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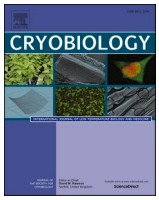

Preserving frozen stallion sperm on dry ice using polymers that modulate ice crystalization kinetics

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ABSTRACT

Cryopreserved semen is routinely shipped in liquid nitrogen. Dry ice could serve as an alternative coolant, however, frozen storage above liquid nitrogen temperatures (LN2, −196 °C) may negatively affect shelf-life and cryosurvival. In this study, we determined critical temperatures for storage of cryopreserved stallion sperm. We evaluated: (i) effects of cooling samples to different subzero temperatures (−10 °C to −80 °C) prior to storing in LN2, (ii) stability at different storage temperatures (i.e., in LN2, dry ice, −80 °C and −20 °C freezers, 5 °C refrigerator), and (iii) sperm cryosurvival during storage on dry ice (i.e., when kept below −70 °C and during warming). Furthermore, (iv) we analyzed if addition of synthetic polymers (PVP-40, Ficoll-70) modulates ice crystallization kinetics and improves stability of cryopreserved specimens. Sperm motility and membrane intactness were taken as measures of cryosurvival, and an artificial insemination trial was performed to confirm fertilizing capacity. We found that adding PVP-40 or Ficoll-70 to formulations containing glycerol reduced ice crystal sizes and growth during annealing. Post-thaw sperm viability data indicated that samples need to be cooled below −40 °C before they can be safely plunged and stored in LN2. No negative effects of relocating specimens from dry ice to LN2 and vice versa became apparent. However, sample warming above −50 °C during transport in dry ice should be avoided to ensure preservation of viability and fertility. Moreover, addition of PVP-40 or Ficoll-70 was found to increase sperm cryosurvival, especially under non-ideal storage conditions where ice recrystallization may occur.

1. Introduction

Cryopreserved sperm is increasingly being used in equine breeding. Frozen specimens are routinely stored and transported in liquid nitrogen or in its vapor phase, at −196 to about −150 °C. Not only is this costly, but it also involves safety and maintenance issues. Furthermore, liquid nitrogen is not always easily available in remote locations [1]. Solid carbon dioxide, i.e., dry ice, has been successfully used for (pellet) freezing [2,3] and short-term storage of sperm from various species [4–8]. Also, mechanical freezers can be employed for storage at temperatures ranging from −80 to −70 °C [9,10]. Storage at such relatively high subzero temperatures, however, may negatively affect stability and cryosurvival [11]; especially when samples are stored above or close to the glass transition temperature of the preservation formulation [12–14]. This is explained by the fact that molecular mobility and chemical reaction rates increase with increasing temperature, and devitrification and recrystallization progress over time [15].

Preservation of cells at ultralow temperatures typically requires addition of cryoprotective agents and controlled/programmable slow cooling rates, e.g., to temperatures below −60 °C; for prolonged storage at liquid nitrogen temperature [16–18]. Permeating cryoprotective agents (CPAs) like glycerol and dimethyl sulfoxide mitigate the damaging effects of solution effects injury and help to avoid intracellular ice formation [19]. In addition, CPAs modulate ice (re)crystallization kinetics [20,21], and facilitate controlled freezing-induced cellular dehydration [22,23]. The latter occurs due to an osmotic gradient generated through an increased solute concentration in the unfrozen extracellular milieu. So-called equilibrium or programmable cooling takes into account the rate and temperature range at which particularly water can move across cellular membranes to control cellular

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dehydration [24–26]. It is assumed that transport processes come to a standstill when the temperature reaches −30 °C, and that dehydration at this point should be sufficient to avoid intracellular ice formation but within osmotic tolerance limits to avoid solution effects injury [24,27]. Critical temperature ranges and the optimal cooling rate for cryopreservation are defined by intrinsic cellular properties (e.g., membrane composition and permeability characteristics, cytoplasmic content, cytoskeletal architecture), but also affected by medium supplements (i.e., permeating solutes, sugars and/or polymers).

In addition to modulating the damaging effects of osmotic gradients, during cooling, CPAs decrease molecular mobility by means of increasing the medium viscosity [19,26,28]. Viscosity drastically increases below the glass transition temperature (Tg), while the temperature below which a vitreous state is formed depends on the relative contents of all high and low molecular weight solutes in the preservation solution. Low molecular weight CPAs typically have a low Tg, i.e., around −80 °C [13,14]. Synthetic polymers, like polyvinylpyrrolidone, can be used to increase the medium viscosity and Tg [13,29], while also serving as a bulking agent (i.e., separating cells in the formulation) [28]. Moreover, they inhibit ice crystal growth, both during storage and when employing slow warming rates. Antifreeze and ice binding proteins that can be found in nature typically exhibit higher ice recrystallization inhibition activity [20,30,31], however, the advantage of synthetic polymers is that they are easily available.

In this study, we have evaluated the feasibility of using dry ice for transport of cryopreserved stallion semen doses in Styrofoam boxes. More specifically, we determined critical temperature ranges during cooling and rewarming that potentially result in viability losses. Sperm motility and membrane intactness were taken as measure of cryosurvival, while an artificial insemination trial was performed to confirm fertilizing capacity. In addition, it was tested if added synthetic polymers (PVP-40, Ficoll-70) are effective in inhibiting ice crystal growth and therewith stabilizing samples under non ideal storage conditions.

