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Trehalose-Enhanced Fluidity of the Goat Sperm Membrane and Its Protection During Freezing

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ABSTRACT

In an attempt to find a suitable freezing method for goat semen, two experiments were conducted to study the influence of trehalose on the cryopreservation of goat spermatozoa. In experiment 1, goat spermatozoa were frozen in trehalose extender (0.375 M) alone (100%) or at different combinations of trehalose with Tris-citric acid-glucose (TCG) extender (0%, 25%, 50%, 75%). Final concentrations of 20% (v:v) egg yolk and 4% (v:v) glycerol were employed in the extenders (osmolality = 370, pH = 7). Sperm motility was assessed using a computer-assisted sperm analysis system and acrosome integrity was assessed using fluorescein isothiocyanate-labeled peanut agglutinin (FITC-PNA). The sperm-motility parameters improved significantly by increasing the concentration of trehalose ($P < 0.05$) and significantly high recovery rates for the motility parameters were also achieved by a high concentration of trehalose ($P < 0.05$). Motility of the frozen-thawed spermatozoa after a 3-h incubation improved significantly with increasing concentrations of trehalose in the extender ($P < 0.05$). The 75% and 100% trehalose extenders yielded a significant increase in the percentage of spermatozoa with intact acrosome ($P < 0.05$). In experiment 2, merocyanine 540/Yo-Pro staining was used to study the influence of trehalose on membrane fluidity compared with that of sucrose and TCG. Percentage of cells with high merocyanine fluorescence was significantly higher in spermatozoa treated with trehalose than sucrose or TCG ($P < 0.05$), indicating a significantly highest membrane fluidity of sperm samples extended with trehalose solution. We thus conclude that the substitution of a Tris-citric acid diluent composition with trehalose significantly improves the freezability of goat spermatozoa. Furthermore, the cryoprotective effects of trehalose observed in this study may be due to enhanced sperm membrane fluidity before freezing.

fertilization, gamete biology, in vitro fertilization, sperm, sperm capacitation

INTRODUCTION

Water plays an important role in the maintenance of the structural and functional integrity of biological membranes. The removal of this water by dehydration or freezing often results in vast structural and functional alteration in the biological membranes. Nevertheless, many organisms exposed to such conditions, which could result in these changes to membranes, accumulate compounds that prevent deleterious alteration to membranes during reduced-water states. One of these compounds, trehalose, is a disaccharide of glucose commonly found in high concentrations in many organisms capable of surviving complete dehydration. These organisms include yeast and fungal spores [1] as well as some batrachians [2].

The plasma membrane is a key component of the cell and must be maintained during freezing conditions if the cell is to be kept alive. It has been demonstrated with artificial membranes, such as unilamellar vesicles, that damage measured by intermixing and fusion can be reduced by a series of cryoprotectants, with trehalose and sucrose being more protective than glycerol [3–5]. Thus, these sugars probably play a key role in preventing deleterious alteration to the membrane during reduced-water states.

In addition, sugars have several functions in sperm extender, including providing energy substrate for the sperm cell during incubation, maintaining the osmotic pressure of the diluent, and acting as a cryoprotectant [6]. The beneficial effects of the addition of trehalose to the extender on the postthaw viability of mammalian sperm cells have been reported in many studies [2, 7–11] and the cryopreservation of human fetal skin has also been improved by combining trehalose with a permeating cryoprotectant [12]. Several other studies have shown that trehalose is effective in preserving dried cells [13, 14] and liposomes [15].

On the other hand, studies on goat semen cryopreservation are few. Despite solving the problem of the detrimental effect of the seminal plasma on the viability of goat spermatozoa in diluents containing egg yolk [16, 17] or in milk-based media [18], the viability of frozen-thawed goat spermatozoa has remained low compared with that of other species. Thus, the development of an appropriate freezing extender for goat spermatozoa is crucial in order to enhance goat reproduction. Although some sugar-containing diluents such as sodium citrate-glucose-yolk [19] and glucose-fructose-raffinose yolk [20] have been studied for their potential use in freezing goat spermatozoa, to the best of our knowledge, no studies have been conducted to date to investigate the influence of trehalose on the viability of frozen-thawed goat spermatozoa.

