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Two-step accelerating freezing protocol yields a better motility, membranes and DNA integrities of thawed ram sperm than three-steps freezing protocols

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ABSTRACT

The present study compares a protocol that mimics freezing of ram semen in static nitrogen vapor with two protocols with an initial low cooling rate in the first step, followed by higher cooling rates where ice nucleation occurs. Semen ejaculates, obtained from twelve adults rams, were diluted with TEST-based extender and frozen with either Protocol 1 (three-step decelerating cooling): from +5 °C to −35 °C (40 °C/min), from −35 °C to −65 °C (17 °C/min), and then from −65 °C to −85 °C (3 °C/min); or Protocol 2 (three-step accelerating cooling): from +5 °C to −5 °C (4 °C/min), from −5 °C to −110 °C (25 °C/min), and then from −110 °C to −140 °C (35 °C/min); or Protocol 3 (two-step accelerating cooling), from +5 °C to −10 °C (5 °C/min), and then from −10 °C to −130 °C (60 °C/min). Post-thaw sperm quality was reduced for all protocols ($p < .05$) compared with fresh semen. Post-thaw percentages of sperm motility characteristics and sperm with intact plasma membrane, intact acrosome, and intact mitochondrial membrane were greater using Protocol 3 than Protocol 2 ($p < .05$) and Protocol 1 ($p < .01$). In addition, the post-thaw percentage of sperm with fragmented DNA was lower ($p < .05$) using Protocol 3 compared with Protocol 1. The present results indicate that a cooling rate of 60 °C/min around and after the time point of ice nucleation provided better post thaw survival and function of ram sperm than lower (and/or decelerating) cooling rates.

1. Introduction

Cryopreservation of semen is used in order to facilitate long-term storage and transportation. This is of special importance for use in artificial insemination of domestic animals, including sheep. Ram sperm cells are susceptible to various stresses during cryopreservation [1], and ultrastructural, biochemical and functional damage occurs in many ram sperm cells during the freezing-thawing process [28]. Usually no more than 50% of sperm cells survive cryopreservation, providing low fertility rates [39,40]. In addition to cell death, the remaining survived sperm cells may have damaged sperm organelles and membranes and the biological efficiency reduced (e.g., sperm capacitation and acrosome reaction) [4]. Cryoinjury during freeze-thawing, can be caused by factors such as thermal shock, ice formation, dehydration, increased salt concentration and osmotic shock [29,42].

Studies on freezing methods for ram semen have considered factors such as the base-diluents [22,24,36], methods of addition and concentration of cryoprotectant agents [10,21,40], and freezing rates [3,8,11,26] to achieve high quality sperm after thawing [35].

The cooling rate is important for sperm survival. As extracellular ice formation takes place, the cells and the dissolved salts are excluded from the ice and become concentrated in the 'unfrozen fraction' remaining between the growing ice masses. Therefore, the osmotic strength of the unfrozen fraction increases, causing an efflux of water from the cells, resulting in cell shrinkage. Both high and low cooling rates are detrimental to the cell [30]. Higher cooling rate induces intracellular ice formation [23,31,44], while too low cooling rate may cause excessive dehydration of the cell and the cells membranes are exposed for longer

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periods to the pockets of hypertonic solutions, with possible deleterious effects such as protein and lipid extraction and the generation of reactive oxygen species (ROS) [25]. The velocity at which ice forms during sperm cryopreservation is strongly dependent on the freezing protocol. Extracellular ice or changes in the composition of the external solution brought about by the conversion of water into the ice are believed to be a cause of sperm damage during the cryopreservation process [29]. Also, the size and shape of ice crystals and ice masses depend on the cooling rate [5]. Thus, the optimal cooling rate has to be regarded as a compromise between these opposing effects mentioned above to achieve minimal cryoinjury and maximize viability and fertility of sperm samples after thawing. Fiser and Fairfull (1984) [17] reported a comprehensive study on the effect of cooling rates and the interaction of cooling rate and glycerol concentration on survival and intactness of ram spermatozoa. More recent studies on the optimal cooling rate for ram semen were reviewed by Dalal et al. (2018) [9]. However, these studies have not addressed different cooling rates in the separate phases of the freezing program, most notably the phases prior to and after ice nucleation.