2. Materials and methods

2.1. Analysis of effects cryoprotective agents on ice recrystallization

Effects of CPAs on ice crystal formation and growth kinetics of frozen preservation formulations were examined using the so-called splat assay [32,33]. Formulations containing various concentrations and combinations of glycerol (GLY), polyvinylpyrrolidone-40 (PVP-40, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and Ficoll-70 (GE17-0310; Sigma-Aldrich) were prepared in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Fifteen-μL solution was dropped from ~1 m height, to obtain a thin frozen film once the sample hits a glass cover slip on an aluminum block of −80 °C. The aluminum block was pre-cooled in a mechanical freezer or by using dry ice, and droplets were released within a tube (diameter: 5 cm, length: 1 m) positioned above the block. After splatting, frozen specimens were stored at −10 °C in a cool-box (Engel, Sawafuji Electric, Japan) for 24 h, to facilitate ice crystal growth just below the melting temperature. For microscopic observations, specimens were transferred to a temperature-controlled sample holder, connected to a liquid nitrogen pump system (Linkam Scientific Instruments, Tadworth, Surrey, UK). The sample temperature was held at −10 °C and an ordinary stereomicroscope (Stemi SV8; Carl Zeiss Microscopy GmbH, Jena, Germany) was used to inspect samples at 10 × 6.4 magnification. The microscope stage with sample holder was contained and purged with dry air to prevent condensation (Pneudri MiDAS; Parker-Hannifin, Cleveland, OH, USA). Microscopic images were taken using a smartphone connected to the eye piece, and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). For each solution three frozen specimens were prepared and analyzed, and the area was determined for 200 ice crystal grains per sample (i.e., 600 grains per frozen solution).

2.2. Work involving animals

The institutions at which work involving animals were performed are registered and licensed as artificial insemination centers, according to regulations of the European Union. All procedures were carried out in accordance with German animal welfare legislation and approved by the Lower Saxony State Office for Consumer Protection and Food Safety. This study only involved procedures also performed for commercial purposes by our institutions, and therefore no further approval was needed.

2.3. Semen collection and processing

Semen was collected from ten stallions of the Hanoverian breed (3−16 years), 1−3 ejaculates each, using a phantom and an artificial vagina (both model 'Hannover'; Minitüb, Tiefenbach, Germany), with a mare being present for stimulation. The artificial vagina was prepared with a disposable inner liner (Minitüb) and sterile glass bottle attached containing INRA-82 semen diluent [34]. Milk filter (Eimermacher Gruppe, Nordwalde, Germany) was used for reducing the gel fraction and dirt. Prior to use, all materials were warmed in an incubator set at 37 °C. Vaseline was used to improve lubricity. Directly after collection, the sperm concentration was determined, using a Nucleocounter (SP-100TM; ChemoMetec, Kaiserslautern, Germany) or hemocytometer (type 'Improved Neubauer'). Semen diluted with at least an equal volume of INRA-82 was subjected to centrifugation (600×*g*, 10–12 min, at ~22 °C). The recovered sperm pellet was resuspended in fresh INRA-82, to two-fold the desired final sperm concentration (i.e., 100×10^6 or 400×10^6 sperm mL^{-1}).

INRA-82 has a pH of 6.8–7.0 and osmolality of 300–330 mOsm kg^{-1}, and is prepared by mixing equal volumes of glucose-saline solution and 0.3 % ultra-heat-treated skim milk. Glucose-saline solution consists of 50 g L^{-1} glucose monohydrate, 3.0 g L^{-1} lactose monohydrate, 3.0 g L^{-1} raffinose, 0.5 g L^{-1} sodium citrate dihydrate, 0.82 g L^{-1} potassium citrate monohydrate, 9.52 g L^{-1} HEPES, 1.0 g L^{-1} penicillin, 1.0 g L^{-1} amikacin and 5 mg L^{-1} amphotericin.

2.4. Sperm cryopreservation, cooling to different subzero temperatures, storage and warming

INRA-diluted sperm samples were divided in three equal parts to test different cryopreservation diluents and thermal processing treatments on the same ejaculate. INRA-82 supplemented with two-fold the desired final concentration of cryoprotective agents was slowly added to an equal volume of sperm diluted in INRA-82 [35]; finally resulting in 50×10^6 or 200×10^6 sperm mL^{-1}, 2.5 % (v/v) clarified egg yolk, 2.5 % (v/v) glycerol and no or 7.5 % (w/v) polymers added. For the latter, the synthetic polymers PVP-40 and Ficoll-70 were tested. Samples with 200×10^6 sperm mL^{-1} were prepared to assess storage stability in dry ice. To determine critical temperatures during cooling, and storage stability in mechanical freezers, a 50×10^6 sperm mL^{-1} concentration was used allowing accurate sperm motility determinations using computer assisted sperm analysis. For all formulations that were tested, multiple cryopreserved samples in straws were prepared as split samples to directly compare outcomes for different storage conditions.

