Theriogenology 83 (2015) 144–151

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effects of glycerol and sugar mixing temperature on the morphologic and functional integrity of cryopreserved ram sperm

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ARTICLE INFO

Article history:
Received 16 December 2013
Received in revised form 8 September 2014
Accepted 8 September 2014

Keywords:
Trehalose
Cooling process
Plasma membrane domains
Frozen-thawed ram spermatozoa

ABSTRACT

Sperm deep freezing procedures for ram semen have considerable variations regarding the steps being employed for cooling, freezing, and addition of cryoprotectants. In this work, we evaluated the effects of the addition of glycerol and/or the disaccharides sucrose and trehalose to hypertonic diluents either before or after cooling from 30 °C to 5 °C in Merino Australian ram semen cryopreservation. Using optical and transmission electron microscopy techniques, we assessed that glycerol was beneficial to the cooling process independently of its addition at 30 °C or 5 °C in terms of sperm membrane integrity in different regions of the plasma membrane (acrosomal region, 14.5% higher integrity; postacrosomal region, 8.0% higher integrity [$P < 0.01$]; hypoosmotic swelling test [HOST], 10.8% higher integrity [$P < 0.001$]). Disaccharides were necessary for a better cryopreservation in liquid nitrogen, and the best procedure was their addition after cooling at 5 °C (12% higher sperm motility [$P < 0.001$]; 8% higher acrosome integrity, [$P < 0.05$]; 9.5% higher plasma membrane integrity assessed by HOST [$P < 0.001$]). Trehalose showed a greater preservation cryoprotectant capacity than sucrose, as indicated by sperm motility after thawing (8.1% greater [$P < 0.01$]) and by the integrity of the intermediate piece (20% greater [$P < 0.05$]). From these results, we conclude that the best procedure for ram semen cryopreservation in hypertonic disaccharide-containing diluents is the addition of glycerol and trehalose after the cooling process, at 5 °C.

1. Introduction

Sperm freezing requires the use of cryoprotectants that help to prevent cell injuries caused even by the controlled decrease of temperature. Glycerol is among the most used cryoprotectants for ram semen freezing [1]. Glycerol is able to enter the spermatic cell, triggering osmotic effects [2,3] and preventing the nucleation and growth of ice crystals [4]. In this way, glycerol protects the cell during the crystallization phase [5]. The temperature at which glycerol is added has been a matter of controversy about the benefits on spermatozoa protection; some works state that a greater protection is achieved when it is added at 30 °C [6], whereas others suggest glycerol addition at 5 °C [7]. As nonpenetrating agents, trehalose and sucrose generate a hypertonic medium that produces cellular dehydration and diminishes the formation of intracellular ice [8–10].

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0093-691X/$ – see front matter © 2015 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.09.007

Trehalose also stabilizes the cellular membrane, acting at the polar heads of phospholipids and avoiding the dehydration and rehydration during the phase transitions owing to temperature changes [11]. At a molecular level, trehalose replaces the hydrogen bonds between water and the phosphate group of the phospholipid by its own hydroxyl groups [12].

To evaluate the effects produced by the cryopreservation process on sperm cell quality, several parameters based on optical microscopy imaging are currently used. However, the structural details about sperm cell or membrane integrity are better assessed using high-resolution transmission electron microscopy [13]. Söderquist et al. [14] stated that the incidence of membrane damage, assessed by electron microscopy, was similar to that depicted by the supravital fluorescence dyes. Transmission electron microscopy is the most accurate method for detecting submicroscopic damage and locating lesions after sample treatment [15]. The assessment of spermatic plasma membrane and acrosome integrity by transmission electron microscopy allows characterizing the types of ultrastructural changes at the sperm cell head. The plasma membrane is the main site for injuries owing to sperm cell freezing [16]. López Armengol et al. [17] proposed a systematic and objective method for analyzing ultrastructural features of the sperm cell head accounting for different categories of the plasma membrane status in different regions, including the acrosomal and postacrosomal regions, as well as the presence or absence of acrosomal contents in the anterior segment. Based on this methodologic approach, spermatic integrity parameters can be defined using transmission electron microscopy.

The aim of this study was to determine the most adequate temperature for the addition of glycerol and the disaccharides trehalose and sucrose during ram spermatozoa cryoprocessing, through the analysis of their morphologic and functional integrity.

2. Material and methods

All chemical reagents employed were of the highest commercially available purity. Tris was purchased from Invitrogen (Carlsbad, CA, USA); citric acid, glucose, glycerol, trehalose and sucrose were purchased from Sigma–Aldrich Co. (Saint Louis, MO, USA).

2.1. Animals, semen collection, and initial evaluation

Five adult Merino rams aged between 5 and 6 years were used; the animals were housed in open pens under uniform nutritional conditions, at Facultad de Ciencias Agrarias, Universidad Nacional del Comahue (38° 51′ S, 68° 04′ W, Río Negro, Argentina). Semen was collected using an artificial vagina during the breeding season (March to June). Animal welfare conditions were ensured in accordance with institutional statements. Immediately after collection, ejaculates were placed in a bath at 30 °C and evaluated for sperm volume, concentration, and mass motility. Only samples with appropriate concentration ($>3 \times 10^9$ cell/mL), volume (>0.3 mL), and mass motility index (>3) were included in the study.

