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Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender ☆

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ABSTRACT

The objective of this study was to examine the interaction of different concentrations of trehalose [0 (T0), 50 (T50) or 100 (T100) mM] and glycerol [5% (G5) or 7% (G7)] on post-thawed quality of ram semen, cryopreserved in a soybean lecithin (SL)-based extender. Twenty-eight ejaculates were collected from four rams and diluted with six trehalose/glycerol combinations: T0G5, T50G5, T100G5, T0G7, T50G7, and T100G7. Sperm motility (CASA), membrane integrity (eosin/nigrosin) and functionality (HOST), abnormal forms, capacitation status (CTC), mitochondrial activity (rhodamine 123), apoptotic features (Annexin V/propidium iodide) and lipoperoxidation (malondialdehyde production) were evaluated after thawing. Extender T100G5 yielded the highest results for total and progressive motility, sperm velocity, normal morphology, functional membranes, active mitochondria and membrane integrity, with $P < 0.05$ in general, except for T50G7 ($P > 0.05$). The combinations T0G5, T0G7 and T100G7 yielded the lowest post-thaw quality. We could not detect significant changes in other kinematic parameters, capacitation status or lipoperoxidation. We conclude that, in our SL-based extender, a combination of 100 mM trehalose and 5% glycerol was the most adequate combination to achieving post-thawing quality in our soybean lecithin-based extender, and our results support that a synergistic effect among trehalose and glycerol exists. We suggest that other combinations could improve these results.

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Introduction

Sugars play an important role during the cryopreservation of biological material, not only due to their osmotic effects, but also to the interaction with the phospholipid bilayers at the low hydration conditions occurring during the freezing process, contributing to stabilize them [16]. Sugars can also depress the membrane phase transition temperature of dehydrated lipids, preventing or delaying this phase transition, and thus reducing shedding of components and membrane fusion. Therefore, sugars have been considered for sperm cryopreservation, not only as a source of energy for the spermatozoa (glucose or fructose), but also to prevent structural and sub-structural damage of sperm during this dehydrated reduced-water state [1,21].

Many authors have been used isoosmotic extenders for cryopreservation of ram semen. However, hyperosmotic extenders using different sugars and sugar concentrations have been shown to improve ram semen quality after the freeze–thawing process [4,5,27,52,53]. Among them, trehalose is a non-penetrating disaccharide that seems to protect cells both by increasing the tonicity of the extender and by stabilizing the plasma membrane, possibly due to direct interaction with phospholipid polar head groups of membrane phospholipids [15]. Trehalose seems to be more efficient than other sugars for protection of spermatozoa in cryopreservation media, and many authors have reported its beneficial effect for semen cryopreservation in different species, such as ram [4,27,38], goat [1,2], bull [13,55], boar [25] and mouse [51]. In contrast, several studies have reported no significant positive effect of trehalose for cryopreserving spermatozoa from stallion [49], Iberian red deer [21], European brown hare [30], rooster [33] and emu [48].

☆ Experiment was designed by M. Zhandi and A. Towhidi. Semen freezing and post thawed sperm evaluations were done by A. Najafi, M. Sharafi, A. Akbari Sharif and M. Khodaei Motlagh. Manuscript was written by A. Najafi, M. Sharafi, M. Zhandi and F. Martinez-Pastor.

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Whereas sugars are non-permeating cryoprotectants, other substances such as glycerol penetrate within the cell, stabilizing the intracellular components [18,42]. The effects of these substances are multiple, preventing intracellular ice formation, contributing to stabilize lipids and proteins in the sperm membrane and increasing dehydration as well as membrane fluidity [22,24]. It seems that combining of penetrating and non-penetrating cryoprotectants in semen extender would be better than that of single cryoprotectant [10]. Previous reports have been shown the combination effect of trehalose and glycerol in egg yolk-based or LDL-based extenders [25,32]. Although we could expect a synergic effect between glycerol and trehalose, due to their different properties, few studies have tested the interaction of different concentrations of these two cryoprotectors [20,31,50].

Moreover, to our knowledge, there are no reports about the synergistic effects of trehalose and glycerol in a soy lecithin (SL)-based extender for cryopreservation of ram semen. Therefore, the objective of this study was to examine the combined effect of different concentrations of trehalose and glycerol in a SL-based extender on some ram sperm parameters after the freeze–thawing process.

Materials and methods

Chemicals

Unless otherwise indicated, all chemicals used in this study were obtained from Sigma (St. Louis, MO, USA), and Merck (Darmstadt, Germany).