The cooling rate inside the sample that is frozen is not constant due to the release of latent heat of fusion. Thus, the freezing rate inside the straw may be different than expected from the programmable biological freezer, due to the heat generated by ice formation. Indeed, the cooling rate right after the dissipation of heat of fusion may be much higher than the programmed cooling rate [43].

In static liquid nitrogen (LN_2) vapor freezing methods, quite high cooling rate in the freezing phase after ice nucleation can be perfectly obtained, if required, by choosing a small distance between the straws and the surface of LN_2 [12]. However, the cooling rate of the cooling phase from the holding temperature (+5 °C) until ice nucleation (<−20 °C) will then also be high, which could cause damage due to 'cold shock'. Indeed, traditional freezing of ram semen in static LN_2 vapor provides a relatively high initial cooling rate prior to ice nucleation, while the cooling rate after ice nucleation decreases at lower temperatures [2,15]. Previous reports suggested that the use of static LN_2 vapors to freezing ram sperm was more harmful to sperm integrity and functionality than controlled freezing methods with the low initial cooling rate (e.g., 0.5–5 °C/min) in the temperature range where the ice nucleation occurs [3,38]. In contrast, many protocols provide a low initial cooling rate in the phase prior to ice nucleation (e.g., Refs. [15,32]), which may help prevent cold shock. The study by Esteso et al. (2018) [15] indeed showed better post thaw sperm quality of Iberian ibex semen frozen with a controlled rate freezer with a low cooling rate between +5 an −5 °C, followed by a higher cooling rate, than semen frozen in static LN_2 vapor at similar overall cooling rates.

Therefore, the aim of the present study was to compare three different freezing protocols in a controlled-rate freezer, of which Protocol 1 mimics the static LN_2 vapor freezing method [15], featuring a relatively high cooling rate in the phase prior to ice nucleation. In contrast, protocols 2 and 3 had a low cooling rate in the first cooling phase, in order to minimize cold chock, followed by higher programmed cooling rates in the temperature-range in which ice nucleation and ice growth occur (−25 and −60 °C/min in Protocols 2 and 3, respectively).

2. Materials and methods

2.1. Animal, semen collection and initial evaluation

Semen was collected over October to November from 4 to 6 year-old twelve adult Merino ram males housed at INIA Department of Animal Reproduction (Madrid, 40° 25′N). All animals were fed with grain, barley straw and dry alfalfa supplements. Water, vitamins and mineral blocks were available ad libitum. All animals were handled according to procedures approved by the INIA Ethics Committee, and the research was performed in accordance with the Spanish Policy for Animal Protection (RD53/2013), which conforms to European Union Directive 86/609 regarding the protection of animals used in scientific experiments.

A total of ninety-eight semen ejaculates were collected in nine collection session carried out weekly using an artificial vagina. The rams were previously trained with an intact oestrus-induced ewe and then an ovariectomized female was used as a "teaser" to stimulate ram sexual behaviors. The volume of each ejaculate was measured in a graduated conical glass tube in milliliters (mL). Briefly, after collection, the raw semen was diluted 1:1 (v/v) with Tris/Tes/Glucose (TTG) solution (210.6 mM Tes, 95.75 mM Tris, 10.09 mM Glucose, 0.54 mM Streptomycin, and 2.14 mM Penicillin; 324 mOsm/kg, pH 7.1) pre-warmed at 37 °C. All the materials, including the artificial vagina and glass collecting tube were maintained at 37 °C before collections. These fresh-extended semen samples with TTG solution were transported to the research laboratory immediately after collection at 37 °C and initial motility and status of sperm membranes were evaluated prior to processing (see below). Sperm concentration was determined using a photometer (SDM 1, Minitube, Germany). Those ejaculates with a volume of 0.75–2 mL, a sperm motility higher than 70%, a score higher than 3 (mass motility scale of 0–5), and a sperm concentration higher than 3.5×10^9 sperm/mL were used in the subsequent experimental work.

