scale of 1 to 5 (1, 2, 3, 4 or 5 = 10 to 25%, 25 to 50%, 50 to 70%, 70 to 90 or 90 to 100% of the motile spermatozoa), viability (% using eosin-nigrosin staining) and morphologically normal acrosome (%). Ejaculates showing >70% motility and having  $>3 \times 10^9$  sperm/ml concentration were used for freezing. The assessment of freeze-thawed spermatozoa included motility, progressive motility, viability, morphologically acrosome integrity and the hypo-osmotic-swelling-test (HOS-test). To evaluate motility and progressive motility, a sample of the diluted spermatozoa was placed under a cover slip in the centre of a pre-warmed (37°C) slide which was transferred to a heated microscope stage set at 37°C and subjectively assessed by phase contrast microscopy ( $\times 400$  magnification). The rate of motility and progressive motility were determined in percentages. Viability was performed using a modification of the eosin-nigrosin stain procedure described by Evans and Maxwell (1987). A mixture of 10  $\mu$ l of diluted spermatozoa and 10  $\mu$ l eosin-nigrosin stain was smeared on a slide and allowed to air dry in a dust-free environment. Two hundred spermatozoa from different microscopic fields were examined under a bright-field microscope using a 400 $\times$  objective, and the number of non-stained (viable) spermatozoa was counted.

The morphologically acrosome abnormality was assessed by viewing a wet mount of diluted spermatozoa fixed in buffered Formalin-Citrate solution as described by Weitze (1977). A drop of the fixed spermatozoa was placed on a slide and covered with a cover glass. The slides were examined by phase-contrast microscopy using a 100 $\times$  oil immersion objective and white light. Spermatozoa ( $n = 200$ /slide) were examined and the percentage with normal acrosomes determined. The hypo-osmotic swelling test (HOS-test) was used to evaluate the functional integrity of the sperm membrane, and was performed by incubating 20  $\mu$ l of semen with 200  $\mu$ l of a 100 mOsm hypo-osmotic solution (9 g fructose+4.9 g sodium citrate/L distilled water) at 37°C for 60 min. After incubation, 100  $\mu$ l of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm was counted in at least 5 different microscopic fields. The percentages of sperm with swollen and curled tails were classified according to the description used by Revell and Mrode (1994).

### Experimental procedure

The basic semen extender was prepared and kept in a

**Table 1.** Macroscopic and microscopic characteristics of seminal plasma of Markhoz goat spermatozoa

Seminal characters	n	Mean	SEM	Min - Max
Volume (ml)	84	1.11	0.03	0.6-2.1
Sperm concentration ( $10^9$ ml <sup>-1</sup> )	84	4.17	0.04	3.3-4.9
Mass activity (1-5)	84	4.64	0.06	3-5
Motility (%)	84	86.56	0.60	75-95
Progressive motility (%)	84	81.67	0.57	70-90
Viability (%)	56	88.53	0.73	82-95
Normal acrosome (%)	56	88.51	0.52	86-93

water bath at 37°C, on the day of semen collection. Semen samples were pooled and diluted (1:4) before freezing. In experiment 1, 50, 75 or 100 mM trehalose and 40, 60 or 80 mM sucrose were added to the diluents. Control diluents were used without trehalose or sucrose. Because the extenders which contained 100 mM trehalose and 80 mM M sucrose were marked as the best and the diluents with 50 mM trehalose and 40 mM sucrose showed the lowest results, the second experiment was designed to investigate the combinational effect of trehalose (T) with sucrose (S) as follow: (T<sub>100</sub>+S<sub>80</sub> and T<sub>50</sub>+S<sub>40</sub>). The extended semen was then packaged in French straws (0.25 ml). The open end of the filled straws was sealed with polyvinyl chloride powder, and permitted to equilibrate at 5°C for 2 h before being exposed to liquid nitrogen vapor (4-5 cm from the LN2 surface level) for 10 min. The straws were then stored in liquid nitrogen for 24 h following which they were thawed in a water bath at 37°C for 30 seconds. The frozen-thawed semen was then assessed for motility, progressive motility, viability, morphologically acrosome abnormality and hypo-osmotic swelling test.

#### Statistical analysis

The experiments were conducted as a completely randomized design, and statistical analysis of data was performed by the GLM procedure of SAS (1996). All percentage data were arcsine transformed before statistical analysis. Back-transformed data are reported as mean±SEM. A probability level of  $p \leq 0.05$  was considered significant.