Semen collection, processing and extender preparation

Semen were collected from four mature Zandi ram (3 and 4 years of age), of superior genetic merit and proven fertility. A total of 28 ejaculations (seven ejaculates for each ram) were collected twice a week from each ram using an artificial vagina, during the breeding season (autumn). The primary criteria for evaluation of sperm were: volume of 0.75–2 mL; semen concentration above than 3×10^9 sperm/mL; progressive motility higher than 70%; less than 10% abnormal sperm. To eliminate individual differences, semen were pooled and processed for extension.

The basic extender used in this study was composed of 27.1 g/L Tris, 10 g/L fructose, and 14 g/L citric acid. Soybean lecithin was added to the basic extender at 1% (wt/vol). The osmolarity and pH of this base extender were set at 320 mOsm and 7.2, respectively. The base extender was supplemented either with 5% (G5) or 7% (G7) glycerol and either 0 mM (T0), 50 mM (T50) or 100 mM (T100) trehalose. Each pooled ejaculate was split into six equal aliquots and diluted (37 °C) with each of the six extenders: T0G5, T50G5, T100G5, T0G7, T50G7 and T100G7, for a total of six experimental groups. Diluted samples were loaded into 0.25 mL French straws (IMV, L'Aigle, France) at a final concentration of 4×10^8 sperm/mL and equilibrated at 4 °C for a period of 2 h. After equilibration, the straws were horizontally frozen in liquid nitrogen vapors (5 cm above liquid nitrogen) for 12 min, and then plunged into liquid nitrogen for storage. For sperm evaluation, straws were thawed individually at 37 °C for 30 s in a water bath. Sperm evaluation was performed on all semen samples immediately after thawing.

Semen evaluation

Analysis of standard semen parameters

Motility and motion parameters of sperm were estimated by computer-assisted sperm motility analysis (CASA; IVOS version 12; Hamilton-Thorne Biosciences, MA, USA). The following variables were analyzed: total motility (TM, %); progressive motility (PM, %); average path velocity (VAP, μm/s); straight-line velocity (VSL, μm/s); curvilinear velocity (VCL, μm/s); amplitude of lateral head displacement (ALH, μm); beat/cross frequency (BCF, Hz); linearity (LIN, %); straightness (STR, %).

Viability was assessed by means of the eosin–nigrosin stain method [17]. 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 at ×400 (CKX41; Olympus, Tokyo, Japan). Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

The evaluation of functional membrane integrity of sperm was performed by using the hypo-osmotic swelling test (HOST), considering positive those spermatozoa with curled or swollen tails [44]. In brief, 25 mL semen was added to 200 mL of hypo-osmotic solution (100 mOsm/L, 57.6 mM fructose and 19.2 mM sodium citrate). After 30 min incubation, the mixtures was homogenized and evaluated under a phase-contrast microscope (CKX41; Olympus, Tokyo, Japan). A total of 200 spermatozoa were counted in at least five different microscopic fields at ×400. The percentage of spermatozoa with swollen and curved tails was recorded.

For the evaluation of total abnormalities in the semen samples, at least three drops of the semen were pipetted into 1.5 mL tubes, containing 1 mL Hancock's solution [45]. One drop of this mixture was placed on a microscope slide and covered with a cover slip. The percentage of sperm abnormalities was recorded by counting a total of 200 sperm under a phase-contrast microscope. Hancock's solution was prepared by mixing 62.5 mL formalin (37% formaldehyde), 150 mL of sodium saline solution, 150 mL of buffer solution and 500 mL of double-distilled water. Sodium saline solution: 9.01 g NaCl in 500 mL of double-distilled water; buffer solution: (1) 21.7 g $Na_2HPO_4 \times H_2O$ in 500 mL of double-distilled water; (2) 22.254 g KH_2PO_4 in 500 mL of double-distilled water; 100 mL of (1) and 80 mL of (2) were mixed to obtain 180 mL of buffer solution.

Chlortetracycline (CTC) staining

Chlortetracycline staining was used for the evaluation of capacitation status as described by Perez et al. [40] with a little modification [19]. A CTC working solution (750 mM) was freshly prepared in a buffer containing 20 mM Tris, 130 mM NaCl, and 5 mM D,L-cysteine at a pH of 7.4. Five microliters of semen were mixed with 20 mL CTC working solution. After 20 s, the reaction was stopped by the addition of 5 μL glutaraldehyde (1% (v/v) in 1 M Tris–HCl, pH 7.8). Smears were prepared on a clean microscope slide, covered with a cover glass, sealed with nail varnish, and kept in the dark at 4 °C. The evaluations were carried out using an epifluorescent microscope (Nikon PCM 2000™ Personal Laser Scanning Confocal Microscope, USA) at ×1000. Two hundred spermatozoa were classified into three categories: uniform fluorescent head (uncapacitated: CTC-F), fluorescent-free band in the post-acrosomal region (capacitated: CTC-B), and non-fluorescent head or a thin fluorescent band in the equatorial segment (acrosome-reacted: CTC-AR).