2.2. Processing and freezing procedures

The fresh-extended semen samples of each semen ejaculate were mixed with TEST-based extender [freezing medium composed of TTG solution, 6% egg yolk (v/v), and 5% glycerol (v/v)] at room temperature, to reach final concentration of 100×10^6 sperm/mL (4.8% glycerol final concentration). Semen samples were then placed in a beaker with 30 mL of water at room temperature, and maintained at this temperature for 5 min before transfer to a refrigerator at 5 °C where they were maintained for a further 3 h (1 h of cooling time plus a further 2 h of holding time). Aliquots of these samples where then loaded into 0.25 mL French straws (IMV, L'Aigle, France). All straws were filled with a volume of approximately 220 μL and a small air bubble of approximately 10 μL inside and finally sealed. Thereafter, the straws were frozen using a programmable biological freezer (Computer Freezer-IceCube 1810, Minitüb, Tiefenbach, Germany). A total of 236 straws were frozen using the following three freezing protocols (Fig. 1):

Protocol 1 (n = 78 straws from 36 semen ejaculates of 12 males): Three-step decelerating cooling rate (similar to conventional freezing in static LN_2 vapor [15]): from +5 °C to −35 °C at 40 °C/min, from −35 °C to −65 °C at 17 °C/min, from −65 °C to −85 °C at 3 °C/min, and then transfer into LN_2 to cool to −196 °C.

Protocol 2 (n = 79 straws, from 31 semen ejaculates of 12 males): Three-step accelerating cooling rate: from +5 °C to −5 °C at 4 °C/min, from −5 °C to −110 °C at 25 °C/min, from −110 °C to −140 °C at 35 °C/min, and then transfer into LN_2 to cool to −196 °C.

Protocol 3 (n = 79 straws, from 31 semen ejaculates of 12 males): Two-step accelerating cooling rate: from +5 °C to −10 °C at 5 °C/min, from −10 °C to −130 °C at 60 °C/min, and then transfer into LN_2 to cool to −196 °C.

The temperatures of the freezing chamber (T_{ch}) and inside the straws (T_{in}) were assessed. For the latter purpose, the thermocouple (1.5 mm diameter) of the biological freezer (temperature sensor of sample) was introduced to a dummy straw (1.6 mm diameter, IMV, France) containing freezing medium (TEST). When the temperature inside the straws approached the fixed temperature of the freezing chamber (5 °C), each protocol was started (time 'zero'). Consequently, the thermocouple inside the dummy straw allows to register the occurrence of ice nucleation and to roughly estimate the duration of the subsequent dissipation of the latent heat of fusion from each freezing protocol (Fig. 2).

Both straws freezing process and semen collection session procedures from each Protocol were carried out weekly. All frozen sperm samples were thawed after three months by placing the straws in a water bath at 37 °C for 30 s. The contents were poured into dry Eppendorf tubes and

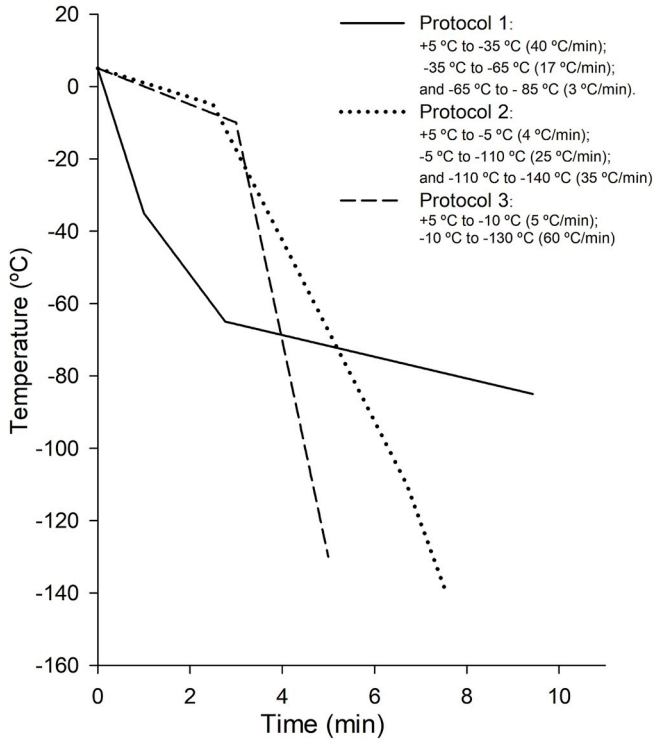

Fig. 1. Three programmable freezing protocols for the cryopreservation of ram semen using two- or three-step controlled cooling rates.

incubated for 5 min at 37 °C. Sperm motility, status of sperm membranes and DNA fragmentation were subsequently evaluated.