2.2. Semen processing

After evaluation in the raw state, ejaculates were pooled to reduce individual differences [18], split into 10 equal portions, and diluted in a two-step procedure [19]. In a first step, semen was diluted to 2×10^9 cell/mL at 30 °C in a base extender containing Tris (240 mmol/L), citric acid (76 mmol/L), glucose (22 mmol/L), and egg yolk (10% v/v). According to the objective of the work, appropriate solutions containing the cryoprotectants glycerol and/or trehalose or sucrose were added to this mixture at 30 °C or after cooling at 5 °C into a programmable chamber with 1 °C/min cooling rate. In this way, final dilutions of 1×10^9 spermatozoa/mL were achieved in solutions equivalent to Salamon's diluent [20] containing 4% glycerol; Salamon's diluent plus trehalose 100 mOsm [21], and Salamon's diluent plus sucrose 100 mOsm. The combinations of these three diluents and the two temperatures of cryoprotectant addition resulted in 10 procedures described in Table 1.

After the dilution and cooling processes, the mixtures were stabilized at 5 °C for 90 minutes. Then, they were packaged in 0.25 mL straws (IMV Technologies, L'Aigle, France), frozen in liquid nitrogen vapor (−100 °C) for 10 minutes, and finally stored in liquid nitrogen (−196 °C).

Thawing was performed in 20 seconds at 37 °C. The contents of the straws were diluted 1:10 in isotonic medium Dulbecco PBS and allowed to equilibrate for 20 minutes before evaluation.

2.3. Functional and morphologic evaluation of sperm cells

Sperm cells were evaluated at three different times: Before dilution (fresh semen), before freezing (refrigerated state), and after freezing-thawing (post-thawed state). Evaluations included functional and morphologic parameters using optical microscopy, and ultrastructural integrity by transmission electron microscopy.

2.3.1. Optical microscopy

Sperm cell quality parameters were evaluated *in vitro* by means of an inverted microscope Nikon Eclipse Ti-S. Sperm cell motility [22], acrosome integrity by phase contrast

Table 1
Dilution and cooling scheme.

Treatment	Diluent	Temperature of addition (°C)	
		Glycerol	Disaccharide
G30	S	30	—
G30/T30	ST	30	30
G30/S30	SS	30	30
G5/T30	ST	5	30
G30/T5	ST	30	5
G5/S30	SS	5	30
G30/S5	SS	30	5
G5	S	5	—
G5/T5	ST	5	5
G5/S5	SS	5	5

Cryoprotectants were added at 30 °C immediately before initiating cooling or at 5 °C after cooling and stabilization at this temperature during 90 minutes.
Abbreviations: G, glycerol; T, trehalose; S, sucrose.

microscopy, plasma membrane functional integrity by hypoosmotic swelling test (HOST) [23], cell viability with eosin-nigrosin staining and 4 hours post-thawing resistance at 37 °C assessed by sperm cell motility were determined [24,25].

2.3.2. Morphologic studies by transmission electron microscopy

Aliquots of 1×10^8 frozen-thawed spermatozoa were processed for transmission electron microscopy [17], using a JEM 1200 EX II transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV. Observations were made in the same ultrathin sections for each sample, taking care not to analyze different sections from the same spermatozoon and to avoid describing the same cell repeatedly.

2.3.2.1. Analysis of sperm cell heads. For the 10 treatments, 1000 sperm cell heads were analyzed in longitudinal, transverse, and tangential sections. Three variables were determined [17]: (1) Presence/absence of plasma membrane in acrosomal region, (2) presence/absence of plasma membrane in postacrosomal region, and (3) acrosome integrity in the anterior segment (Fig. 1A, B).

2.3.2.2. Analysis of sperm tails. Intermediate piece sections ($n = 840$) of the 10 treatments were analyzed. Two categories were considered: Intact plasma membrane and altered plasma membrane (dilated or disrupted + plasma membrane absent). This variable was expressed as frequency of intermediate pieces with plasma membrane intact of the total of intermediate pieces observed (Fig. 1C–F).

2.4. Statistical analysis

The evaluation of frozen–thawed semen was performed in five independent trials, and three thawed samples were analyzed for each extender. A factorial model was applied, considering the main effects of sugar (no addition, sucrose, and trehalose), glycerol addition temperature (5 °C and 30 °C), and sugar addition temperature (5 °C and 30 °C), including in the analysis all possible interactions between factors and considering that the effects of lack of sugar cannot be crossed with sugar temperature factor. The parameters evaluated by optical microscopy were analyzed by ANOVA followed by Fisher least squares difference *post hoc* test. Analysis of membrane damage by transmission electron microscopy was assessed by a χ^2 test from absolute values. Correlations between variables of optical and transmission electron microscopy were performed by Pearson's coefficient *r*.

3. Results

The values of parameters evaluated at raw state (fresh semen), expressed as means ± standard error of the mean, were sperm motility (82.5% ± 7.5%), acrosome integrity (91.5% ± 3.5%), HOST (73.5% ± 3.5%), and cell viability (79% ± 4%).