The mechanism by which trehalose protects cells subjected to dehydration or freezing seems to involve its stabilizing effects on both cellular proteins and membranes; however, the nature of this mechanism remains unclear. The effects of freeze damage on cell membrane fluidity have been speculated on in several studies [21–23]. We hypothesized that the extension of spermatozoa with trehalose before freezing could increase sperm membrane fluidity, rendering the spermatozoa capable of enduring freeze-thawing damage. The present study was therefore conducted to examine the influence of trehalose supplementation on the

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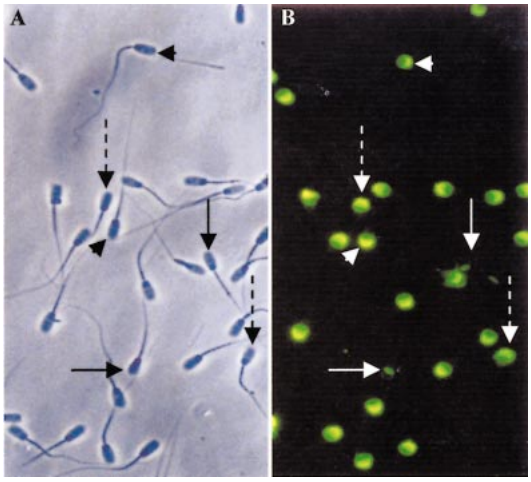

FIG. 1. Photograph of goat sperm acrosome stained with FITC-PNA. **A**) Image obtained by phase contrast microscope. **B**) The same field of image **A**, obtained by fluorescence microscope. Arrowhead indicates intact acrosome; arrow with dashed line indicates partially damaged acrosome; and arrow with solid line indicates lost acrosome. Magnification ×350.

viability of frozen-thawed goat spermatozoa, evaluated in terms of motility and acrosome integrity. Furthermore, we investigated the effects of trehalose on membrane fluidity before freezing. In addition, to explore whether this effect is a property of all disaccharides or a property peculiar to trehalose, the effect of trehalose on goat sperm membrane fluidity was compared with that of sucrose.

MATERIALS AND METHODS

Semen Extenders

The basic extender used in this study was Tris (hydroxymethyl-aminoethane)-citric acid-glucose (TCG) solution as defined by Salmon and Ritar [20] and consisting of Tris (375.0 mM), citric acid (124.0 mM), and glucose (41.0 mM). Trehalose solution (0.375 M) was added to TCG solution to give the following trehalose extenders: 0% (only TCG), 25%, 50%, 75%, and 100% (v:v) (only trehalose). Furthermore, sucrose solution was also prepared and used in the membrane fluidity test. These solutions were adjusted to pH 7.0 with Tris, and the osmolality was adjusted to 375 mOsm. Trehalose and TCG solutions were used for semen cryopreservation. The egg yolk was separated from the albumin and 20% (v:v) egg yolk was added to both the trehalose and the TCG solutions. Egg yolk lipids were solubilized by adding 0.035% (wt:vol) sodium dodecyl sulphate (SDS) to each solution. The solutions were centrifuged at 15 000 × *g* for 1 h and the supernatants were aspirated and filtrated through a 0.45-μm membrane filter (Millipore SA, Molsheim, France).

Semen Collection, Evaluation, and Dilution

Buck semen was obtained from a Shiba goat stud trained to mount a dummy at Hiroshima University. Semen was collected each morning of the experiment using an artificial vagina that was fixed on the dummy and then transported to the laboratory within 15 min of collection in an insulated container at 30°C. The semen was diluted with TCG (free of egg yolk and glycerol) and then evaluated for motility and sperm concentration. Only semen samples with adequate motility (more than 70%) and sperm concentration (more than 3×10^{10} sperm/ml) were used for semen freezing. Semen was divided into equal volumes and diluted with TCG (free of egg yolk and glycerol) and then centrifuged twice at 1600 × *g* for 30 min each time to remove the seminal plasma. The supernatant was then removed, and the sperm pellets were used for freezing.