Sperm samples were slowly cooled, at ~0.1 °C min^{-1}, to 5 °C. This was done by incubating samples in 15- or 30-mL TPP tubes (Sarstedt, Nümbrecht, Germany) in a beaker filled with room temperature water (of ~22 °C), placed in a temperature-controlled handling cabinet (IMV technologies, L'Aigle, France) set at 5 °C. After reaching 5 °C (after ~90 min), sperm samples were filled into 0.5-mL straws (IMV). Straws were transferred to metal racks and placed in a controlled rate freezer (Mini Digit Cool; IMV) for cooling at 50 °C min^{-1} down to −140 °C, after which straws were plunged and stored in liquid nitrogen.

To assess critical temperature ranges during cooling, straws were cooled on racks placed in a polystyrene box (height × width × depth: 20 × 60 × 40 cm) filled with liquid nitrogen (height: 3 cm). Samples were cooled in the vapor phase of liquid nitrogen, ~3.5 cm above the liquid level, at a cooling rate of ~30 °C min^{-1}. The cooling profile was measured using a T-type thermocouple (Fluke, Everett, WA, USA) inserted in a straw, to monitor the times needed to reach different subzero temperatures after which straws were plunged in liquid nitrogen (Fig. 1A).

Sperm samples were stored in liquid nitrogen for at least 24 h, where after they were analyzed or used for storage experiments on dry ice (described below), a mechanical freezer (−80 °C, −20 °C) or a fridge (5 °C). Prior to analysis, warming was done by singly plunging frozen straws in a water bath set at 37 °C, and removing after 30 s incubation (Fig. 1B).

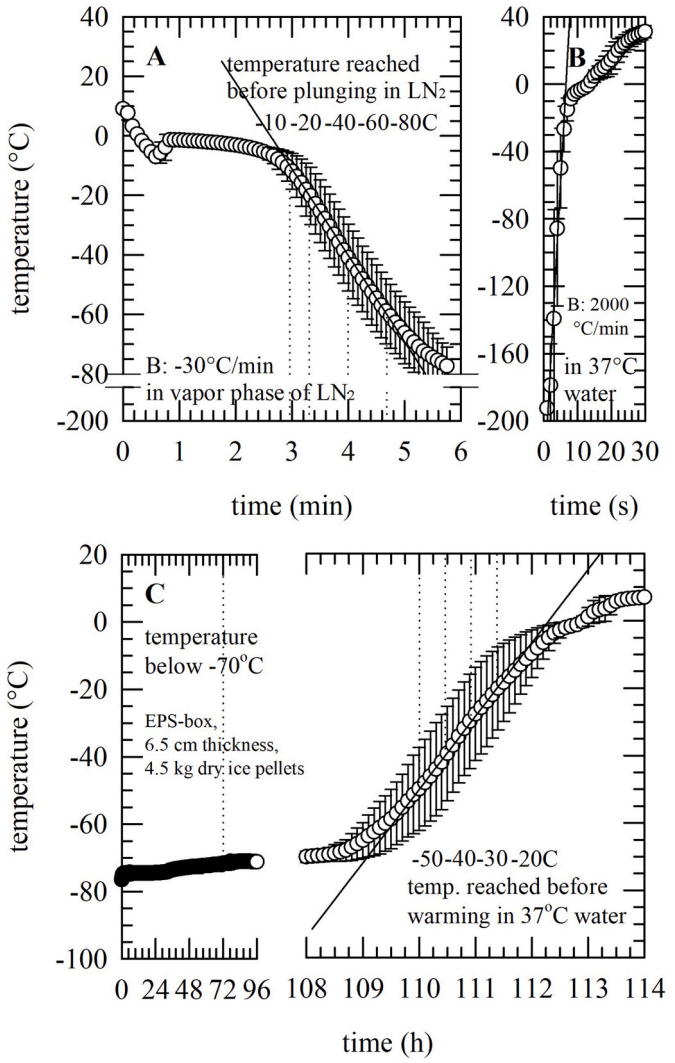

Fig. 1. Temperature profiles of sperm samples during: (A) cooling from 5 to below −80 °C in the liquid nitrogen vapor phase, (B) warming of cryopreserved specimens upon plunging in a 37 °C water bath, and (C) storage of cryopreserved specimens in dry ice for up to 5 d. The sample temperature within a straw was monitored versus time using an inserted thermocouple. During cooling, in liquid nitrogen vapor, specimens were plunged in liquid nitrogen when reaching temperatures lower than −80 °C (i.e., after ~10 min), as well as at higher subzero temperatures (dashed lines: cooling down to −10, −20, −40 and −60 °C). When stored on dry ice, specimens remained below −70 °C for up to 72–96 h, whereas longer storage durations resulted in dry ice evaporation and increasing temperatures (dashed lines: warming up to −50, −40, −30 and −20 °C). Average ± standard deviations are presented, determined from three independent measurements.