Malondialdehyde (MDA) concentrations

The thiobarbituric acid reaction was used for measurement of MDA, which is indicative of lipid peroxidation in phospholipids of the sperm membrane. This method was adapted from Placer et al. [41]. Briefly, 1 mL of diluted sperm (250×10^6 mL^{-1}) were mixed with 1 mL of cold 20% (w/v) trichloroacetic acid to precipitate protein. The precipitate was pelleted by centrifuging (900*g* for

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15 min), and 1 mL of the supernatant was incubated with 1 mL of 0.67% (wt/vol) thiobarbituric acid in a water bath at 100 °C for 10 min. After cooling, the absorbance was determined by a spectrophotometer (UV-1200, Shimadzu, Japan) at 532 nm.

Flow cytometry

Flow cytometry analises were carried out with a FACSCalibur flow cytometer (Becton Dickinson, San Khosoz, CA, USA). Annexin-V fluorescence and Rhodamine-123 fluorescence were detected on detector FL1, and PI fluorescence was detected on detector FL3. For each sample, 10,000 events were collected.

Mitochondrial activity

Mitochondrial activity was assessed using Rhodamine 123 (R123; Invitrogen TM, Eugene, OR, USA) and PI. Briefly, 10 μL of R123 solution (0.01 mg/mL) were added to 500 μL of tris-diluted semen samples (50×10^6 sperm/mL) and incubated for 20 min at room temperature in the dark. Samples were then centrifuged at 1200g for 10 min and the sperm pellets were re-suspended in 500 μL tris buffer and 10 μL PI (1 mg/mL) were added to sperm suspension. The percentage of sperm with functional mitochondria was identified by R123 high fluorescence and no PI fluorescence.

Phosphatidylserine translocation assay

The detection of phosphatidylserine translocation was carried out by means of a commercial kit and according to the manufacturer's instructions (Immune Quality Products (IQP), Groningen, The Netherlands). Briefly, samples were washed in calcium buffer and diluted to 1.0×10^6 sperm/mL in calcium buffer. Then, 10 μL Annexin V-FITC was added to 100 μL sperm suspension and incubated for 20 min. Afterward, 10 μL propidium iodide (PI) was added to the sperm suspension and incubated for 10 min. The sperm were classified to three groups: viable non-apoptotic cells, negative for Annexin-V and excluding PI (A^-/PI^-); cells presenting signs of early apoptosis (externalized phosphatidylserine), binding Annexin-V but still excluding PI (A^+/PI^-); and dead spermatozoa, stained with PI (PI^+).

Statistical analysis

Data were analyzed in the R statistical environment [54]. Data were analyzed using linear mixed-effects models. The mathematical model included main effects (trehalose and glycerol) and their interactions. Statistical differences between the various treatment group means were determined by Tukey's test. Differences with values of $P < 0.05$ were considered to be statistically significant. Results are shown as mean ± SEM, unless indicated.

Results

Glycerol and trehalose concentration affected many of the studied post-thawing parameters. In all the cases were the models were significant, the interaction glycerol × trehalose was significant, suggesting a synergic effect of some combinations. Indeed, the combination of 100 mM trehalose and 5% glycerol (T100G5) and 50 mM trehalose and 7% glycerol (T50G7) yielded the highest post-thawing quality, whereas T0G5, T0G7 and T100G7 yielded the lowest quality.

Motility

The results of sperm motility and kinematic parameters are shown in Fig. 1 and Table 1. Total motility in T100G5 and T50G7 extenders was significantly higher than the other groups, above 50% (Fig. 1a). T50G5 and T0G7 had intermediate motility (around 50%), and T0G5 and T100G7 showed the lower motility values, averaging below 50%. Progressive motility of post-thawed sperm was significantly higher in the T100G5 extender (27.9% ± 1.2), with T50G7 showing a slightly lower average value (24.6% ± 1.3), but not significantly different (Fig. 1b). The rest of the extenders yielded progressive motility values significantly lower than T100G5. The three sperm velocities, VCL, VAP and VSL, showed a similar pattern, with T100G5 yielding higher average values than the rest of the extenders, and T50G7 following next (Fig. 1c–e). For the rest of the kinematic parameters (ALH, BCF, LIN and STR), no differences were found among extenders (Table 1).