2.3. Sperm analysis

Sperm motility analysis was assessed using a CASA system running Sperm Class Analyzer® 1999 v.4.0 software (Microptic S.L., Barcelona, Spain) coupled to a Nikon Eclipse model 50i phase contrast microscope (negative contrast) as previously described by Galarza et al. (2018) [19]. Values were recorded for the percentage of sperm motility (SM), percentage of progressive sperm motility (PSM), straight line velocity (VSL, μm/s), linearity (%LIN), straightness (%STR), and the amplitude of lateral head displacement (ALH, μm).

Plasma, acrosome and mitochondrial membrane status were assessed using a triple association of fluorescent probes - propidium iodide (PI, Sigma P4170), fluorescein isothiocyanate-conjugated peanut *(Arachis hypogaea)* agglutinin (PNA-FITC, Sigma L7381), and Mitotracker Green *FM*® (MITO, Invitrogen M7514) - according to Galarza et al. (2019) [20]. A total of 200 sperm cells per slide were examined using a Nikon Eclipse E200 epifluorescence light microscope (Nikon Instruments Inc,. New York, NY, USA) with a triple-band pass filter (40 × magnification with an excitation: 450–490 nm, and emission: 520 nm) and eight subpopulations of cells quantified, i.e., those showing in percentages: (1) intact plasma membrane/intact acrosome/intact mitochondrial membrane (IPIAIM); (2) intact plasma membrane/intact acrosome/damaged mitochondrial membrane (IPIADM); (3) intact plasma membrane/damaged acrosome/intact mitochondrial membrane (IPDAIM); (4) intact plasma membrane/damaged acrosome/damaged mitochondrial membrane (IPDADM); (5) damaged plasma membrane/intact acrosome/intact mitochondrial membrane (DPIAIM); (6) damaged plasma membrane/intact acrosome/damaged mitochondrial membrane (DPIADM); (7) damaged plasma membrane/damaged acrosome/intact mitochondrial membrane (DPDAIM); and (8) damaged plasma membrane/damaged acrosome/damaged mitochondrial membrane (DPDADM). In addition, the total percentage of cells presenting an intact plasma membrane (IPM: IPIAIM + IPIADM + IPDAIM + IPDADM), intact acrosomal membrane (IAM: IPIAIM + IPIADM + DPIAIM + DPIADM), and intact mitochondrial membrane (IMM: IPIAIM + IPDAIM + DPIAIM + DPDAIM) were calculated.

DNA fragmentation of frozen-thawed ram sperm was assessed by TUNEL assay. For this purpose, we used the In Situ Cell Death Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol with few modifications. In brief, after thawing semen samples were fixed in 4% formaldehyde (v/v) in PBS solution at a