2.5. Storage of cryopreserved sperm on dry ice, warming to different subzero temperatures

For storage of straws with cryopreserved sperm on dry ice (i.e., between dry ice pellets), polystyrene insulation boxes were used; with 26 × 15 × 15 cm (height × width × depth) inner dimensions and a wall thickness of 6 cm (Schaumaplast GmbH, Lüchow, Germany). A total of 4.5 kg dry ice pellets (Cleangas GmbH, Willingen, Germany) was used per box. Straws (≤7) were packaged using two goblets (with 10 and 13 mm diameter, inserted in each other, with openings in opposite position). Packaged straws were placed on a layer of dry ice (height: ~1 cm) where after the remaining dry ice was added (height: ~15 cm). The sample temperature during storage was monitored using a data logger (HH520; Omega, Manchester, UK), with a thermocouple inserted in a mock straw (i.e., filled with INRA-82). The temperature was recorded every 6 min, up to 5 d. Samples remained below −70 °C for ~96 h, whereas longer storage durations resulted in dry ice evaporation and warming (Fig. 1C). After storage in dry ice for 72 h (i.e., considered safe), straws were either taken for sperm analysis or transferred into liquid nitrogen for further storage (>24 h) and analysis thereafter. In addition, to determine critical temperatures during warming, straws containing cryopreserved sperm were stored in dry ice for longer durations where after they were thawed (37 °C, 30 s) and analyzed.

2.6. Assessment of sperm plasma membrane intactness and motility

Percentages of plasma membrane intact sperm were determined before and after cryopreservation, by staining sperm with propidium iodide (PI) and Hoechst-33342 (H33342) and observation using fluorescence microscopy (BX60; Olympus, Hamburg, Germany). To 100 µL sperm sample (50–200 × 10^6 sperm mL^{-1}), 4 µL 10 mg mL^{-1} H33342 was added followed by 5 min incubation at 37 °C, where after 4 µL 5 mg mL^{-1} PI was added and samples were incubated for 5 additional min. From this an aliquot of 20 µL was taken and mixed at ~22 °C with an equal amount of fixation solution (100 mM trisodium citrate, 1.5 % formaldehyde) for preparing dried smears (5 µL) on microscope slides. Percentages of membrane intact sperm (i.e., sperm with blue/H33342-fluorescence, not exhibiting red/PI-fluorescence) were determined via inspecting a minimum of 200 sperm per specimen, in 3 fields, using a 10 × 20 magnification. Outcomes of these manual counting analyses were validated using Nucleocounter measurements (data not shown).

Sperm motility characteristics were assessed using computer-assisted sperm analysis (CASA, Androvision; Minitüb). The setup included a microscope with heated motor-controlled stage, camera (60 frames s^{-1}), and accompanying software. Settings were according to instructions provided by the manufacturer. Sperm motility was assessed after 5−10 min incubation at 37 °C (500 µL; microtubes in a heated metal block). Three-µL sample was loaded into a chamber of a 20 µm four-chamber slide (Leja products BV, Nieuw-Vennep, Netherlands), and a minimum of 4 fields and 200 sperm were inspected. Sperm were classified motile when they had an average path velocity greater than 20 µm s^{-1}, and progressively motile when this velocity was greater than 40 µm s^{-1}. Supplementing specimens with PVP-40 and Ficoll-70 resulted in an increased medium viscosity, and in some occasion particles/condensates interfered with CASA. Therefore, sperm motility was also evaluated subjectively via phase contrast microscopic observations using 5 µL sample aliquots on ordinary slides at 10 × 20 magnification. These evaluations were performed by the same experienced person, and outcomes (with using ordinary diluents) were validated using CASA (data not shown).

2.7. Artificial insemination studies

Cryopreserved sperm stored for 24−72 h in dry ice (i.e., mimicking

frozen shipment, not above −70 °C) was tested with artificial insemination, and outcomes were compared with using straws stored in liquid nitrogen (at about −196 °C). For both storage conditions, twelve mares with a normal breeding history were inseminated, using semen originating from two stallions of proven fertility. Only specimens in standard freezing extender not supplemented with polymers were tested (as used for commercial purposes). Prior to insemination, when exhibiting decreased uterine edema and follicles reaching a minimum size of 35 mm, mares were treated with human chorionic gonadotropin (1500 IU) to stimulate ovulation at an estimated time point about 36 h later. At the time point this treatment was done, a cryopreserved insemination dose was transferred from liquid nitrogen to dry ice and stored there for at least 24 h. Mares were checked regularly, to time the insemination maximally 6 h after ovulation. One insemination dose consisting of ~800 × 10^6 sperm (4 or 8 straws) was applied deep into the uterine horn, as routinely done with cryopreserved sperm. Four to 12 h after insemination, mares were flushed with saline and treated with oxytocin (20 IU). After 14–16 d, mares were examined using transrectal ultrasound imaging for determining successful artificial insemination and being pregnant.