Membrane integrity, functionality and abnormal forms

The proportion of spermatozoa with membrane integrity (eosin/nigrosin stain) and functionality (hypo-osmotic swelling test) was higher in the T100G5 extender (Fig. 2a and b), being this difference significant except with T50G7, which followed closely. The proportion of abnormal forms showed an inverted pattern, obtaining the lower average values in the T100G5 and T50G7 extenders (Fig. 2c). T50G5 yielded also a lower proportion of abnormal forms, being not significantly different than T100G5 or T50G7. In these analyses, T0G5, T0G7 and T100G7 yielded the lower sperm quality.

Capacitation status

Sperm capacitation results, determined using CTC staining, are shown in Table 2. Most spermatozoa belonged to the B pattern (capacitated). No significant effects of glycerol or trehalose were found, although T100G5 and T50G7 extenders yielded a slightly lower proportion of acrosome-reacted sperm (AR), together with a higher proportion of uncapacitated sperm (F).

Lipid peroxidation

The different extenders had no significant effect on MDA yield after the freeze–thawing process. The overall MDA production was 7.0 ± 0.2 nmol per 10^8 spermatozoa. T100G5 and T50G7 yielded slightly lower mean values (6.2 ± 0.5 and 6.2 ± 0.6, respectively).

Mitochondrial activity

The proportions of viable spermatozoa with active mitochondria for each extender are showed in Fig. 2d. T100G5 showed the higher results (57.0% ± 3.5), significantly higher than the rest of the extenders except T50G7 (48.3% ± 0.8).

Phosphatidylserine translocation

Fig. 3 shows the results of the Annexin V/PI analysis. T100G5 yielded the largest proportion of live spermatozoa without phosphatidylserine translocation (48.2% ± 3.8; Fig. 3a), being significantly higher than T0G5 (25.3% ± 3.9), T0G7 (31.5% ± 3.3) and T100G7 (29.8% ± 3.0). T50G5 and T50G7 yielded a non-significant lower mean value. However, there was no effect neither of glycerol nor trehalose on the proportion of live spermatozoa with externalized phosphatidylserine (overall 27.7% ± 0.9; Fig. 3b). The distribution of proportions of dead spermatozoa (PI^+; Fig. 3c), was almost

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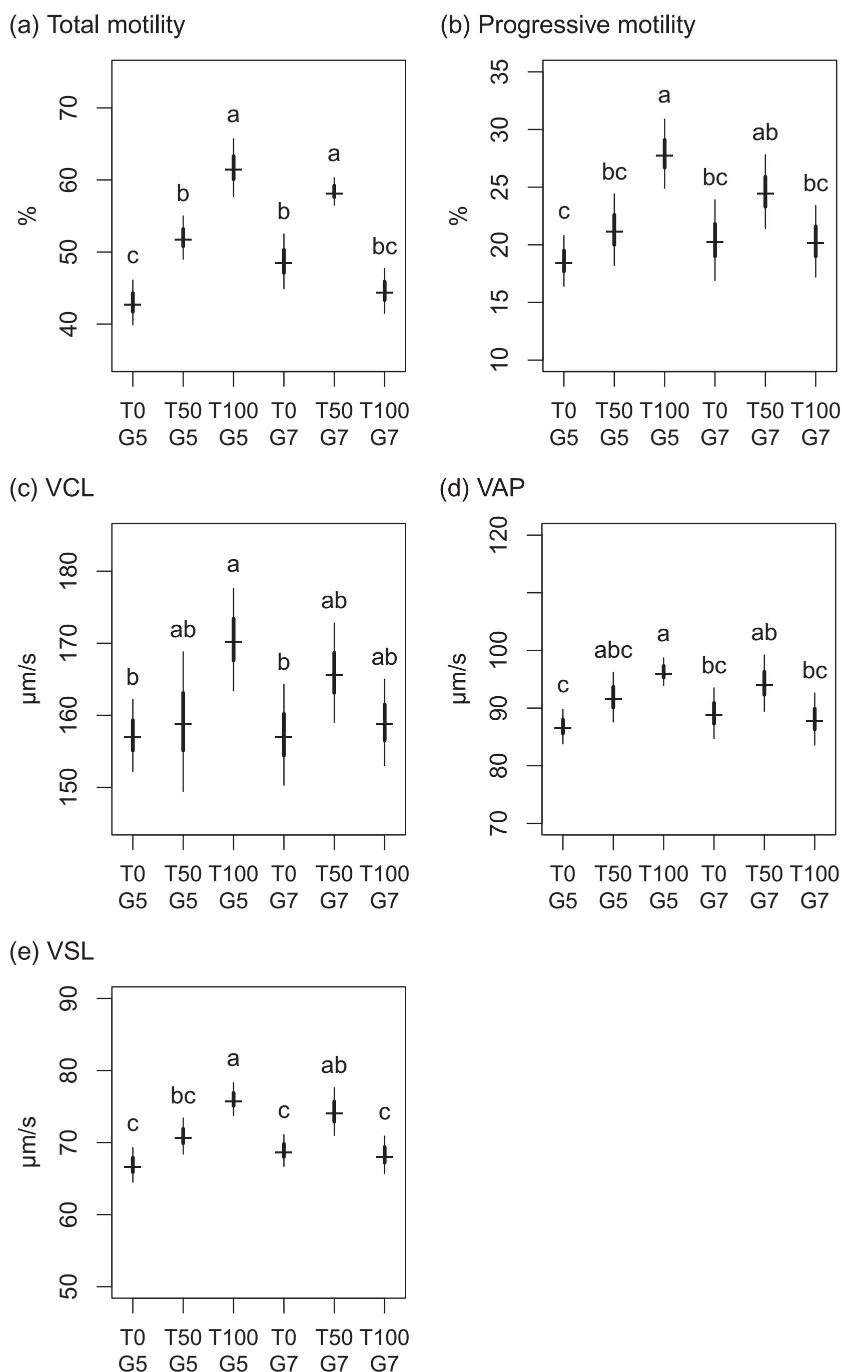