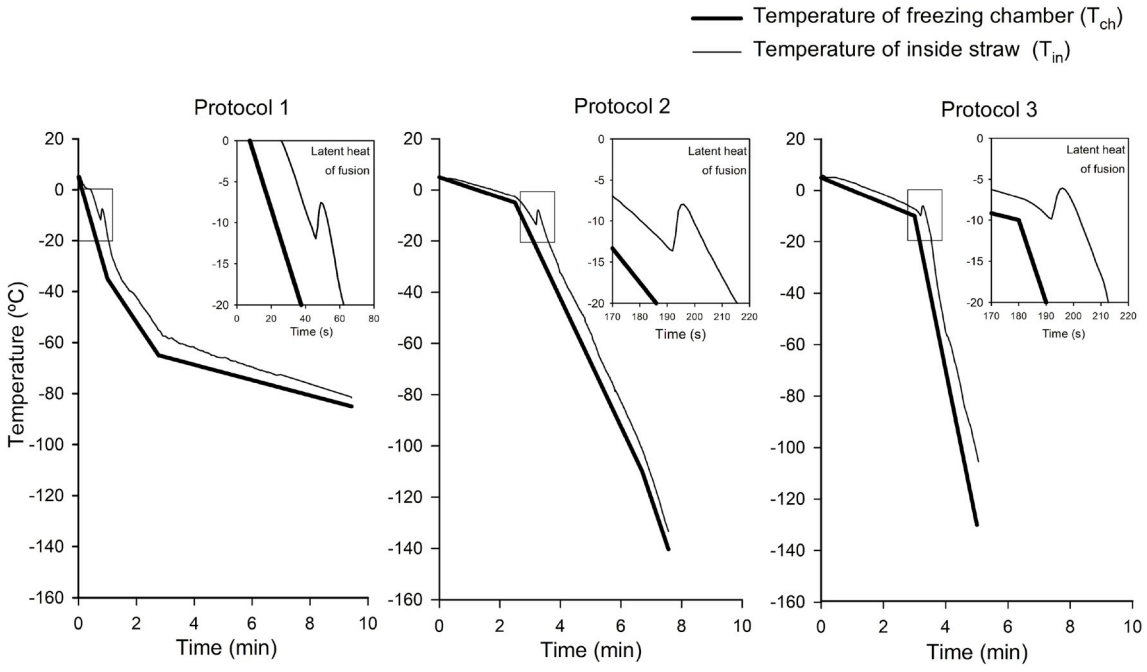

Fig. 2. Time courses of programmed temperature in the freezing chamber (T_{ch}, thick line) and typical examples of time courses of measured temperature inside the straws (T_{in}, thin line) in the different freezing protocols. The duration time (s) and temperature at which latent heat of fusion (ice nucleation) occurs inside straws are shown in the small box (thin line) of each freezing protocol.

concentration of 5×10^6 sperm/mL for 30 min. Then 10 μL of each samples were placed on a glass slide previously demarcated by Liquid-repellent slide marked pen for staining procedures and leave to dry on a thermic plate set at 37 °C. The slides were permeabilized with 0.1% Triton X-100 (v/v) (Sigma X100) for 5 min in a humidified chamber at room temperature and washed with PBS. Subsequently, the slides were incubated for 1 h in a humidified chamber in the dark at 37 °C with the TUNEL reaction mixture, which contained terminal deoxynucleotidyl transferase (TdT) plus TMR-Red label. After labeling, samples were washed with PBS, stained with Hoechst 33342 (1 mg/mL), mounted with Fluoromount aqueous mounting medium (Sigma F4680) and sealed with nail polish. Then, the samples were analyzed immediately by Nikon Eclipse E200 epifluorescence microscope [40 × magnification, with an excitation: 510–560 nm and emission: 590 nm for TUNEL; and an excitation: 330–380, and emission: 420 nm for Hoechst]. As positive control for TUNEL (TUNEL +), sperm were treated with DNase I, 1 mg/ml, for 60 min at room temperature, fixed on the slides and then treated with TUNEL reaction mixture. In each sample at least 200 sperm were evaluated. Each microscopic field was evaluated first under UV-light to determine the total number of sperm and then under green-light to determine the positive TUNEL sperm.

2.4. Statistical analysis

The results are presented as mean ± SEM. All statistical analyses were performed using Statistica software for windows v.12 (StatSoft Inc. Tulsa, OK, USA). Sperm variables that showed non-normal distributions, as determined by Shapiro-Wilk test, were transformed to *arcsine* (percentages values) or log*10* (numeric values) before analysis. The effects of cooling rate protocols on post-thawed sperm quality were then compared by one-way ANOVA using the General Linear Model procedure. In addition, the male factor as covariable was included in this analysis due to variability between some rams. When ANOVA revealed a significant effect, the values were compared by Tukey's multiple range test. Significance was set at $p < .05$.

3. Results

Typical examples of temperature time courses of the freezing protocols are showed in Fig. 2. Ice nucleation occurred in the first ramp in Protocol 1 and in the second ramp in Protocols 2 and 3, at registered T_{in} of approximately −10 to −14 °C. The onset of ice nucleation occurred at 47 s (at −11,95 °C in first-step to 40 °C/min), 193 s (at −13.63 °C in second-step to 25 °C/min), and 192 s (at −9,86 °C in second-step to 60 °C/min) for protocols, 1, 2 and 3, respectively. In addition, the apparent durations of the dissipation of heat of fusion (here defined as the time between ice nucleation and return to the same T_{in}) were 8 s, 13 s, and 10 s, for protocols, 1, 2, and 3, respectively.