3.1. Effects of different procedures after cooling

In the refrigerated state, cooling tolerance for the different procedures did not show differences for sperm motility, acrosome integrity, or cell viability (Table 2). In turn, cooling tolerance for HOST indicated that the absence of disaccharides led to higher membrane integrity percentages compared with the addition of nonpermeating sugars ($P < 0.001$). Cooling tolerance for plasma membrane assessed by transmission electron microscopy parameters showed results in the same trend, with greater preservation in the absence versus presence of disaccharides for plasma membrane in the acrosomal and postacrosomal regions ($P < 0.01$; Table 2). Acrosome integrity in the anterior segment and plasma membrane integrity in the intermediate piece did not show any differences between the diluents or temperature of addition of the cryoprotectants.

3.2. Effects of different procedures observed after freezing–thawing

After thawing, the parameter sperm motility showed no effects for the temperature of glycerol addition. In turn, when the temperature of disaccharide addition was considered, a highly significant improvement of about 10% to 12% in the sperm motility was observed for sugar addition at 5 °C versus 30 °C ($P < 0.001$; Fig. 2I). A highly significant improvement (8.1%) in sperm motility was

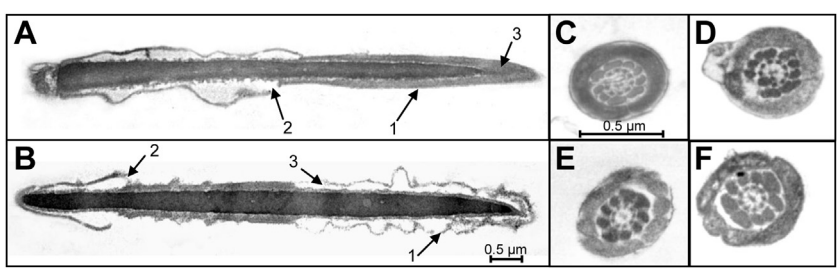

Fig. 1. Damage categories determined by transmission electron microscopy in ram spermatozoa. Longitudinal sections of sperm heads showing (A): (1) Absence of plasma membrane in the acrosomal region, (2) absence of plasma membrane in postacrosomal region, and (3) intact acrosome in the anterior segment. (B): (1) Absence of plasma membrane in the acrosomal region, (2) absence of plasma membrane in postacrosomal region, and (3) damaged acrosome with partial loss of its content. Transversal sections of intermediate pieces showing (C) intact plasma membrane, (D) dilated plasma membrane, (E) disrupted plasma membrane, and (F) absent plasma membrane.

Table 2
Cooling tolerance parameters in ram sperm diluted and cooled by different procedures.

Treatment	Parameter							
	Optical microscopy (percent values)				TEM (frequency values)			
	MOT	ACR	HOST	VIAB	PM-AR	PM-PAR	IA–AS	PM-IP
No disaccharide								
G30	65.0 ± 4.2	81.0 ± 3.5	59.6 ± 3.8[a]	56.0 ± 5.2	0.70[b]	0.70[b]	0.70	0.57
G5	67.9 ± 3.9	84.4 ± 2.9	52.0 ± 3.8[a]	61.4 ± 5.0	0.64[b]	0.63[b]	0.70	0.60
Trehalose								
G30/T30	61.4 ± 4.6	76.9 ± 2.9	43.8 ± 3.4	52.1 ± 4.1	0.54	0.48	0.63	0.68
G5/T30	61.9 ± 3.1	80.7 ± 2.6	42.0 ± 2.0	52.5 ± 2.8	0.47	0.50	0.60	0.98
G30/T5	61.3 ± 3.2	81.1 ± 2.2	42.6 ± 3.6	54.3 ± 4.0	0.47	0.34	0.62	0.72
G5/T5	64.3 ± 3.7	83.6 ± 2.8	40.3 ± 4.3	52.0 ± 4.1	0.79	0.41	0.58	0.61
Sucrose								
G30/S30	65.0 ± 3.8	79.0 ± 3.3	42.1 ± 2.4	56.0 ± 4.1	0.42	0.35	0.52	0.54
G5/S30	62.5 ± 3.9	79.9 ± 2.8	49.4 ± 3.7	57.0 ± 3.8	0.49	0.53	0.65	0.70
G30/S5	65.6 ± 4.5	82.6 ± 2.0	45.5 ± 2.4	53.5 ± 6.4	0.52	0.60	0.65	0.64
G5/S5	65.0 ± 2.7	81.4 ± 2.6	46.5 ± 3.3	56.0 ± 3.9	0.50	0.67	0.52	0.60

The different procedures are indicated as in Table 1, for glycerol (G), and trehalose (T) or sucrose (S) addition at 30 °C or 5 °C. For optical microscopy parameters sperm cell motility (MOT), acrosome integrity (ACR), hypoosmotic swelling test (HOST), and cell viability (VIAB), mean values ± standard error (SE) from five experiments and triplicate measures are shown.

[a] Significant differences for diluents without disaccharide versus sugar-containing diluents ($P < 0.001$, factorial ANOVA; Fisher's least squares difference test). Frequency values from the five experiments are shown for transmission electron microscopy (TEM) parameters presence/absence of plasma membrane in acrosomal region (PM-AR) and postacrosomal region (PM-PAR), intact acrosome in the anterior segment (IA–AS), and intact/damaged plasma membrane in the intermediate piece (PM-IP).