Fig. 1. Post-thawing motility results for each of the tested extenders, showing the interaction glycerol × trehalose. Data are shown as mean (horizontal lines), one standard error of the mean (thick bars) and 95% confidence intervals (thin bars). Different letters indicate that extenders differ $P < 0.05$.

Table 1
Results of the kinematic parameters ALH, BCF, LIN and STR of ram spermatozoa after cryopreservation in different extenders (mean ± SEM). No significant differences were found among extenders.

Parameter (unit)	Extenders					
	T0G5	T50G5	T100G5	T0G7	T50G7	T100G7
ALH (µm)	8.5 ± 0.3	8.2 ± 0.2	8.1 ± 0.2	8.3 ± 0.2	8.1 ± 0.3	8.4 ± 0.2
BCF (Hz)	25.0 ± 0.6	25.2 ± 0.5	26.8 ± 0.6	25.3 ± 0.6	26.1 ± 0.9	25.5 ± 0.6
LIN (%)	42.4 ± 0.9	44.0 ± 1.1	44.6 ± 1.0	43.6 ± 1.0	44.4 ± 0.6	42.9 ± 0.6
STR (%)	76.3 ± 1.6	77.1 ± 1.1	78.6 ± 1.4	77.0 ± 1.1	77.9 ± 1.3	76.7 ± 1.3

Extenders were SL-based and contained no trehalose (T0), trehalose at 50 mM (T50) or at 100 mM (T100), and glycerol at 5% (G5) or 7% (G7).

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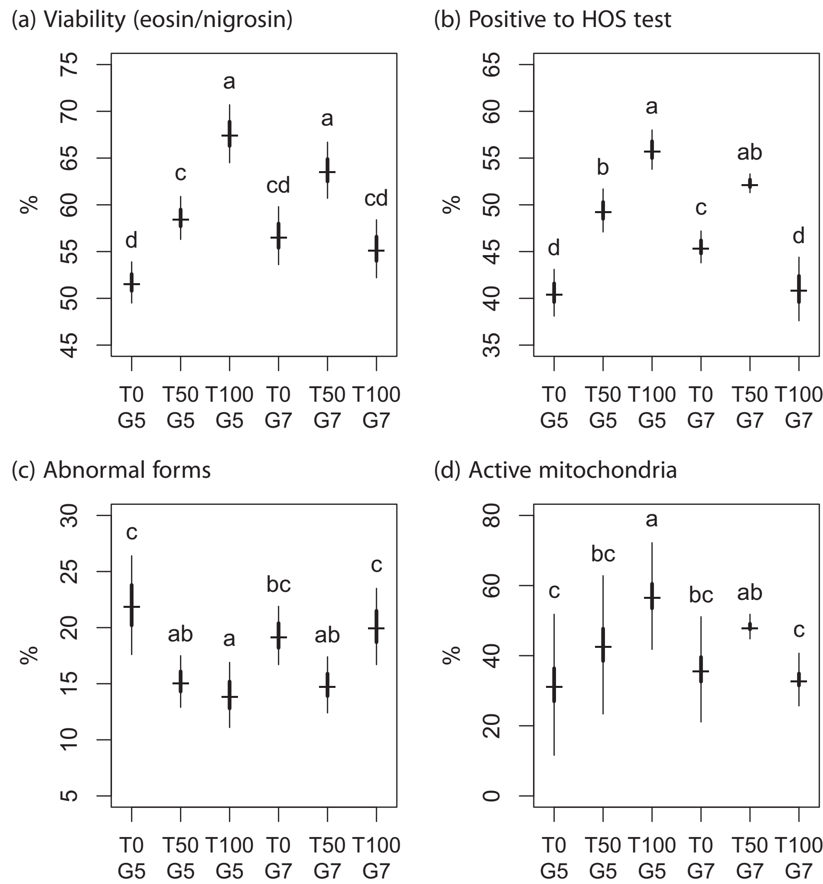