## RESULTS

Macroscopic and microscopic seminal characteristics in primary evaluation are presented in Table 1. The average volume (ml), sperm concentration ( $10^7$ /ml), mass activity (1-5), motility (%), progressive motility (%), viability (%) and rate of morphologically normal acrosome (%) in the ejaculates of Markhoz goat spermatozoa were 1.11, 4.17, 4.64, 86.56, 81.67, 88.53 and 88.51, respectively.

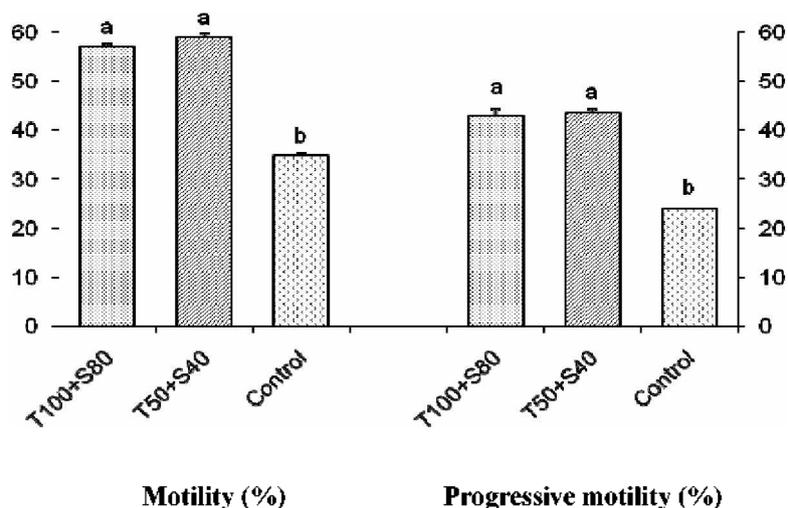
In Exp. 1, the effect of different concentrations of trehalose and sucrose added to the isotonic tris-fructose-citric acid-diluents on the motility and progressive motility, viability, morphologically acrosome abnormality, total abnormality and membrane integrity (HOS-test) of Markhoz goat spermatozoa after freezing and thawing are presented in Table 2. The results showed significant ( $p < 0.05$ ) improvement by increasing both trehalose and sucrose concentrations in extenders used. Furthermore, the results obtained were significantly ( $p < 0.05$ ) higher than for the control extender. The results in Exp. 1 indicated that diluent containing 100 mOsm trehalose was significantly ( $p < 0.05$ ) the best extender, and diluents containing 75 mM trehalose and 80 mM sucrose concentrations proved to be significantly ( $p < 0.05$ ) better than other concentrations of trehalose and sucrose. In the HOS-test, the rate of morphologically membrane abnormality decreased significantly ( $p < 0.05$ ) with increasing concentrations of both trehalose and sucrose contained in diluents.

Based on the results of experiment 1, Figures 1, 2 and 3 show the combination effects of trehalose and sucrose

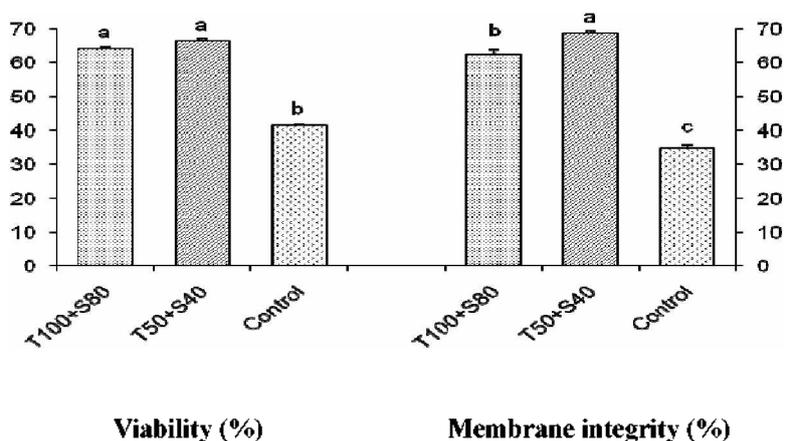
**Table 2.** Effects of trehalose and sucrose on the post-thaw characteristics of Markhoz goat spermatozoa (mean±SEM)