The sperm pellets were resuspended at 30°C in trehalose extenders of each concentration (0%, 25%, 50%, 75%, and 100%) containing egg yolk. The diluted semen was cooled to 5°C within 3 h. The semen was then further diluted 1:1 with each trehalose extender containing 8% glycerol to obtain a sperm concentration of 1×10^8 and was then equilibrated at 5°C for 15 min before freezing.

Freezing and Thawing Procedure

Semen was frozen in pellet form (0.2 ml) on dry ice and was then plunged into liquid nitrogen. Thawing was performed using a thawing solution (TCG without egg yolk and glycerol) at 37°C. Frozen sperm pellets were diluted 1:3 and allowed to equilibrate at this temperature for 10 min before evaluation. Frozen-thawed spermatozoa were evaluated for motility (M%), progressive motility (PM%), and path velocity (VAP).

Thermal Resistance Test

The frozen-thawed spermatozoa were incubated in a water bath at 37°C. Two samples for each treatment were evaluated after 1, 2, and 3 h of incubation to determine the motility parameters (M, PM, and VAP).

Assessment of Sperm Motility

The sperm-motility parameters were assessed immediately after thawing and after 1, 2, and 3 h of incubation using a computer-assisted sperm analysis system (Hamilton Thorne Research, Beverly, MA). The motility analyzer settings were as follows: frame acquired = 30, minimum contrast = 7, minimum size = 11, low size gate = 0.7, high size gate = 1.3, low intensity gate = 0.6, high intensity gate = 1.7, medium VAP = 25, low VAP = 10.

Assessment of Acrosome Integrity

The acrosome status of sperm samples was assessed with fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) using a slightly modified version of the procedure described by Fazeli et al. [24]. Briefly, aliquots (30 μl) of semen samples were smeared onto microscopic slides, air dried, and fixed in absolute methanol for 10 min. Thirty microliters of FITC-PNA (Sigma Chemical Co., St. Louis, MO) solution in phosphate buffered saline (PBS) (100 μg/ml) was spread over each glass slide. The slides were then incubated in a dark and moist chamber for 30 min at 37°C. They were gently rinsed with PBS solution and air dried, then mounted with 10 μl of antifade solution (Molecular Probes, Inc., Eugene, OR) in order to preserve fluorescence. Each smear was then covered with a coverslip and sealed using colorless nail polish. The acrosome status of spermatozoa was monitored and photographed with an epifluorescence microscope (IMT-2; Olympus, Tokyo, Japan) with a set of DMU (Olympus) filters (350×) using an excitation wavelength of 480 nm and emissions of 530 nm. For the same field, a photograph was also taken with a phase-contrast microscope. At least 200 cells per sample were counted and a total of five replicates were examined.

The observed images of goat spermatozoa stained with FITC-PNA were classified into three groups (Fig. 1): 1) spermatozoa displaying intensively bright fluorescence of the acrosomal cap, indicating an intact acrosome; 2) spermatozoa displaying disrupted fluorescence of the acrosomal cap, indicating the process of the breakdown of the acrosomal cap (partially damaged acrosome); and 3) spermatozoa displaying no fluorescence, indicating a complete loss of the outer acrosomal membrane (damaged acrosome). The condition of the last group was determined under phase-contrast illumination.

Assessment of Sperm Membrane Fluidity

Membrane fluidity was assessed using merocyanine 540/Yo-Pro-1 staining as previously described by Harrison et al. [25]. Aliquots of spermatozoa were first stained with Yo-Pro-1 (Molecular Probes) at a final concentration of 50 nM to stain the moribund cells and then incubated at 38°C for 12 min. About 140 μl of the prestained spermatozoa (5×10^6 sperm/ml) were then stained with 30 μl of a 40 μM solution of merocyanine 540 (Sigma) in Tyrode solution freshly prepared from 5 mM stock solution in dimethyl sulfoxide. The stained sperm samples were then analyzed by flow cytometry.