Fig. 2. Membrane integrity (a), membrane functionality (b), abnormal forms (c) and mitochondrial status (d) after thawing samples cryopreserved with the different trehalose and glycerol concentrations. Data are shown as mean (horizontal lines), one standard error of the mean (thick bars) and 95% confidence intervals (thin bars). Different letters indicate that extenders differ $P < 0.05$.

Table 2
Percentage of uncapacitated (F), capacitated (B), and acrosome-reacted (AR) ram spermatozoa after cryopreservation in different extenders (mean ± SEM). No significant differences were found among extenders.

Patterns	Extenders					
	T0G5	T50G5	T100G5	T0G7	T50G7	T100G7
F	13.2 ± 0.8	13.9 ± 0.8	15.0 ± 0.9	13.2 ± 0.9	14.8 ± 1.1	13.2 ± 1.0
B	58.8 ± 1.9	59.9 ± 1.5	60.0 ± 1.1	59.3 ± 1.4	60.2 ± 0.8	60.3 ± 0.9
AR	27.9 ± 2.1	26.2 ± 1.1	25.0 ± 1.6	27.4 ± 2.0	25.0 ± 1.3	26.5 ± 1.3

Extenders were SL-based and contained no trehalose (T0), trehalose at 50 mM (T50) or at 100 mM (T100), and glycerol at 5% (G5) or 7% (G7).

opposite to Fig. 3a, with T100G5 showing the lowest proportion, followed by T50G7 and T50G5 ($P > 0.05$). T0G5 yielded the highest proportion of dead spermatozoa.

Discussion

This study demonstrates a synergistic effect of trehalose and glycerol in a soybean lecithin (SL)-based extender when used to protect ram sperm during the freeze–thawing process. Sperm cryopreservation results in a reduction in viability and fertility of spermatozoa, due to multifactorial cryo-damage [24]. This cryo-damage can be attenuated by modifying the composition of cryoprotectants in the extender [23,29]. Several studies have highlighted the suitability of trehalose as a supplement for cryopreserving semen of many species [4,39,46]. Although several authors have studied the effects of different trehalose concentrations, the interaction of trehalose with other cryoprotectants has not been considered but for few reports [20,31,50]. Moreover, no studies have dealt with the supplementation of SL-based extenders with trehalose.

Therefore, we hypothesized that the combination of trehalose and glycerol at given concentrations in SL-based extender could yield very different and even contrary effects. Our results demonstrated that combining 5% glycerol and 100 mM trehalose resulted in higher post-thawing sperm quality than other combinations, or using glycerol alone, and that the synergistic effect exists (T100G7 was no better than no using trehalose at all). Our results with T100G5 are in agreement with the findings of Aisen et al. [4], and Jafaroghli et al. [27], who found a higher post-thawing quality freezing ram spermatozoa with 3% or 5% glycerol (respectively) and 100 mM trehalose. In fact, Aisen et al. [4], found a lower quality if trehalose was not added to the extenders, or if it was used at higher concentrations (200 and 400 mM). These authors [4,27], carried out fertility trials using 100 mM trehalose vs. only glycerol,