After freezing and thawing, significant reductions were recorded for all sperm quality variables in frozen, compared with fresh sperm, for all freezing protocols used. Pre-freeze and post-thaw values for the sperm motility variables are shown in Table 1. Both SM and PSM percentages were greater ($p < .05$) after freezing with Protocol 3 than Protocol 1 and 2. Moreover, the VSL value was greater ($p < .05$) after freezing with Protocol 3 than Protocol 1.

Data for status of sperm membranes and DNA fragmentation are included in Table 2 and Fig. 3. The cryopreservation process affected sperm plasma membranes and mitochondrial membranes, but the percentage of sperm affected was lower with Protocol 3 followed by Protocol 2, and Protocol 1 was the most deleterious (Table 2 and Fig. 3). In addition, the percentage of sperm with DNA damage after thawing was lower ($p < .05$) with Protocol 3 than with Protocol 1 (Table 2).

Indeed, after cryopreservation, the percentage of sperm with intact plasma, acrosome and mitochondrial membranes (IPIAIM) was greater using Protocol 3 than Protocol 2 ($p < .05$) and Protocol 1 ($p < .01$). Protocol 2 showed a higher ($p < .05$) percentage of IPIAIM than Protocol 1. Lower ($p < .05$) percentages of sperm with damaged plasma membranes were observed after freezing with protocol 3 compared with protocols 1 and 2 in each of the following categories: sperm with damaged plasma membrane, intact acrosome and mitochondrial membrane (DPIAIM), sperm with damaged plasma membrane, intact acrosome and damaged mitochondrial membrane (DPIADM), and sperm with damaged plasma and acrosome membranes, and intact mitochondrial membrane (DPDAIM). Moreover, total values of sperm with intact plasma membrane (IPM, $p < .01$), intact acrosome membrane (IAM, $p < .01$) and intact mitochondrial membrane (IMM, $p < .05$) were greater with Protocol 3 than Protocol 2 and 1 (Fig. 3).

Table 1
Sperm motility variables assessed by CASA, of ram semen before (fresh) and after freezing/thawing using three different freezing protocols.

Sperm parameters	Fresh samples (n = 98)	Protocol 1 (n = 78)	Protocol 2 (n = 79)	Protocol 3 (n = 79)
SM (%)	87.8 ± 0.8^{a}	44.5 ± 1.9^{c}	47.9 ± 2.0^{c}	61.4 ± 1.9^{b}
PSM (%)	34.8 ± 1.0^{a}	18.0 ± 1.0^{c}	20.8 ± 1.1^{c}	27.2 ± 1.0^{b}
VSL (μm/s)	85.1 ± 1.5^{a}	55.1 ± 2.0^{c}	59.2 ± 1.7^{bc}	62.8 ± 2.1^{b}
LIN (%)	51.1 ± 0.7^{b}	63.3 ± 0.8^{a}	63.5 ± 0.8^{a}	63.5 ± 0.7^{a}
STR (%)	66.5 ± 0.7^{b}	75.1 ± 0.7^{a}	76.1 ± 0.6^{a}	75.1 ± 0.5^{a}
ALH (μm)	4.7 ± 0.1^{a}	2.5 ± 0.1^{b}	2.7 ± 0.1^{b}	2.6 ± 0.1^{b}

Data are mean ± SEM. Different superscripts within a same row differ significantly ($p < .05$ for $^{a–b}$, and $^{b–c}$; and $p < .01$ for $^{a–c}$). SM: total sperm motility; PSM: progressive sperm motility; VSL: straight line velocity; LIN: linearity index; STR: straightness; and ALH: amplitude of lateral head displacement.

Table 2
Percentages of sperm in the various categories of membrane integrity (assessed with fluorescent markers PI/PNA-FITC/MITO) and percentage of sperm with fragmented DNA (TUNEL +), in ram semen before (fresh) and after freezing/thawing using three different freezing protocols.