2.8. Statistical analysis

For statistical analysis 'SAS' software (version 7.15; SAS Institute, Cary, NC, USA) was used under supervision of an experienced statistician (Institute for Biometry and Epidemiology of the University of Veterinary Medicine Hannover). In addition Sigmaplot (version 13.0, Systat software; Inpixon GmbH, Düsseldorf, Germany) was used. Data are presented as means ± standard deviations. In addition, were appropriate, values for each stallion are indicated. First, the data were tested for normal distribution, where after either a parametric or a non-parametric test was performed. Analysis of variance (ANOVA) was used to analyze differences in sperm characteristics at different time points using different diluents. In addition, the Tukey test and the Holm-Sidak method were applied for multiple comparisons. Pregnancy data obtained with the insemination trial were analyzed using the Chi-squared ($\chi 2$) test and, due to the small number of mares per group, Fisher's exact test was used where applicable. In all cases, differences were considered statistically significant when the probability of error was less than 5 % (i.e., $p < 0.05$).

3. Results

3.1. PVP-40 and Ficoll-70 reduce ice crystal growth

Fig. 2 shows micrographs of frozen specimens of different compositions, as formed upon instant freezing and warming. Ice crystals appear smaller when specimens are supplemented with increasing glycerol concentrations. Holding specimens at −10 °C, just below the melting temperature, results in ice crystal growth. The rate and extent at which this occurs is taken as an indicator for ice recrystallization occurring during storage and warming of cryopreserved specimens; therefore ice crystal sizes were analyzed after overnight incubation at −10 °C using image analysis. Fig. 2 shows that addition of 7.5 % PVP-40 or Ficoll-70 to 2.5 % glycerol formulations decreases the mean ice crystal grain size as compared to glycerol solution without additives. Furthermore, the size distribution appears more uniform when these polymers are added, and the mean and median crystal sizes more similar. Supplementing specimens with polyethylene glycol does show an inhibitory effect on ice recrystallization (data not shown).

3.2. Cryopreservation requires control of the freezing rate down to −40 °C, and PVP-40 and Ficoll-70 increase cryopreservation outcome

Sperm were diluted in ordinary freezing medium as well as medium supplemented with PVP-40 or Ficoll-70. It was determined if this

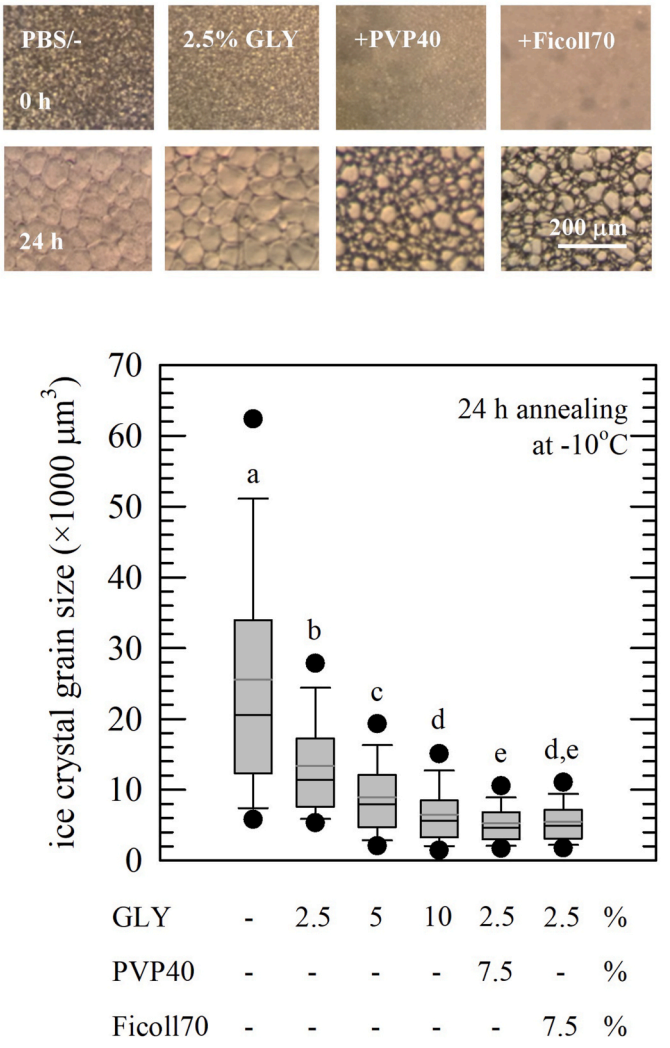

Fig. 2. Ice recrystallization inhibition in diluent supplemented with increasing glycerol (GLY) concentrations, as well as glycerol combined with PVP-40 or Ficoll-70. Thin frozen specimens were obtained via the splat assay, on a block of −80 °C, while ice recrystallization was allowed to occur during overnight incubation at −10 °C. Typical cryomicrographs are shown (upper panel), as well as the outcomes of image analysis (lower panel). For each formulation, ice crystal grain size distributions are presented as box-and-whisker plots (i.e., quartiles 1–4), with also indicated minimum and maximum values (black circles), mean and median values (grey and black lines, respectively). Three repetitions were analyzed, 200 crystals each. Statistical significant differences ($p < 0.05$) are indicated with different letters.

affected the minimum subzero temperature below which specimens need to be frozen for safe cryopreservation and/or cryosurvival. In Fig. 3 it can be seen that, irrespective of the freezing medium used, sperm cryosurvival was similar when samples were frozen down to −40 °C or lower, i.e., before plunging and storing in liquid nitrogen. Nonetheless, percentages of membrane intact sperm post-thaw increased in the order no additives > with PVP-40 > Ficoll-70. The polymer additives were especially effective in increasing sperm viability in the critical temperature range during freezing, from −10 to −40 °C (Fig. 3A). Percentages of motile sperm were found to be lower when using diluents supplemented with PVP-40 (Fig. 3B). As compared to sperm residing in ordinary freezing medium, the velocity at which sperm moved was decreased in media supplemented with PVP-40 and to a lesser extent with Ficoll-70 (data not shown). This can be explained by the increased medium viscosity with addition of these polymers.