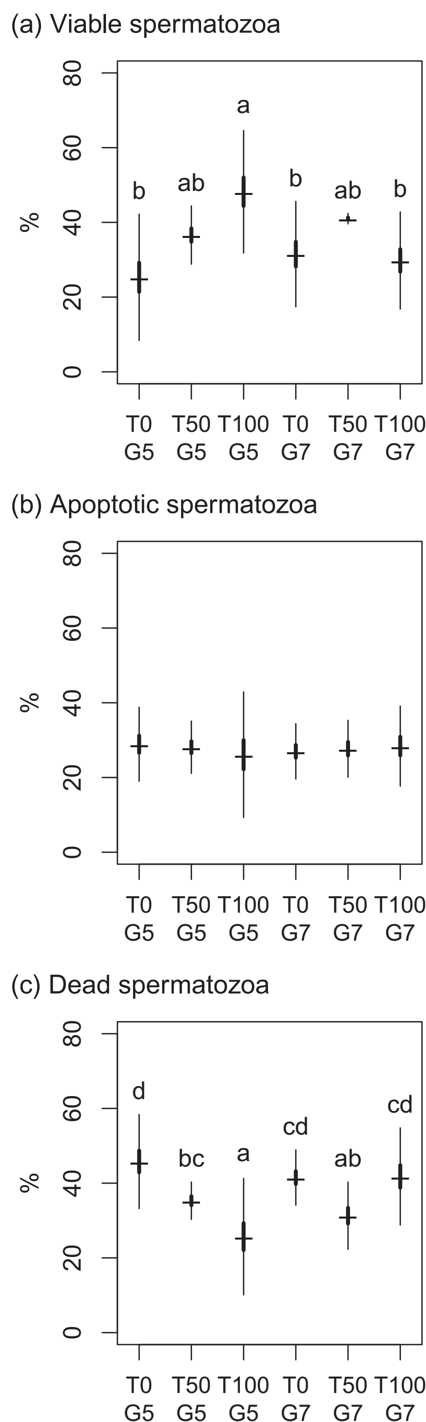

Fig. 3. Results of the Annexin V/propidium iodide stain. The proportions of the three populations detected by flow cytometry are shown for each extender. Data are shown as mean (horizontal lines), one standard error of the mean (thick bars) and 95% confidence intervals (thin bars). Different letters indicate that extenders differ $P < 0.05$.

obtaining higher lambing rates when trehalose was used in the extenders. It is notable that the extender used in our study was based on SL, whereas other studies in ram used egg yolk, but the results were nonetheless comparable. Other authors have not found positive effects of trehalose, but it might be due to using concentrations other than around 100 mM (ram [35]: 435 mM; deer [21]: 40 mM).

In the present study, eosin/nigrosin, HOST and viability results (Annexin V/PI) indicated that T100G5 significantly reduced membrane damage in sperm cells after the thawing. In ram [4,27], goat [29], and buffalo [9,46], membrane integrity was better preserved when trehalose was used at that 100 mM. Sperm freezing may lead to membrane deterioration due to membrane phase transitions occurring in the different regions of the highly specialized regionalized sperm plasma membrane [11,36]. Trehalose stabilizes biological membranes, possibly due to its interaction with the polar heads of the membrane phospholipids [6], therefore modulating membrane fluidity. These events may allow the sperm membrane to withstand damage during freezing [10]. This improvement on membrane integrity and functionality would result in better protection for sperm function, such as sperm motility, which was observed in our study. Interestingly, whereas we obtained good results with T100G5 but not with T100G7, Khalili et al. [29], obtained the highest post-thawing quality when combining nearly 200 mM of trehalose (198.24 mM) and 8% glycerol. This suggests both that there may be important differences between species regarding the optimal trehalose/glycerol concentration, and those positive interactions between trehalose and glycerol concentrations could be occurring at concentrations not tested in our study.

Moreover, it is important to highlight that when we cryopreserved ram semen with 7% glycerol, trehalose was more efficient if used at 50 mM. Interestingly, even if the mean results of T50G7 tended to be lower than those of T100G5, there were no significant differences between both extenders. Glycerol is a membrane-permeating cryoprotectant, which therefore differs very much on its mechanism of action respect to trehalose, and this might explain their interaction, reflecting in a synergistic effect at some concentration ranges. This could explain, apart from between-species differences, why several studies have reported no positive effects of trehalose, and even negative effects at some concentrations [3,8]. Furthermore, our study showed that freezing ram semen in our SL-base extender with 5% glycerol (T0G5) yielded doses of lower quality (HOST) than when using 7% glycerol (T0G7). Despite this superiority of glycerol at 7% in our SL-based extender, just by adding 50 mM of trehalose to the 5% extender, we compensated for this difference (in fact, T50G5 achieved higher results in the HOST than T0G7), and adding 100 mM of trehalose we achieved the highest sperm quality. Therefore, starting from an apparently suboptimal extender and taking advantage of a putative synergistic effect of trehalose and glycerol on ram semen cryopreservation, we could not only achieve good results, but also superior ones.