Flow Cytometric Analysis

Flow cytometric analysis was performed on a FacStar-plus flow cytometer (FACSCalibur, Becton Dickinson and Co., Franklin Lakes, NJ), using excitation at 488 nm with an argon ion laser at a power output of 15 mW. Fluorescence data were collected in logarithmic mode, while forward and sideways light-scatter data were collected in linear mode. The fluorescence of Yo-Pro was detected by the FL1 detector using a 530/30-nm bandpass filter, while the fluorescence of merocyanine 540 was detected by the FL2 detector using a 620-nm longpass filter. Data were collected from 30 000

TABLE 1. Effect of trehalose concentration on the postthawed sperm motility parameters and recovery rate.*

Trehalose (%)	Motility parameters			Recovery rate		
	M%	PM%	VAP (μ/s)	M	PM	VAP
0	62.0 ± 1.8 [a]	49.0 ± 2.2 [a]	81.0 ± 9.3 [a]	0.69 ± 0.03 [a]	0.8 ± 0.05 [a]	1.1 ± 0.02
25	66.0 ± 2.9 [ab]	52.0 ± 3.0 [ab]	89.0 ± 3.2 [ab]	0.75 ± 0.05 [ab]	0.8 ± 0.07 [a]	1.2 ± 0.07
50	69.0 ± 3.1 [bc]	58.0 ± 3.1 [ab]	88.0 ± 2.6 [ab]	0.78 ± 0.05 [abc]	0.8 ± 0.05 [ab]	1.2 ± 0.03
75	73.0 ± 2.5 [bc]	59.0 ± 3.8 [b]	95.0 ± 5.7 [b]	0.83 ± 0.02 [bc]	1.0 ± 0.10 [b]	1.2 ± 0.05
100	78.0 ± 1.8 [c]	61.0 ± 3.1 [b]	96.0 ± 1.5 [b]	0.87 ± 0.02 [c]	0.9 ± 0.05 [ab]	1.1 ± 0.03

*M, Motility; PM, progressive motility; VAP, path velocity. Recovery rate, postthaw/prefreeze values. Values are mean percentages ± SEM.
[a–c], Different superscripts within the same column are significantly different ($P > 0.05$).

events for further analysis with Cell-Quest software (Becton Dickinson). Sideward scatter and the functional sperm concentration were recorded so that only sperm cell-specific events were selected for analysis.

Experimental Design

In experiment 1, we investigated the effects of trehalose incorporated into the diluents on the viability of the frozen-thawed goat spermatozoa. Fresh spermatozoa were frozen in diluents containing different concentrations of trehalose (0%, 25%, 50%, 75%, and 100%). The frozen-thawed goat spermatozoa were evaluated for immediate postthaw motility, progressive motility, path velocity, and recovery rates of these parameters. A further thermal resistance test was performed to assess sperm motility, progressive motility, and path velocity after 1, 2, and 3 h of incubation.

The results of experiment 1 revealed that dilution with 100% trehalose solution was found to yield a significant improvement on the viability of frozen-thawed goat spermatozoa. Thus, in experiment 2, to investigate the mechanism by which trehalose could exert this influence, we studied the membrane fluidity of the goat spermatozoa that were suspended in trehalose, compared the effect of trehalose (0.375 M) with that of disaccharide sucrose (0.375 M). Fresh washed spermatozoa were diluted with either trehalose, sucrose, or TCG, then left for 15 min to be equilibrated before staining with merocyanine 540/Yo-Pro-1, as described above. The stained samples were then subjected to flow cytometric analysis.

Statistical Analysis

Experiments were repeated five times, data were analyzed by analysis of variance (ANOVA), and Fisher protected least-significant difference posthoc test using the STATVIEW Software (Abacus Concepts, Inc., Berkeley, CA). All percentage data were subjected to an arcsin transformation before statistical analysis. Data are expressed as mean ± SEM. A probability of $P < 0.05$ was considered to be statistically significant.

RESULTS

Motility Parameters of the Frozen-Thawed Spermatozoa

The effects of trehalose on the postthaw sperm motility and recovery rate are summarized in Table 1. All trehalose extenders led to higher sperm motility than the TCG solution, with the best results obtained for the sperm sample extended with 100% trehalose solution, which was significantly higher than the results obtained with the TCG solution ($P < 0.05$). Among trehalose extenders, the samples frozen in the diluent supplemented with 25% trehalose solution showed the lowest postthaw motility, which was not significantly different from that of samples frozen in the TCG solution; there were no significant differences in the postthaw motility of samples frozen in the 50%, 75%, or 100% trehalose solutions. Sperm samples frozen in all trehalose solutions showed significantly ($P < 0.05$) higher progressive motility than that of samples frozen in TCG solution (Table 1). Furthermore, the path velocity of the goat spermatozoa frozen in trehalose extenders was significantly ($P < 0.05$) higher than that of those frozen in TCG extender (Table 1).