Fluorescence parameters	Fresh samples (n = 98)	Protocol 1 (n = 78)	Protocol 2 (n = 79)	Protocol 3 (n = 79)
IPIAIM (%)	74.0 ± 0.6^{a}	41.7 ± 1.3^{d}	45.8 ± 1.5^{c}	58.4 ± 1.1^{b}
IPIADM (%)	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
IPDAIM (%)	1.7 ± 0.2	1.7 ± 0.2	1.5 ± 0.2	1.1 ± 0.2
IPDADM (%)	0.0 ± 0.0	0.04 ± 0.0	0.0 ± 0.0	0.01 ± 0.0
DPIAIM (%)	13.3 ± 0.6^{c}	18.1 ± 0.6^{a}	15.5 ± 0.6^{b}	11.8 ± 0.4^{c}
DPIADM (%)	6.5 ± 0.4^{c}	11.8 ± 0.5^{a}	10.6 ± 0.6^{a}	8.4 ± 0.4^{b}
DPDAIM (%)	3.3 ± 0.3^{c}	22.9 ± 0.9^{a}	22.3 ± 1.1^{a}	16.4 ± 0.8^{b}
DPDADM (%)	0.2 ± 0.1^{b}	3.6 ± 0.4^{a}	4.2 ± 0.5^{a}	3.1 ± 0.3^{a}
TUNEL+ (%)	-	7.1 ± 1.2^{a}	5.4 ± 1.1^{ac}	2.3 ± 0.5^{c}

Data are mean ± SEM. Different superscripts within a same row differ significantly ($p < .05$ for $^{a–b}$, and $^{b–c}$; $p < .01$ for $^{a–c}$; and $p < .001$ for $^{a–d}$). IPIAIM: intact plasma membrane/intact acrosome/intact mitochondrial membrane; IPIADM: intact plasma membrane/intact acrosome/damaged mitochondrial membrane; IPDAIM: intact plasma membrane/damage acrosome/intact mitochondrial membrane; IPDADM: intact plasma membrane/damaged acrosome/damaged mitochondrial membrane; DPIAIM: damage plasma membrane/intact acrosome/intact mitochondrial membrane; DPIADM: damaged plasma membrane/intact acrosome/damaged mitochondrial membrane; DPDAIM: damaged plasma membrane/damage acrosome/intact mitochondrial membrane; DPDADM: damaged plasma membrane/damaged acrosome/damaged mitochondrial membrane.

4. Discussion

The results of the present study clearly showed that Protocol 3 was less harmful to ram sperm cells than Protocols 1 and 2, yielding higher values for motility variables, integrity of plasma, acrosome and mitochondrial membranes, and a lower percentage of DNA damage.

The Protocol 3 demonstrated the lowest cryoinjury response which may be attributed to the second-step cooling rate (from −10 to −130 °C at 60 °C/min) that could be sufficiently rapid to minimize exposure to the deleterious effects of unfrozen, extracellular, hypertonic solutions

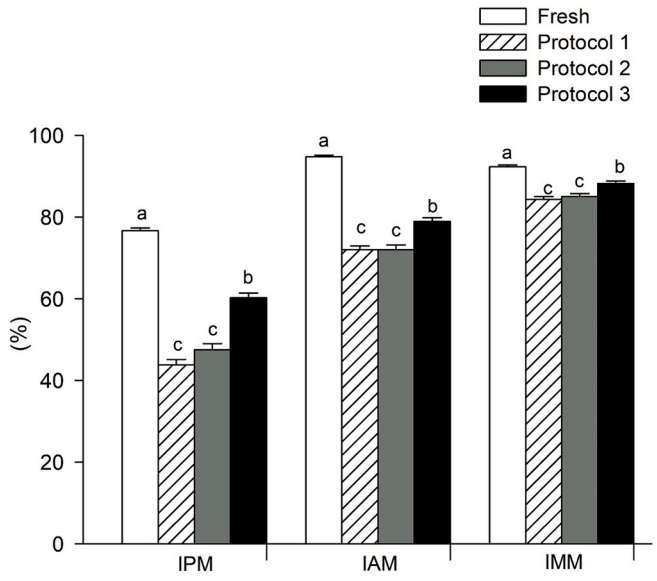

Fig. 3. Percentage of sperm with intact plasma membrane (IPM), intact acrosome (IAM) and intact mitochondrial membrane (IMM) in fresh (n = 98) and in frozen-thawed sperm using three freezing protocols: Protocol 1 (n = 78), Protocol 2 (n = 79), Protocol 3 (n = 79). See main text for details of the three freezing protocols.

during this critical temperature range and ice growth occurs.