Several parameters seemed not to be affected by the extender choice, though. CTC patterns are modified by cryopreservation, due to membrane changes and alteration of the Ca^{2+} homeostasis, a phenomenon that has been termed as "cryocapacitation" [28]. Moreover, phosphatidylserine externalizations (and other apoptotic markers) are enhanced during these stressing protocols [7]. Due to the stabilizing effect of trehalose in biological membranes, we expected that trehalose would reduce cryocapacitation and apoptotic-like features. Nevertheless, neither the post-thawing CTC pattern nor the proportions of membrane-intact (PI^-) spermatozoa with externalized phosphatidylserine were affected by the extender. This observation is in agreement with the findings of sharafi et al. [47], who reported that cryoprotectants had no effect on the CTC staining patterns (uncapacitated, capacitated and acrosome reacted) of sperm in SL-based extender.

Similarly, MDA yield was unchanged by different trehalose or glycerol concentrations. Other studies in ram sperm could not detect any change in lipoperoxidation levels after freezing semen in the presence of 50 or 100 mM of trehalose [12]. Similar results were obtained for goat Atessahin et al. [8], using 50 or 75 mM of trehalose (25 mM increased MDA production). Aisen et al. [3], showed that freezing ram semen with 100 mM trehalose had no ef-

fect on MDA production if measured just after thawing, but after 3 h at 37 °C they detected an increase in control samples but not in trehalose-supplemented samples. Chhillar et al. [14], reported that both trehalose and taurine decreased H_2O_2 and MDA in frozen-thawed bull semen to the levels of fresh semen, and Badr and Hassan [9], reported similar results in buffalo semen. Therefore, the effect of trehalose the oxidative stress concomitant to sperm cryopreservation seems to vary with species, and possibly with the application of different protocols.

Finally, highest quality samples (T100G5) also showed the highest proportion of viable spermatozoa with active mitochondria. Much alike motility and membrane status parameters, extender differences were not strictly related to a given trehalose concentration, but to the specific trehalose/glycerol combinations. Thus, T50G7 showed no significant differences with T100G5, but this higher trehalose concentration could not protect mitochondria if combined with 7% glycerol (T100G7). These observations further support our interaction hypothesis regarding trehalose/glycerol effects. The protection of mitochondria is especially important during sperm cryopreservation. During conventional freezing, ice or osmotic events may damage cell structures [26], and ice crystallization could mechanically induce unregulated events related to apoptosis [34]. Mitochondria are known to play a central role during the execution phase of apoptosis as a decrease in their membrane potential occurs and opening of mitochondrial pores leads to the subsequent release of pro-apoptotic factors [43]. Even though we did not detect an increase on "early apoptotic" spermatozoa (Annexin V^+/PI^-), the decrease of spermatozoa with active mitochondria could be related to the differences on motility and proportion of dead spermatozoa among treatments. Moreover, synthesis of ATP is under control of mitochondrial activity, which damage to mitochondria leading to non-renewal of ATP [37]. Therefore, lack of energy result in depletion in ATP which may be in part responsible for lower sperm motility after the freeze–thawing process [34,37].

Conclusion

We have reported a synergistic effect on the quality of frozen-thawed ram spermatozoa depending on the concentrations of glycerol and trehalose. In fact, in our experiment the highest post-thawing quality was obtained at a glycerol concentration of 5% and a trehalose concentration of 100 mM. Moreover, when glycerol was used at 7% and trehalose was used at 50 mM results were similar. Combining our findings with previous studies [4,25], it seems that moderately high trehalose concentrations, around 100 mM, could present this synergistic effect when combined with relatively low concentrations of glycerol (5% or below), being advisable to use lower trehalose concentrations if increasing glycerol concentration. Moreover, our results invite to test other glycerol/trehalose combinations, exploring other concentration ranges. We suggest that a combination of 5% glycerol and 100 mM trehalose in a SL-based extender could be the starting point to create a suitable extender for ram semen.

Conflict of interest

None of the authors have any conflict of interest to declare.

Authors contributions

Experiment was designed by M. Zhandi and A. Towhidi. Semen freezing and post thawed sperm evaluations were done by A. Najafi, M. Sharafi, A. Akbari-Sharif and M. Khodaei Motlagh. Manuscript was written by A. Najafi, M. Sharafi, M. Zhandi and F. Martinez-Pastor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cryobiol.2013.03.002.

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