Freeze tolerance (i.e., recovery rate) was measured as the ratio of the sperm postthawed motility parameters to the prefreeze values and is shown in Table 1. Increasing the concentration of trehalose in the diluent was found to significantly increase the recovery rate of M%, PM%, and VAP% ($P < 0.05$) with the best values obtained for the sperm samples extended with 100% trehalose solution.

Thermal Resistance Test

The thermo-resistant test revealed that, after 3 h of incubation at 37°C, the highest percentage of motility (Fig. 2) and progressive motility (Fig. 3) were obtained in samples frozen in 100% trehalose extender ($P < 0.05$). Furthermore, after 3 h of incubation, 100% trehalose signifi-

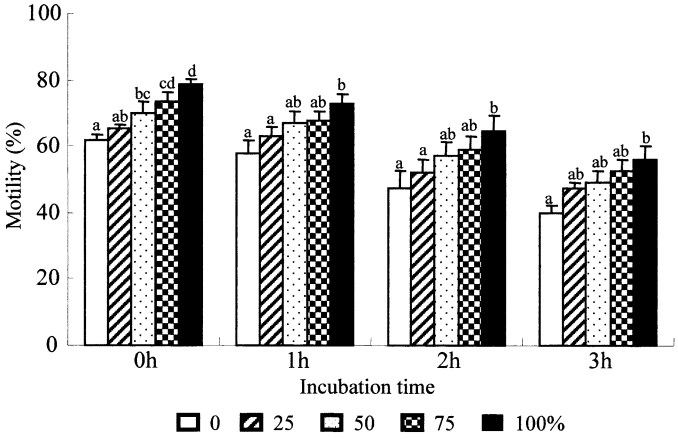

FIG. 2. Motility of goat spermatozoa frozen in different concentrations of trehalose after a 3-h postthawing resistance test at 37°C. Data are represented as the mean ± SEM and different superscript letters over bars represent statistical differences at $P < 0.05$ within the incubation time.

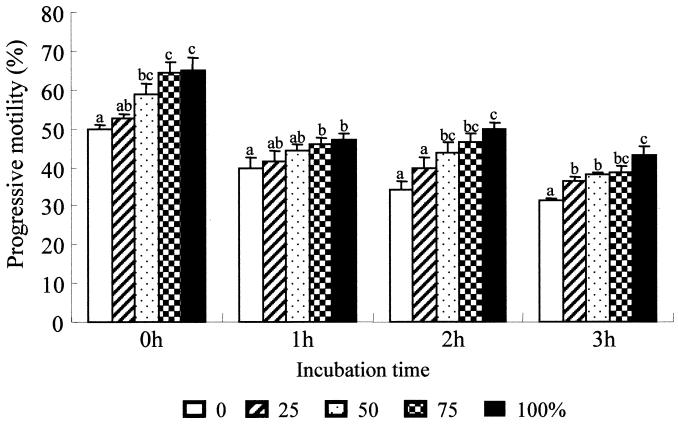

FIG. 3. Progressive motility of goat spermatozoa frozen in different concentrations of trehalose after a 3-h postthawing resistance test at 37°C. Data are represented as the mean ± SEM and different superscript letters over bars represent statistical differences at $P < 0.05$ within the incubation time.

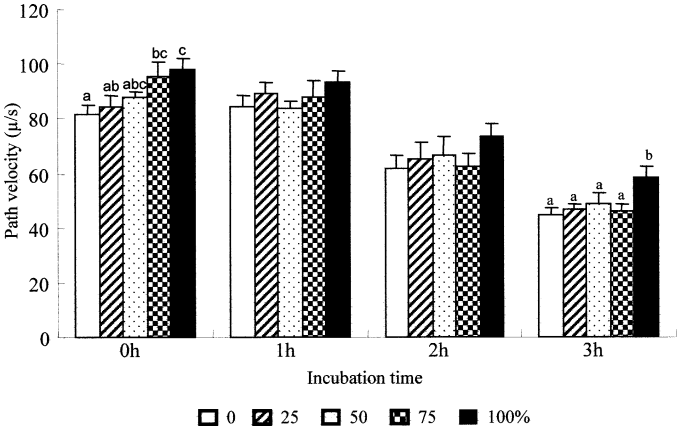

FIG. 4. The path velocity of goat spermatozoa frozen in different concentrations of trehalose after a 3-h postthawing resistance test at 37°C. Data are represented as the mean ± SEM and different superscript letters over bars represent statistical differences at $P < 0.05$ within the incubation time.