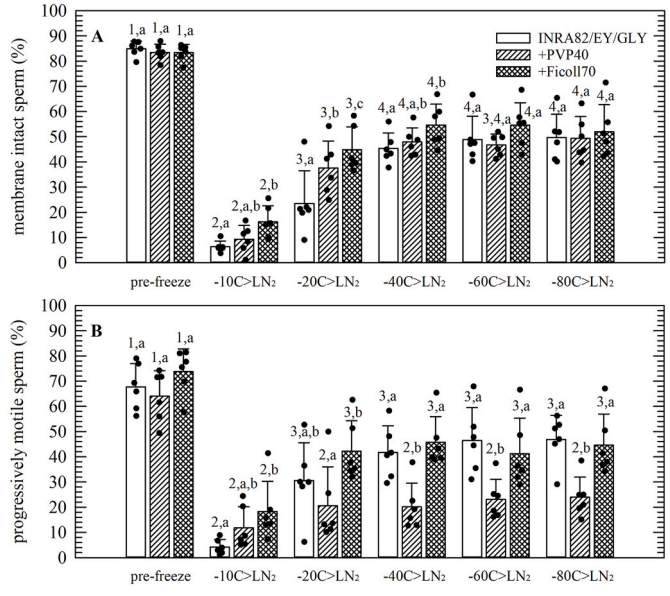

Fig. 3. Percentages of membrane intact (A) and progressively motile (B) sperm before and after cryopreservation, while cooling down to varying subzero temperatures (−10, −20, −40, −60, −80 °C) prior to plunging and storing in liquid nitrogen. For cryopreservation, ordinary diluent was used (INRA82/EY/GLY; white bars), as well as diluent supplemented with PVP-40 (bars with diagonal lines) or Ficoll-70 (bars with crossed lines). Sperm plasma membrane intactness was determined using fluorescence microscopy, after staining with PI/H33342, and motility was evaluated using CASA. Mean values and standard deviations are presented, determined for sperm originating from 6 different stallions, 2 ejaculates each. In addition, the average values determined for each stallion are presented (black circles). Statistical significant differences ($p < 0.05$) amongst time points of analysis and preservation formulations used are indicated with different numbers and letters, respectively.

3.3. PVP-40 and Ficoll-70 modulate ice recrystallization under non ideal storage conditions

Fig. 4A presents studies in which it was determined if storage at elevated subzero temperatures affects sperm cryosurvival. When cryopreserved specimens were stored in a mechanical freezer of −80 °C, for short duration (1 d), sperm membrane intactness and motility after thawing were found to be similar compared to specimens kept in liquid nitrogen (>1 d, −196 °C). Sperm viability post-thaw appeared to be slightly decreased with increasing storage duration (1 month). Overnight storage of frozen specimens at −20 °C, completely abolishes sperm viability. This temperature lies within the critical range where ice recrystallization and crystal growth during storage provoke cellular damage. Interestingly, when frozen specimens are transferred to 5 °C this appears less damaging than storage at −20 °C. Moreover, during slow thawing at 5 °C, sperm viability is increased by adding Ficoll-70. This can be explained by the time frame for ice crystal growth and the inhibiting action of Ficoll-70 on ice recrystallization. In agreement with this, sperm survival was also increased when cryopreserved specimens were rapidly thawed and there after stored overnight at 5 °C; especially when Ficoll-70 or PVP-40 were added.

3.4. Viability of cryopreserved sperm stored on dry ice is maintained when the sample temperature remains below −50 °C

Fig. 4B–C shows that, irrespective of the freezing medium used, sperm cryosurvival is preserved during additional 72 h storage on dry ice, i.e., to similar extents as found when kept in liquid nitrogen. This is evident from the linear correlations found when post-thaw outcomes are plotted against each other, for sperm membrane intactness (54 ± 5 vs 53 ± 5 %; r: 0.90, r^2: 0.81) as well as motility (49 ± 7 vs 50 ± 7 %; r: 0.61, r^2: 0.41). Furthermore, when relocated and stored in liquid nitrogen after storage in dry ice, there are no apparent losses in membrane intactness (55 ± 5 %; r: 0.85, r^2: 0.71) or motility (50 ± 7 %; r: 0.63, r^2: 0.39). It should be noted that subjective motility evaluations revealed higher values in diluents containing polymers, whereas differences amongst diluents derived from computer assisted sperm analysis were less pronounced.