Different semen freezing rates have been studied in rams and other ruminant species with variable results. Kumar et al. (2003) [26] recommended a controlled linear cooling rate for ram semen in the temperature range of ice nucleation and ice formation (−5 to −25 °C) of 30 °C/min. Also Fang et al. (2016) [16] and Vichas et al. (2018) [38] found relatively low optimal cooling rates (25–40 °C/min) for ram sperm. However, Fiser and Fairfull (1984) [17] in an elaborate study, as well as Duncan and Watson (1992) [13] found that higher cooling rates were permissible or better for ram semen. Similarly, for bull semen, it was reported that optimal cooling rates used may be as high as 76–140 °C/min [42].

As explained in chapter Introduction, in traditional static LN_2 vapor freezing, cooling rate, after the dissipation of heat of fusion, may decrease already at still relatively high subzero temperatures. This is the opposite of what may be considered optimal, as Woelders and Chaveiro (2004) [43] argued on the basis of mathematical simulations that cooling rates after ice nucleation can and probably should accelerate. In addition to a decelerating cooling rate, static vapor freezing features a rapid cooling in the phase prior to ice nucleation, which may lead to 'cold shock' damage. Indeed, a number of studies have shown that static vapor freezing of ram semen gave poorer results than controlled rate methods [2,3,38]. Our present study confirms these observations, as post-thaw sperm quality appeared lowest in Protocol 1, which protocol had characteristics of freezing in static LN_2 vapor, but with the better control of a controlled rate freezer.

The factor that sets Protocol 3 apart from Protocols 2 and 1 is its relatively high cooling rate (60 °C/min) after the dissipation of heat of fusion until straws were plunged at −130 °C. This shortens the time it takes before the cells are stabilized by reaching the glass transition (but only little compared with Protocol 2). But, perhaps more importantly, cells may become less strongly shrunken and dehydrated. Moreover, even though the total fraction of ice formed only depends on the subzero temperature, the higher cooling rate may also influence forms and shapes of ice crystals and ice masses [5,7] and therewith possibly the mechanical stresses the cells endure [14].

Freezing and thawing can also affect sperm functionality by alteration of DNA integrity [32] and disruption of mitochondrial membrane function [33]. Damage of the mitochondria can cause a decreased oxidative phosphorylation (ATP synthesis) [41], which can affect sperm motility [6] and sperm motility kinetic variables [3]. Previous reports demonstrated a positive correlation between the mitochondrial function and motility of thawed bull sperm, both after freezing in static nitrogen vapor and after a two-step protocol with an accelerating freezing rate [18]. The results of the present study showed that values of motility variables, the percentage of sperm with intact plasma, acrosome and mitochondrial membranes were greater for semen frozen with Protocol 3 than with Protocols 2 and 1. Even sperm subpopulation with damaged plasmalemma and intact acrosome showed the same percentage of sperm with intact mitochondrial membrane than fresh samples when Protocol 3 is used. Moreover, Protocol 3 showed less cryoinjury of sperm with damaged plasmalemma and acrosome, and intact mitochondrial membrane than Protocols 2 and 1. These findings suggest that Protocol 3 produced less damage to mitochondrial membranes allowing better motility than the other protocols.

Cryopreservation increases apoptosis-like manifestations including mitochondrial membrane potential, caspase activation, membrane permeability and phosphatidylserine externalization [27]. It is suggested that sperm DNA fragmentation is associated with an increase in oxidative stress and ROS production during cryopreservation [37]. Said et al. (2010) [34] proposed that an alteration in the mitochondrial membrane fluidity occurs during cryopreservation, which then would lead to a rise in mitochondrial membrane potential and the release of ROS that cause DNA damage in sperm.

In conclusion, the results of the current study indicate that a cooling rate of 60 °C/min around and after the time point of ice nucleation provided better post thaw survival and function of ram sperm than lower and/or decelerating cooling rates. In addition, the results suggest that a rapid cooling in the initial phase of the protocol (i.e. from the holding temperature of +5 °C to just above ice nucleation temperature) may not be beneficial.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2019.10.007.

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