[b] Significant differences for diluents without disaccharide versus sugar-containing diluents, ($P < 0.01$; χ^2 test).

observed for trehalose versus sucrose or versus no disaccharide addition to diluents ($P < 0.01$); no interactions were observed in sperm motility analysis for the three factors. Acrosome integrity was not modified either by the temperature of addition of glycerol or by the presence or absence of disaccharide (Fig. 2II). The temperature for disaccharide addition, in turn, showed a greater acrosome integrity cryopreservation for 5 °C versus 30 °C ($P < 0.05$). Interaction between factors was not significant. In turn, HOST showed that glycerol addition at 5 °C was higher than 30 °C, improving the cryopreservation for respective treatments in about 5%, independent of the other factors ($P < 0.05$; Fig. 2III). The addition of disaccharide at 5 °C showed a higher HOST value than at 30 °C ($P < 0.001$). The type of disaccharide or its absence did not affect HOST values. No interactions between the three factors were observed for HOST. Sperm cell viability was not affected by glycerol addition temperature (Fig. 2IV). In turn, it was significantly improved by the treatments containing disaccharides compared with no sugar addition ($P < 0.001$). Temperature of disaccharide addition did not affect cell viability. No interactions between factors were found. The 4-hour post-thawing resistance study only showed significant effects for the temperature of disaccharide addition (Fig. 2V); addition at 5 °C caused a significantly greater post-thawing resistance than addition at 30 °C ($P < 0.001$); no significant interactions were detected.

The analysis of freeze–thawing effects studied by transmission electron microscopy showed a significantly higher frequency of intact membranes for plasma membrane in acrosomal region in disaccharide-containing treatments than in treatments containing only glycerol as cryoprotectant ($P < 0.001$; Fig. 3I). No effect was observed for temperature of addition for glycerol or disaccharide. Plasma membrane in the postacrosomal region led to greater preservation in the presence of disaccharides than without disaccharides ($P < 0.05$), and disaccharide addition at 5 °C led to a higher frequency of membrane integrity than at 30 °C ($P < 0.05$; Fig. 3II). No effects were observed for the temperature of addition of glycerol on plasma membrane in the postacrosomal region. Evaluation of acrosome integrity in the anterior segment (Fig. 3III) also showed a highly significant improvement for treatments containing disaccharides versus no addition of disaccharide ($P < 0.001$); no effects were observed for the temperature of addition of glycerol or disaccharides. The evaluation of plasma membrane integrity in the intermediate piece showed that the presence of trehalose in the diluents led to higher frequency of integrity than sucrose or no disaccharide ($P < 0.05$; Fig. 3IV). No effects of the temperature of addition of the cryoprotectants glycerol or disaccharide were observed.

The expression of the parameters, such as cooling or freezing tolerance quotients referred, to the fresh semen values to compensate sample variability [25], showed the same results as the direct percentage values (data not shown). Correlation analysis of optical and transmission electron microscopy parameters measured after freezing–thawing showed a highly significant correlation for post-thawing resistance with sperm motility ($r = 0.81$; $P < 0.01$) and with acrosome integrity ($r = 0.79$; $P < 0.01$). There was also a significant correlation between post-thawing resistance and plasma membrane in post-acrosomal region ($r = 0.63$; $P < 0.05$). Viability associated with membrane integrity was positively correlated with plasma membrane in the acrosomal region ($r = 0.73$; $P < 0.05$) and intact acrosome in the anterior segment ($r = 0.70$; $P < 0.05$), which were in turn correlated between themselves ($r = 0.73$; $P < 0.05$). Finally, plasma membrane integrity in acrosomal and postacrosomal regions showed a highly significant positive correlation ($r = 0.88$; $P < 0.001$).