cantly ($P < 0.05$) preserved path velocity of the frozen-thawed goat spermatozoa.

Acrosomal Status of the Frozen-Thawed Spermatozoa

The intact acrosome percentage was found to improve significantly with the addition of trehalose in the diluents (Fig. 5). The samples frozen in 75% or 100% trehalose obtained the highest intact acrosome percentages, which were significantly different from those of sperm samples frozen in TCG solution ($P < 0.05$). However, low concentrations of trehalose (25% and 50%) also provided some minor protection for the frozen-thawed acrosome.

Membrane Fluidity

Fresh goat spermatozoa (suspended in TCG, sucrose, or trehalose solution) stained with merocyanine 540/Yo-Pro-1 and analyzed on a flow cytometer showed distinct subpopulations (Fig. 6A): cells that were stained with Yo-Pro were classified as dead sperm (window D), regardless of their merocyanine staining; cells unstained with Yo-Pro (live cells) showing a low merocyanine fluorescence signal were identified as low merocyanine live; and cells with more than the minimal merocyanine 540 signal were identified as high merocyanine live. Cells stained with Yo-Pro were rejected for analysis when the histogram plot was applied

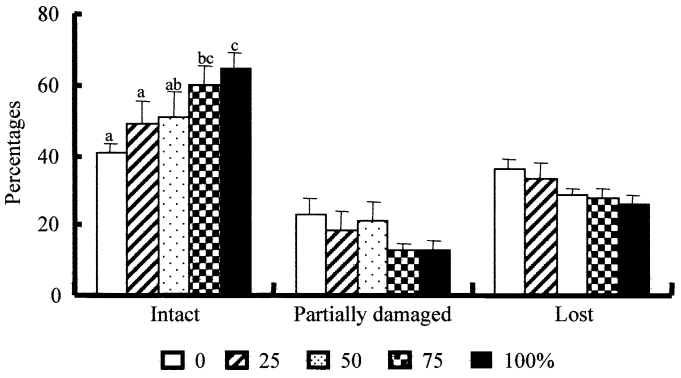

FIG. 5. Effect of different concentrations of trehalose on acrosome status of the frozen-thawed goat spermatozoa. Data are represented as the mean ± SEM and different superscript letters over bars represent statistical differences at $P < 0.05$ within each parameter.