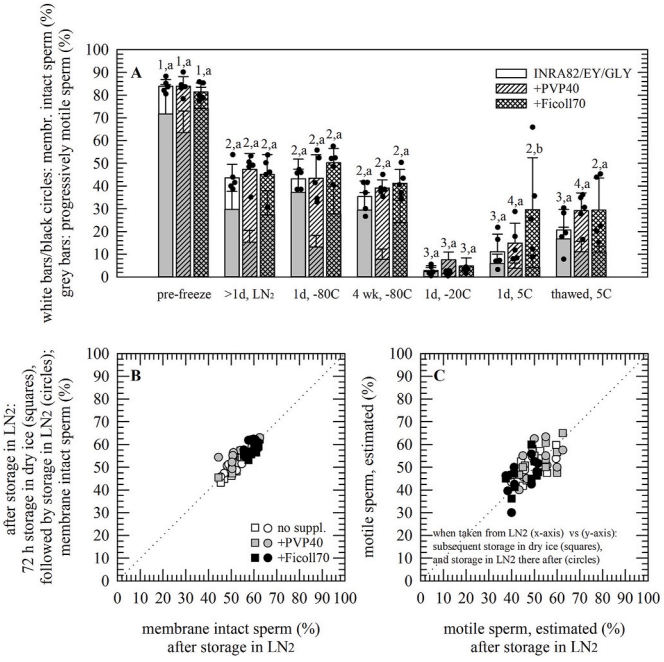

Fig. 4. Percentages of viable sperm before and after cryopreserved storage in liquid nitrogen (>1 d), or a mechanical freezer of −80 °C (1 d, 4 wk) or −20 °C (1 d). In addition, sperm viability post-thaw was analyzed after overnight storage in a 5 °C fridge (1 d), both when transferred while frozen or after ordinary thawing (A). Furthermore, correlations are presented on sperm viability post-thaw for different storage conditions (B,C). Straws from the same ejaculate were kept in liquid nitrogen (i.e., x-axis values), transferred to dry ice for 72 h storage (y-axis values; squares) and transferred to liquid nitrogen again thereafter (y-axis values; circles). For cryopreservation, ordinary diluent was used (INRA82/EY/GLY; bars without lines, white squares and circles), as well as diluent supplemented with PVP-40 (A: bars with diagonal lines, grey squares and circles) or Ficoll-70 (bars with crossed lines, black squares and circles). Sperm plasma membrane intactness was determined using fluorescence microscopy after PI/H33342 staining (A: white bars, B), whereas motility was analyzed using CASA (A: grey bars) as well as via subjective evaluation (C). In panel A, mean values and standard deviations are presented, determined for sperm originating from 5 different stallions, 2 ejaculates each (white and grey bars). In addition, average percentages of membrane intact sperm determined for each stallion are presented (black circles). Statistical significant differences ($p < 0.05$) amongst time points of analysis and preservation formulations used are indicated with different numbers and letters, respectively (i.e., for plasma membrane intactness data). In panel B–C, data belonging to sperm originating from 10 different stallions are presented.

Prolonged storage and dry ice evaporation eventually leads to warming of frozen specimens. Upon attaining distinct temperatures, sperm samples were taken, thawed, and analyzed (Fig. 5). As described above, when stored for 72 h in dry ice with temperatures kept below −70 °C, sperm cryosurvival was similar to values found for samples in liquid nitrogen. Sperm membrane intactness and motility post-thaw start to decrease when the sample temperature reaches −50 °C prior to rapid warming. Losses were more pronounced when the sample temperature reached higher subzero storage temperatures, especially for motility values in ordinary freezing medium. The percentage of motile sperm dropped below 35 % when specimens reached −40 °C (i.e., >110 h), prior to rapid warming.