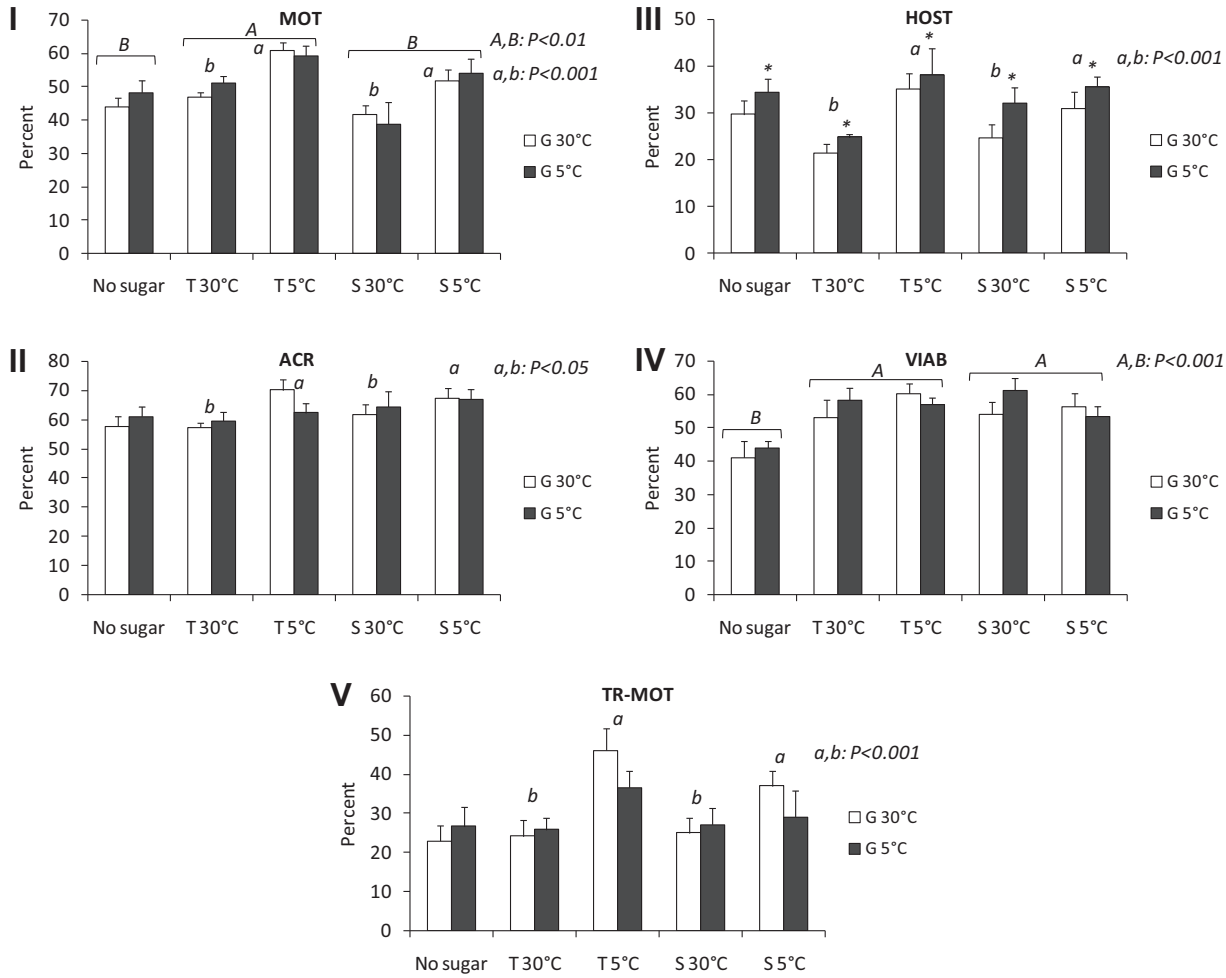

Fig. 2. Effects of different semen processing and diluents on sperm quality parameters assessed by optical microscopy after freeze-thawing. Ram semen from five males was diluted in glycerol (G) alone (no disaccharide), or the disaccharides trehalose (T) or sucrose (S), added either at 30 °C or at 5 °C (after semen cooling). Mean values ± standard error of the mean are shown from 15 doses in five replicated assays. Parameters were determined after freezing in liquid nitrogen and thawing: (I) sperm motility (MOT), (II) acrosome integrity (ACR), (III) HOST, (IV), sperm viability (VIAB), and (V), sperm motility after 4 hours. post-thawing resistance at 37 °C (TR-MOT). Significant differences determined by factorial ANOVA followed by Fisher's lowest significant differences test: *A,B*, differences within the factor "disaccharide"; *a,b*, differences for the factor "disaccharide addition temperature." *P < 0.05 for "glycerol addition temperature." No interactions between factors were observed.

4. Discussion

We evaluated in this study the effects of glycerol and disaccharides and the temperature of their addition on the cryoprotecting capacity of semen diluents. Historically, the addition of glycerol at 4 °C to 5 °C [8] was preferred by most workers, but some found no differences between its addition at either 32 °C or 3 °C or at 22 °C or 5 °C [6,7]. In this work, there were no differences between 30 °C or 5 °C for the incorporation of glycerol, except for HOST, where plasma membrane showed greater preservation if glycerol was added at 5 °C. Glycerol causes structural damage during prefreeze processing, and it has been suggested that glycerol should be added no more than 20 to 30 minutes before freezing. Effective cryoprotection after short contact (5–10 seconds) with glycerol has been demonstrated for bull and boar, and also for ram semen, which supports the earlier view that penetration of glycerol into the cell is very fast [1]. This polyalcohol causes a molecular reorganization at plasma membrane during cooling [26]; for this reason, HOST could be the parameter showing higher sensitivity to addition at 30 °C. With regard the effects of different procedures immediately after cooling, the absence of disaccharides during the refrigeration step showed a higher preservation results for the plasma membrane-related parameters (HOST, cell viability, and plasma membrane integrity in acrosomal region and postacrosomal region). These results are comparable with those found by Aguado et al. [27]. These authors compared ram semen diluted in a skim milk extender or in trehalose-containing extender, maintained at 5 °C for 24 hours, demonstrating that HOST and fertility were lower for the disaccharide-containing diluents. At 5 °C, the sperm cell metabolism does not stop completely, lipid peroxidation occurs, and the plasma