Characteristic	Trehalose (mM)			Sucrose (mM)			Control
	50	75	100	60	80	40	
Motility (%)	43.54±0.72 <sup>db</sup>	50.36±0.54 <sup>b</sup>	52.36±0.60 <sup>a</sup>	44.77±0.63 <sup>d</sup>	47.00±0.82 <sup>c</sup>	42.22±0.82 <sup>e</sup>	35.45±0.69 <sup>f</sup>
Progressive motility (%)	33.95±0.65 <sup>c</sup>	39.22±0.62 <sup>a</sup>	40.13±0.40 <sup>a</sup>	33.86±0.69 <sup>c</sup>	36.27±0.70 <sup>b</sup>	32.54±0.75 <sup>c</sup>	24.55±0.55 <sup>d</sup>
Sperm viability (%)	51.55±0.93 <sup>c</sup>	55.36±0.61 <sup>ab</sup>	57.63±0.67 <sup>a</sup>	51.75±0.80 <sup>c</sup>	54.11±0.92 <sup>b</sup>	48.74±0.93 <sup>d</sup>	43.07±0.83 <sup>e</sup>
Acrosome abnormality (%)	10.11±0.47 <sup>b</sup>	9.15±0.47 <sup>ab</sup>	8.05±0.47 <sup>a</sup>	9.16±0.47 <sup>ab</sup>	8.66±0.47 <sup>a</sup>	10.33±0.47 <sup>b</sup>	16.11±0.49 <sup>c</sup>
Total abnormality (%)	15.30±0.60 <sup>b</sup>	14.60±0.83 <sup>bc</sup>	13.23±0.55 <sup>c</sup>	14.22±0.85 <sup>bc</sup>	14.73±0.57 <sup>bc</sup>	15.68±0.61 <sup>b</sup>	25.23±0.57 <sup>a</sup>
HOS-test (%)	45.72±0.94 <sup>c</sup>	49.32±0.81 <sup>b</sup>	54.54±0.70 <sup>a</sup>	44.77±0.61 <sup>c</sup>	48.45±0.52 <sup>b</sup>	40.36±0.78 <sup>d</sup>	35.65±0.74 <sup>e</sup>

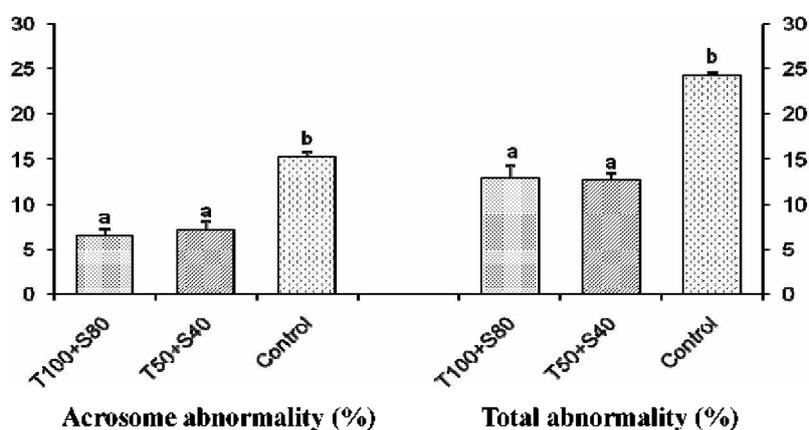
Values with different letter(s) in the same row are significantly different ( $p < 0.05$ , Duncan-test).



**Figure 1.** Effects of tris-extenders containing trehalose and sucrose on motility and progressive motility of Markhoz goat spermatozoa after freezing and thawing. Similar letters (a, b) indicate no significant differences ( $p < 0.05$ , Mean $\pm$ SEM, Duncant's-test).



**Figure 2.** Effects of tris-extenders containing trehalose and sucrose on sperm viability and membrane integrity of Markhoz goat spermatozoa after freezing and thawing. Similar letters (a, b) indicate no significant differences ( $p < 0.05$ , Mean $\pm$ SEM, Duncant's-test).



**Figure 3.** Effects of tris-extenders containing trehalose and sucrose on acrosome abnormality and total abnormality of Markhoz goat spermatozoa after freezing and thawing. Similar letters (a, b) indicate no significant differences ( $p < 0.05$ , Mean $\pm$ SEM, Duncant's-test).