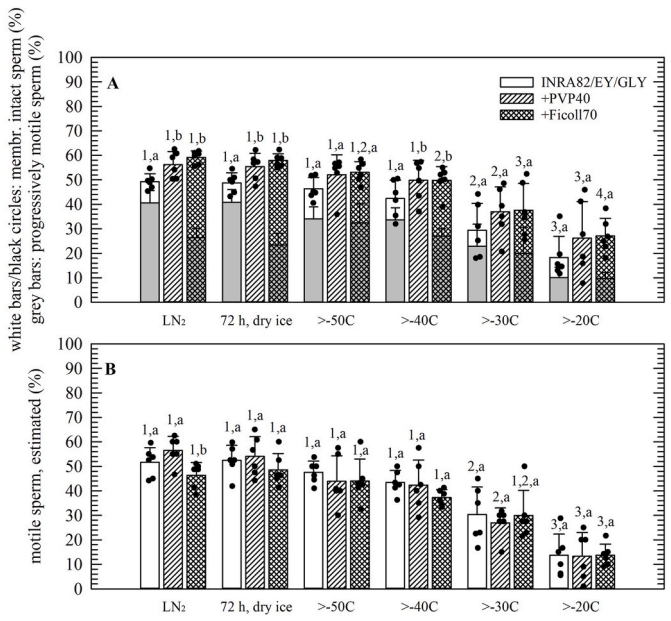

Fig. 5. Percentages of membrane intact (A) and (progressively) motile (A, B) sperm after cryopreservation and storage in liquid nitrogen (LN_2), 72 h in dry ice, as well as longer storage in dry ice causing increased temperatures (i.e., warming to: −50, −40, −30, −20 °C) prior to thawing (30 s at 37 °C) and analysis. For cryopreservation, ordinary diluent was used (INRA82/EY/GLY; bars without lines), as well as diluent supplemented with PVP-40 (bars with diagonal lines) or Ficoll-70 (bars with crossed lines). Sperm plasma membrane intactness was determined using fluorescence microscopy, after staining with PI/H33342 (A; white bars), motility was evaluated using CASA (A; grey bars) as well as via subjective evaluations (B: white bars). Mean values and standard deviations are presented, determined for sperm originating from 6 different stallions, 2 ejaculates each (white and grey bars). In addition, average percentages of membrane intact (A) and motile (B) sperm determined for each stallion are presented (black circles). Statistical significant differences ($p < 0.05$) amongst time points of analysis and preservation formulations used are indicated with different numbers and letters, respectively. In panel A, statistical differences are indicated for plasma membrane intactness data.

3.5. Fertility potential of cryopreserved sperm is maintained after storage on dry ice

To test feasibility of using dry ice for transport of stallion sperm doses for artificial insemination purposes, a trial was performed using sperm cryopreserved in ordinary freezing medium (i.e., without addition of PVP-40 or Ficoll-70). Fertility of cryopreserved sperm samples/straws stored on dry ice was compared with that of samples stored in liquid nitrogen. Sperm originating from two different stallions was tested. When artificial insemination was done using straws stored 1−3 d in dry ice, 7 out of 12 mares were found to be pregnant (i.e., 58 %). This is not significantly different from values found for samples kept in liquid nitrogen (10 out of 12 mares, 83 %).

4. Discussion

In this study, we analyzed critical temperature trajectories for stallion sperm cryopreservation, evaluated the feasibility of using dry ice for transport of insemination doses, and determined if synthetic polymers can be employed to improve storage stability. We found that sperm needs to be cooled at a controlled rate to temperatures below −40 °C, where after straws can be safely plunged and stored in liquid nitrogen. This is in agreement with transfer temperatures found by others, for a variety of cell types [36–39]. This can be explained by controlled freezing-induced cellular dehydration until transport processes come to a standstill; while limiting the incidence of intracellular ice formation and damage due to solution effects [16,17,22,23,27]. Furthermore, we determined that sperm cryosurvival is not affected when specimens are stored for short durations on dry ice or in a mechanical freezer, i.e., when kept below −50 °C. A small decline in sperm viability was seen after 4 weeks storage at −80 °C, similarly as reported for sperm from other species [5,10]. However, addition of the synthetic polymers PVP-40 and Ficoll-70 resulted in higher cryosurvival, and appeared to counteract this decline to some extent.

Ficoll and PVP are often used for embryo and oocyte vitrification [40,41]; because they enhance thermal stability of preservation solutions by inhibiting devitrification [42]. The positive effect of such compounds depends both on the concentration as well as the molecular weight of the polymer used [13]. In the current work, we pursued studies with PVP-40 and Ficoll-70, because of their previously described thermophysical characteristics and benefits for equilibrium cooling and storage of cryopreserved cells at −80 °C [13,42]. We confirmed that both polymers are effective in inhibiting ice crystal growth [20]. Moreover, when combined with glycerol, we show that presence of PVP-40 and Ficoll-70 both enhance stabilization of cryopreserved semen under non-ideal storage conditions, e.g., exposure to elevated subzero temperatures, and during slow rewarming rates [29]. It remains to be determined if stallion sperm velocity is restored after removal or dilution of polymer-containing preservation solutions, and if PVP-40 and Ficoll-70 interfere with sperm fertilizing capacity. For PVP, there are contradicting views regarding sperm and embryo toxicity effects; which can possibly be attributed to use of too high concentrations or presence of impurities in the polymer source [43–46].

In agreement with findings of others [7], we found that cryopreserved sperm can be held for ~3–5 d at around −70 °C, when using 4–5 kg dry ice pellets in thick-wall expanded polystyrene boxes; without losing fertilizing potential. Longer storage durations are also feasible, however, need addition of more/fresh dry ice [8]. Furthermore, polystyrene boxes should not be sealed and contained in a larger cardboard-box, to prevent accumulation of carbon dioxide gas (and possibly medium acidification) [1,47]. No negative effects of relocating specimens from dry ice to liquid nitrogen and vice versa became apparent.

Taken together, we show that dry ice could serve as an alternative coolant for transport or temporary storage of cryopreserved semen doses. Furthermore, we have identified critical temperature ranges for storage and transport of cryopreserved stallion sperm: samples need to be cooled below −40 °C before they can be safely plunged and stored in liquid nitrogen, while warming above −50 °C during transport in dry ice should be avoided. When kept below −70 °C during transport, upon arrival, cryopreserved samples can be safely used for artificial insemination or relocated to liquid nitrogen. Moreover, addition of polymers that exhibit ice recrystallization activity (i.e., PVP-40, Ficoll-70) can be used to stabilize sperm viability under non-ideal storage and transport conditions.

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