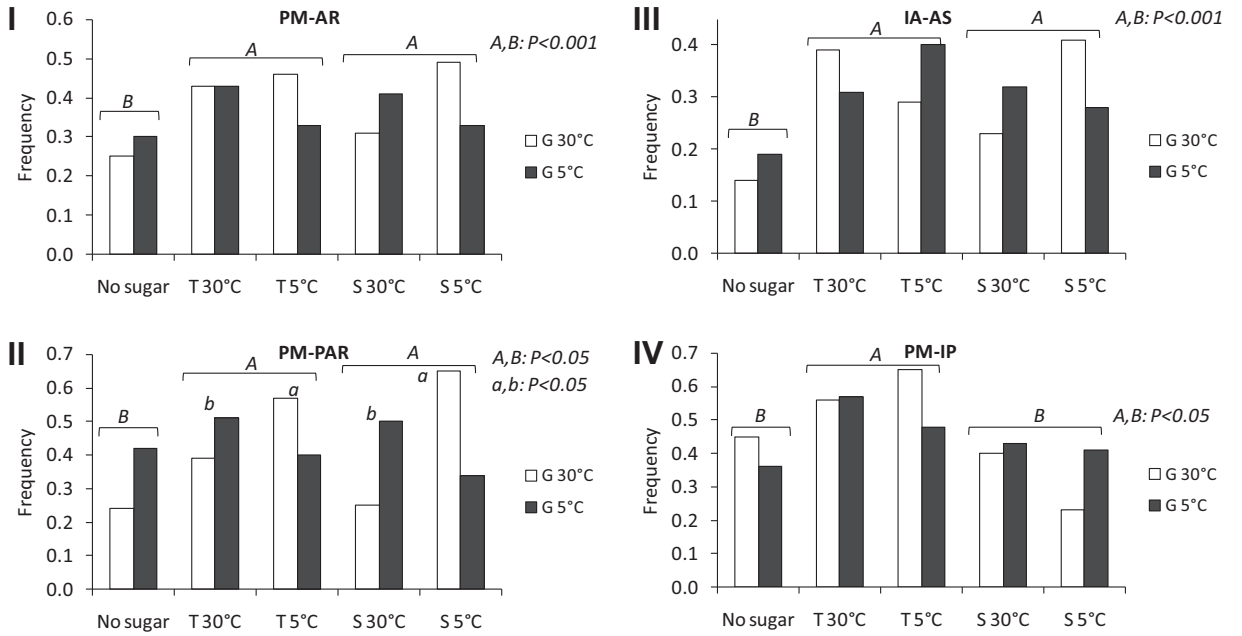

Fig. 3. Effects of different semen processing and diluents on sperm quality parameters assessed by transmission electronic microscopy after freeze-thawing. Ram semen was diluted in glycerol (G) alone (no disaccharide), or the disaccharides trehalose (T) or sucrose (S), added either at 30 °C or at 5 °C (after semen cooling). Parameters were determined after freezing in liquid nitrogen and thawing. Frequency values from 1000 observations are shown. Statistical differences determined by χ^2 frequency analysis for (I) presence of plasma membrane in acrosomal region (PM-AR), (II) presence of plasma membrane in postacrosomal region (PM-PAR), (III) intact acrosome in the anterior segment (IA–AS), and (IV) integrity of plasma membrane in the intermediate piece (PM-IP). Different capital letters *A,B* indicate differences within the factor "disaccharide"; *a,b* indicate differences for the factor "disaccharide addition temperature."

membrane shows physical and physiological modifications [28]. The osmotic regulation in spermatozoa at low temperatures may impair motility and affect membrane integrity, owing to structural damages in the lipidic bilayer of the membrane [29]. In the chilled state, cold shock affects cellular functions, causing loss of selectivity in plasma membrane and acrosome membrane permeability [30]. In this state, injuries to the plasma membrane observed in the anterior segment were expansion and disruption, whereas the acrosome injuries were swollen or exhibited acrosome reaction [22]. In this sense, the hypertonic conditions owing to the presence of disaccharides could have increased this damage. However, the inclusion of trehalose in extenders without glycerol may improve sperm motility during the cooling process [31].

On the other hand, the results described herein show that the presence of trehalose and sucrose during the freezing process improves the cell viability, plasma membrane integrity in acrosomal and postacrosomal regions, acrosome integrity in the anterior segment, and plasma membrane integrity in the intermediate piece at thawing. The addition of disaccharides in semen extenders for cryopreservation has demonstrated to be beneficial in many species such as ram [25,31,32], goat [33], bull [34], and mice [35]. Disaccharides such as trehalose and sucrose promote cell dehydration and influence the crystallization pattern of water [36], contributing to the reduction in the formation of ice crystals [9,11]. These nonpenetrating cryoprotectants increase the osmotic pressure of the extracellular fluid and hence draw water out of the spermatozoa, decreasing the risk of ice crystal formation and physical damage. However, they may exacerbate the problem of dehydration and increase in solute concentration [37]. The protection derived from the sugar increases with its concentration, provided that the total osmolarity remains under 500 mOsm [38]. In ideal cooling protocols, dehydration in hypertonic media allows sperm to support negative temperatures for long periods of time [39].

The procedures including trehalose showed higher sperm quality parameters compared with those using sucrose as cryoprotectant, when sperm motility and plasma membrane integrity in the intermediate piece were analyzed. The relation between both parameters is in accordance with the condition that the middle piece remains well preserved after freezing–thawing to fuel an adequate motility. Ram sperm can tolerate hyperosmotic diluents, and a range of sugar concentration from 50 to 100 mmol/L may be successfully incorporated in the diluents for semen cryopreservation [9]. It was demonstrated that 100 mmol/L of trehalose or raffinose significantly decreased the occurrence of sperm abnormalities, compared with other sugars [32]. Glycine-betaine, proline [31], and trehalose [24] are thought to interact directly with membrane lipids, altering their phase transition behavior and hydration state. However, these amphiphatic substances have only proved effective in the presence of glycerol and egg yolk [40].