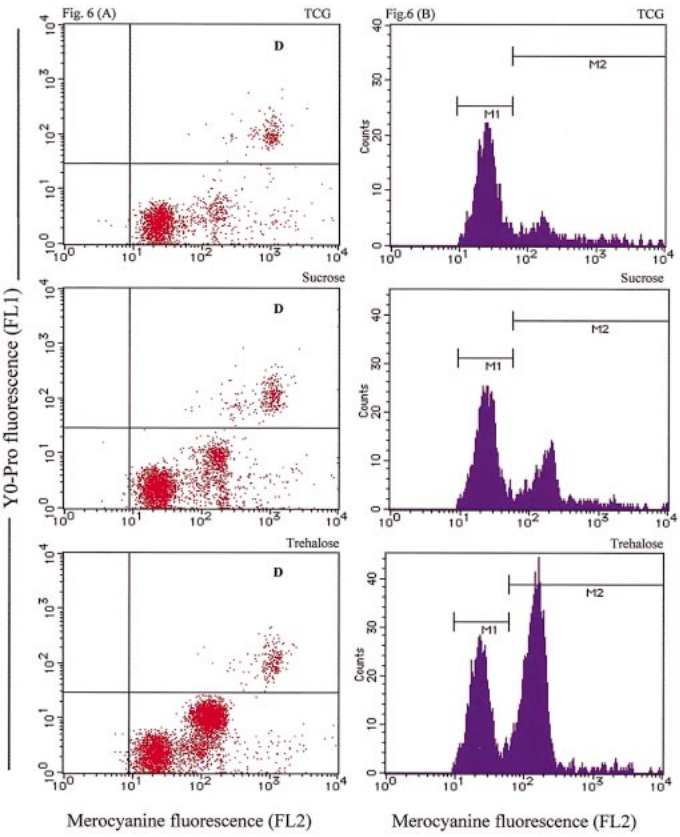

FIG. 6. Flow cytometric plots of merocyanine/Yo-Pro-stained goat spermatozoa. **A**) Dot plots of merocyanine/Yo-Pro-stained cells, (**B**) Histogram-plots of merocyanine-stained cell. D, Dead spermatozoa; M1, live cells showing low merocyanine stain; M2, live cells showing high merocyanine stain.

(Fig. 6B). Cells that showed low merocyanine 540 fluorescence (M1) were considered to have low membrane fluidity, while highly fluorescent (M2) sperm cells were considered to have a highly fluid membrane. When spermatozoa were diluted with trehalose solution, 51.0% of the spermatozoa showed high merocyanine 540 fluorescence (Fig. 7) that was significantly higher than when spermatozoa were diluted with sucrose (35.0%) or TCG (30.0%). No

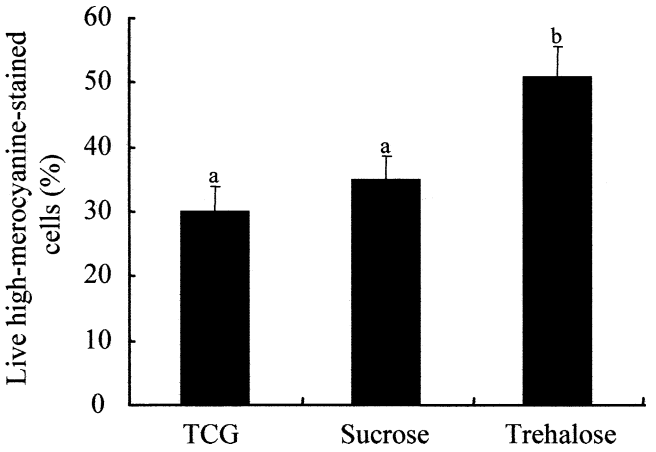

FIG. 7. Percentage of live high-merocyanine-staining cells after dilution with TCG, sucrose, and trehalose. Data are represented as the mean ± SEM and different superscript letters over bars represent statistical differences at $P < 0.05$ within the extenders.

significant difference was observed in membrane fluidity between spermatozoa diluted with sucrose and those diluted with TCG.

DISCUSSION

The results of the present study show that the addition of high concentrations of trehalose to sperm extender provide the best protection with regard to postthaw motility parameters, recovery rates, thermal resistance, and acrosome integrity, with the best results obtained for the 100% trehalose extender. Supplementation of the diluent by trehalose has been shown to have varying effects on freeze tolerance [8, 9].

Several investigators have found that the incorporation of trehalose in sperm diluents protects the spermatozoa of many species against freeze damage. Woelders et al. [26] demonstrated that an isotonic sugar medium in which Tris-citrate components were substituted with sucrose and trehalose is significantly superior to a Tris-citrate egg yolk medium in preserving the motility and acrosome integrity of bovine spermatozoa. Moreover, in a comparison of raffinose and trehalose, Storey et al. [27] showed that trehalose brings about a significantly better recovery rate in intact mouse spermatozoa. In addition, Aisen et al. [2] observed that trehalose significantly improves the viability of ram spermatozoa assessed for motility and acrosome integrity, with the best results obtained for a trehalose + EDTD extender. Molinia et al. [8] conclude that the motility of frozen-thawed ram spermatozoa is higher in the presence of sucrose or trehalose than in the presence of glucose when glycerol is not incorporated in the diluent; whereas when glycerol is employed in the diluent, no differences were observed among the various sugar types. Frozen-thawed dog spermatozoa have also been found to be protected by the supplementation of trehalose in the diluent [10].