( $T_{100}+S_{80}$  and  $T_{50}+S_{40}$ ) and "isotonic" tris extender on post-thawed sperm characteristics. In regard to motility, progressive motility, viability, morphologically acrosome abnormality, total abnormality and membrane integrity (HOS-test) the results showed that  $T_{100}+S_{80}$  ( $57.00\pm 1.1$ ,  $43.00\pm 1.63$ ,  $64.00\pm 1.46$ ,  $6.50\pm 0.7$ ,  $12.80\pm 1.45$  and  $62.50\pm 1.4$ , respectively) and  $T_{50}+S_{40}$  ( $58.80\pm 0.60$ ,  $43.60\pm 0.91$ ,  $66.20\pm 0.63$ ,  $7.17\pm 0.90$ ,  $12.60\pm 0.82$  and  $68.66\pm 0.7$ , respectively) extenders were significantly ( $p<0.05$ ) better than the results of isotonic control diluents ( $34.80\pm 1.05$ ,  $24.10\pm 0.83$ ,  $41.40\pm 1.05$ ,  $15.20\pm 0.50$ ,  $24.30\pm 0.29$  and  $34.66\pm 1.1$ , respectively).

## DISCUSSION

Cryopreservation adversely affects the cryosurvival of spermatozoa and under the best experimental conditions about 50% of motile sperm can survive the freeze-thaw process (Watson, 2000). Irreversible damage during this process reduces motility and fertility of spermatozoa and has been attributed to different reasons (Watson and Martin, 1975). One of the main reasons for reduced quality of spermatozoa is intracellular ice crystallization during cryopreservation (De Leeuw et al., 1993), which has been minimized when isotonic extenders are supplemented with non-penetrated sugars.

The present study investigated whether the presence of trehalose and sucrose would improve the quality of Markhoz goat sperm after freezing and thawing. The results demonstrated that with increasing of both trehalose and sucrose concentrations, all post-thawed sperm characteristics were improved significantly. In agreement with our finding, Abdelhakem et al. (1991), Molinia et al. (1994a) and Aisen et al. (2002) reported that hypertonic extender gave the highest post-thaw motility of ram sperm. Farshad and Akhondzadeh (2008) suggested that the cryopreservation of goat sperm in hypertonic sucrose diluents was better than isotonic extenders. Aboagla and Terada (2003) reported that freezing of goat sperm in the presence of 375 mM trehalose resulted in significantly greater post-thaw total motility and progressive motility than in the absence of trehalose. Woelders et al. (1997) demonstrated that an isotonic sugar medium in which Tris-citrate components were substituted with sucrose and trehalose was superior to a Tris-citrate-egg yolk extender for preserving the motility of bovine spermatozoa. Story et al. (1998) showed that trehalose significantly improved the viability of mouse spermatozoa. Yildiz et al. (2000) showed that trehalose and sucrose significantly improved the viability and motility of dog spermatozoa.

Furthermore, we found that combinations of trehalose and sucrose resulted in higher percentages for sperm quality

after cryopreservation, although the differences amongst various combinations were not significant. We are not aware of any reports on the effects of combinations of trehalose and sucrose on the post-thaw attributes of goat spermatozoa; however, synergistic effects of sucrose and glycerol on cryopreservation of marine embryos have been reported by Honadel and Killian (1988) who also suggested that trehalose may be an alternative to sucrose.

The exact mechanism by which trehalose and sucrose preserve the sperm membrane is not known, but it is theorized that these sugars probably play a key role in preventing deleterious alteration to the membrane during reduced water states (Aboagla and Terada, 2003). Furthermore, Liu et al. (1998) and Aboagla and Terada (2003) hypothesized that trehalose and sucrose penetrate into the plasma membrane of the spermatozoa and form hydrogen bonds with the polar head groups of phospholipids. Thereby, they also create an osmotic pressure, inducing cell dehydration, increased membrane fluidity and a lower incidence of intracellular ice formation (Molinia et al., 1994a; Aisen et al., 2002). However, the cryoprotective ability of sugars on sperm may depend on their molecular weight (Molinia et al., 1994b) and the type of buffer used (Abdelhakeam et al., 1991).

In conclusion, the results of the present study indicated that, in agreement with other findings, goat sperm can tolerate hypertonic trehalose and sucrose solutions better than isotonic extenders during the freezing period. In particular, these positive effects have been shown for acrosome integrity as an important characteristic for the fertilization ability of sperm. Furthermore, although sucrose and trehalose influence sperm motility and viability, more data is needed on pregnancy rate, acrosome reaction and IVF to ascertain the real effect.

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