Most researchers focused their attention on the addition of disaccharides at 30 °C [31] or at 37 °C [33,41]. In turn, in this work we observed that disaccharide addition to the diluents led to higher sperm quality parameters at 5 °C than at 30 °C, particularly for sperm motility, acrosome

integrity evaluated by optical and transmission electron microscopy, HOST, and post-thawing resistance. It is probable that disaccharide addition at 5 °C causes a lesser hyperosmotic shock than at 30 °C, because of a lesser metabolic rate in sperm cell and a shorter lapse of exposure to deleterious action. In accordance with Salamon and Maxwell [1], it was found that the main changes that occurred during liquid storage included reduction in motility and morphologic integrity of spermatozoa.

Highly significant correlations were found between some parameter responses. Some of these correlations indicate that plasma membrane-related parameters assessed by optical microscopy are coincident with ultrastructural changes observed under transmission electron microscopy in specialized domains of the plasma membrane. According to Dott et al. [42], the high correlation between cell viability and presence of plasma membrane in the head at the acrosomal and postacrosomal region indicates that the structural evaluation with eosin-nigrosin staining is positive when the plasma membrane is disrupted. Eosin staining is in fact evaluating membrane integrity, which is functionally associated with cell viability. Eosin binds to the DNA of sperm that have a disrupted postacrosomal plasma membrane [43]. Thermoresistance was logically correlated with sperm motility after thawing, but also correlated with morphologic parameters, such as acrosome integrity and the presence of plasma membrane in postacrosomal region, confirming the need of intact membranes to maintain cell viability. Other changes, in turn, are only observable under transmission electron microscopy because the alterations evaluated by optical microscopy require more generalized damage; this may be the reason why no correlations were found between other expected pairs of parameters, such as acrosome integrity evaluated by optical microscopy and presence of plasma membrane in acrosomal and postacrosomal region or an intact acrosome evaluated by transmission electron microscopy, or between plasma membrane integrity in the intermediate piece and HOST.

4.1. Conclusion

The best treatment to process the ram ejaculates for deep freezing seems to be the simultaneous addition of glycerol and disaccharide after cooling to 5 °C, using trehalose as nonpermeating sugar.

Acknowledgments

Supported by Grant 04/A 109 from the Universidad Nacional del Comahue. Valeria Pelufo holds a doctorate fellowship from Chubut Province. The authors thank Air Liquide (Neuquén, Argentina) for the liquid nitrogen supply. We thank Professor Alejandra Dabrowski for her careful revision of English style of the manuscript.