In spite of this evidence in favor of the beneficial effects of trehalose, not all studies have found the same results. Chen et al. [28] report that trehalose caused only minor improvement in bull sperm survival, and Liu et al. [29], in a study on the freezability of bull spermatozoa in TCG extender containing up to 25% (v:v) trehalose or sucrose, conclude that replacing part of TCG-containing egg yolk with these sugars had no beneficial effects. The low concentrations of trehalose (0.05, 0.1 M) used by these researchers may account for the low improvement in sperm survival because trehalose in high concentrations was used by the many authors who found significant protection against freeze damage [10], and we hypothesize that the high concentrations of trehalose used in the present study could account for the successful cryopreservation of goat spermatozoa.

The precise mechanism by which trehalose protects spermatozoa during the freezing-thawing process remains to be elucidated and the exact mechanism of fast-cooling damage remains a subject of debate. Nevertheless, for reproductive purposes, it is important that the sperm cell be able to perform, as well as withstand a rapid loss of a large fraction of its intracellular water during freezing. The observed protection of the sperm by trehalose indicates that the presence of this sugar may render the membrane less vulnerable to the rapid physical and morphological changes that occur during the rapid efflux of water. It is assumed that sugars contribute to the stability of the membranes, as has been found in a number of other cells dehydrated during freeze drying or low-rate freezing and thawing [3, 30, 31].

The presence of sugars in the diluents is likely to affect the pattern of crystallization, and the shape and width of the channels of unfrozen solution [32], which could perhaps relieve or prevent fast-cooling damage to the spermatozoa. The presence of sugars leads to a lower salt concentration in the unfrozen water, consequently reducing the effects of the solution. It is also possible that the sugars may help prevent injurious eutectic freezing by trapping salts in an increasingly viscous or even glass-like phase [32]. Additionally, Woelders et al. [26] and Crowe et al. [33] suggest that the cryoprotective effect of trehalose results primarily from the formation of a hydrogen bond between the sugar hydroxyl group and the phospholipid polar head group, thus substituting for the water molecules under dehydration.

It is conceivable that the ability of trehalose to insert itself into bilayer phospholipids could modulate membrane fluidity, rendering the sperm membrane more able to withstand damage from freezing. The contribution of trehalose to membrane fluidity has been suggested by Iwashi et al. [34], and Lee et al. [35] state that the fluidity of lipids together with bilayer stabilization at the head group contribute to membrane viability in anhydrobiotic organisms. In addition, Giraud et al. [21] report that the recovery rate of viable or motile spermatozoa after freezing and thawing is superior for spermatozoa with high membrane fluidity before freezing.

In the present study, we used merocyanine 540 as a probe to explore whether or not trehalose could influence sperm membrane fluidity. Merocyanine 540 has been shown to stain cell membranes more intensely if their lipids are in a higher state of disorder, preferentially staining the fluid domains of the outer leaflet of the lipid bilayer [36], and such staining has been widely used to detect changes in membrane lipids in several cell types [37, 38]. Our results show that the percentage of cells with high merocyanine fluorescence is significantly higher in spermatozoa treated with trehalose. It is known that the differences between high- and low-affinity binding to merocyanine reflect differences in the composition or structural organization of the membrane [38]. Taken together with this information, our results suggest that trehalose plays a major role in increasing membrane fluidity before freezing, leading to greater endurance of the spermatozoa against freeze-thawing damage. The relationship between membrane fluidity and adaptation to low temperature remains to be elucidated; however, stabilization of the biological membranes during freezing requires depression of transition temperature and consequent maintenance of the constituent lipids in a liquid crystalline. Crowe et al. [39] conclude that, in the presence of trehalose, dry lipids have a transition temperature similar to that of fully hydrated lipids, while lipids dried without trehalose have a higher transition temperature. In addition, the membrane-phase transition temperature is known to be lowered by trehalose during freeze drying of yeast cells [40]. On the basis of this evidence, we speculate that an increase in membrane fluidity could enhance the depression of membrane transition temperature, allowing the sperm membrane to endure low-temperature effects.

In conclusion, 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. In addition, with respect to membrane fluidity, our findings confirm the protective effects of trehalose during freezing of goat spermatozoa.

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