References

[1] Salamon S, Maxwell WM. Storage of ram semen. Anim Reprod Sci 2000;62:77–111.
[2] Salamon S, Maxwell WM. Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. Anim Reprod Sci 1995;37:185–249.
[3] Nauk VA, Lansberg ES, Sheikin VN. Cryogenic changes in ram spermatozoa [in Russian]. Ovtsevodstvo 1970;10:25–7.
[4] Farrant J, Lee H, Walter CA. Effects of interaction between cooling and rewarming conditions on survival of cells. In: Elliot K, Whelan J, editors. The freezing of mammalian embryos. Ciba Foundation Symposium 52 (New Series). Amsterdam: Elsevier; 1977. p. 49–67.
[5] Morris GJ, Farrant J. Interactions of cooling rate and protective additive on the survival of washed human erythrocytes frozen to −196°C. Cryobiology 1972;9:173–81.
[6] Evans G, Maxwell WM. Salamon's artificial insemination of sheep and goats. Sydney: Butterworths; 1987.
[7] Colas G. Effect of initial freezing temperature, addition of glycerol and dilution on the survival and fertilizing ability of deep-frozen ram semen. J Reprod Fertil 1975;42:277–85.
[8] Khalili B, Farshad A, Zamiri MJ, Rashidi A, Fazeli P. Effects of sucrose and trehalose on the Freezability of Markhoz goat spermatozoa. Asian-Aust J Anim Sci 2009;22:1614–9.
[9] Aisen EG, Medina VH, Venturino A. Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenology 2002;57:1801–8.
[10] Purdy PH. A review on goat sperm cryopreservation. Small Rumin Res 2006;63:215–25.
[11] Crowe JH, Carpenter JF, Crowe LM. The role of vitrification in anhydrobiosis. Annu Rev Physiol 1998;60:73–103.
[12] Tang M, Waring AJ, Mei Hong M. Trehalose-protected lipid membranes for determining membrane Protein Structure and Insertion. J Magn Reson 2007;184:222–7.
[13] Fawcett DW. The mammalian spermatozoon. Dev Biol 1975;44:394–436.
[14] Söderquist L, Madrid-Bury N, Rodríguez-Martínez H. Assessment of ram sperm membrane integrity following different thawing procedures. Theriogenology 1997;48:1115–25.
[15] Plummer JM, Watson PF. The quantitative ultrastructural assessment of head membrane damage in boar spermatozoa subjected to varying degrees of cold shock. Anim Reprod Sci 1988;16:265–75.
[16] Watson PF. Recent developments and concepts in cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995;7:871–91.
[17] López Armengol MF, Jurado SB, Pelufo V, Aisen EG. A quantitative ultramorphological approach for systematic assessment of sperm head regions. An example in rams. Cryobiology 2012;64:223–34.
[18] Gil J, Lundeheim N, Söderquist L, Rodriguez-Martinez H. Influence of extender, temperature, and addition of glycerol on post-thaw sperm parameters in ram semen. Theriogenology 2003;59:1241–55.
[19] Aisen EG, Alvarez HL, Venturino A, Garde JJ. Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology 2000;53:1053–61.
[20] Salamon S, Visser D. Effect of composition of tris-based diluent and of thawing solution on survival of ram spermatozoa frozen by the pellet method. Aust J Biol Sci 1972;25:605–18.
[21] Aisen E, Pelufo V, Malcotti V, Morello H, Medina V, Venturino A. Trehalose improves ram semen cryopreservation when it is present in conventional extenders. Reprod Dom Anim 2008;43:70.
[22] Aisen EG. Reproducción Ovina y Caprina. Buenos Aires: Intermédica; 2004.
[23] Jeyendran R, Van Der Ven H, Perez-Pelaez M, Crabo B, Zaneveld L. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other characteristics. J Reprod Fertil 1984;70:219–28.
[24] Molinia FC, Evans G, Casares PI, Maxwell WM. Effect of monosaccharide and disaccharides in Tris-based diluents on motility, acrosome integrity and fertility of pellet frozen ram spermatozoa. Anim Reprod Sci 1994;36:113–22.
[25] Aisen E, Alvarez H, Venturino A, Larreguy D, Garde JJ, Vazquez I. Efecto comparativo de diluyo-conservadores de diferente composición y tonicidad sobre la criopreservación del semen ovino. Invest Agr Prod Sanid Anim 1995;10:223–31.
[26] Hammerstedt RH, Graham JK. Cryopreservation of poultry sperm: the enigma of glycerol. Cryobiology 1992;29:26–38.
[27] Aguado MJ, García-Cervigón M, Manso A, Pérez-Guzmán MD, Garde J, Montoro V. Estudio preliminar del poder fecundante del semen de ovino manchego mantenido durante 24 horas en refrigeración. Producción Ovina y Caprina 1998;23:521–4.
[28] Maxwell WM, Stojanov T. Liquid storage of ram semen in the absence or presence of some antioxidants. Reprod Fertil Dev 1996;8:1013–20.

[29] Meyers SA. Spermatozoal response to osmotic stress. Anim Reprod Sci 2005;89:57–64.
[30] Medeiros CM, Forell F, Oliveira ATD, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? Theriogenology 2002; 57:327–44.
[31] Sánchez-Partida LG, Maxwell WM, Paleg LG, Setchell BP. Proline and glycine betaine in cryoprotective diluents for ram spermatozoa. Reprod Fertil Dev 1992;4:113–8.
[32] Jafaroghli M, Khalili B, Farshad A, Zamiri MJ. The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. Small Rumin Res 2011;96:58–63.
[33] Aboagla EM, Terada T. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol Reprod 2003;69:1245–50.
[34] Foote RH, Chen Y, Brockett CC, Kaproth MT. Fertility of bull spermatozoa frozen in whole milk extender with trehalose, taurine, or blood serum. J Dairy Sci 1993;76:1908–13.
[35] Storey BT, Noiles EE, Thompson KA. Comparison of glycerol, other polyols, trehalose, and raffinose to provide a defined cryoprotectant medium for mouse sperm cryopreservation. Cryobiology 1998;37: 46–58.
[36] Nicolajsen H, Hvidt A. Phase behaviour of the system trehalose–NaCl–water. Cryobiology 1994;31:199–205.
[37] Steinmann H. Use of trehalose as a cryoprotectant in the freezing of stallion semen. A laboratory study. Germany: Thesis, Tierärztliche Hochschule Hannover; 1996.
[38] Koshimoto C, Mazur P. The effect of the osmolality of sugar-containing media, the type of sugar, and the mass and molar concentration of sugar on the survival of frozen-thawed mouse sperm. Cryobiology 2002;45:80–90.
[39] Woelders H. Fundamentals and recent development in cryopreservation of bull and boar semen. Vet Q 1997;19:135–8.
[40] Holt W. Fundamental aspects of sperm cryobiology: the importance of species and individual differences. Theriogenology 2000; 53:47–58.
[41] Najafi A, Zhandi M, Towhidi A, Sharafi M, Sharif AA, Motlagh MK, et al. Trehalose and glycerol have a dose-dependent synergistic effect 3 on the post-thawing quality of ram semen cryopreserved in a soybean 4 lecithin-based extender. Cryobiology 2013;66: 275–82.
[42] Dott HM, Foster GC. A technique for studying the morphology of mammalian spermatozoa which are eosinophilic in a differential 'live/dead' stain. J Reprod Fertil 1972;29:443–5.
[43] Foster ML, Love CC, Varner DD, Brinsko SP, Hinrichs K, Teague S, et al. Comparison of methods for assessing integrity of equine sperm membranes. Theriogenology 2011;76:334–41.