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Dimethyl formamide improves the postthaw characteristics of sex-sorted and nonsorted stallion sperm

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ABSTRACT

Cryopreserved, sex-sorted stallion sperm has been shown to have poor fertility. During this study, the effects of cryoprotectant (glycerol [GLY] and dimethyl formamide [DMF]), cryoprotectant equilibration time (0, 30, 60, 90, or 120 minutes), and cryoprotectant concentration (2%, 3%, or 4% vol/vol) on stored sex-sorted and stored nonsorted stallion sperm were evaluated. Total motility, viability, and DNA integrity (determined using sperm chromatin structure assay) of sperm were assessed after thawing. Equilibration for 90 minutes improved total motility (33.8%) compared with 0 (28.5%) or 120 minutes (29.8%; $P < 0.05$), though viability was higher after 120 minutes (33.1%) compared with 0 (30.5%) or 30 minutes (31.0%; $P < 0.01$). The viability of nonsorted sperm decreased as cryoprotectant concentration increased ($P < 0.001$), and total motility of nonsorted sperm was higher when DMF alone was used (15.8%, 16.6%, and 24.0% for GLY, GLY and DMF, and DMF respectively; $P < 0.001$). Sex sorting was detrimental to the postthaw quality of sperm; at 45 minutes after thawing, total motility of nonsorted sperm was higher than that of sex-sorted sperm (37.4% vs. 5.6%; $P < 0.001$), the viability of sex-sorted sperm was lower than that of nonsorted sperm (12.4% vs. 30.0%; $P < 0.001$, averaged over postthaw time), and sex-sorted sperm had higher detectable DNA fragmentation index (DFI) (63.6% vs. 11.3%, $P < 0.001$) and mean DFI (285.1 vs. 211.3, $P < 0.001$) than nonsorted sperm. The viability of sex-sorted sperm was improved by GLY and DMF or DMF compared with GLY (22.6%, 25.3%, and 19.3%, respectively; $P < 0.05$), and the DNA integrity of sex-sorted sperm was improved by the use of DMF compared with GLY (detectable DFI, 60.2 vs. 66.8, $P < 0.05$; and mean DFI, 280.9 vs. 289.2, $P < 0.05$, respectively). In conclusion, postthaw characteristics of stored sex-sorted and stored nonsorted stallion sperm were improved by the use of DMF as a cryoprotectant, though the parameters to benefit differed between sorted and nonsorted sperm.

1. Introduction

The commercial application of flow cytometric sex sorting to the horse industry has been limited by the poor fertility of sex-sorted, cryopreserved stallion sperm [1,2]. Although fertility of fresh or liquid stored, sex-sorted stallion sperm is acceptable [3], its reduced longevity introduces a number of limitations. These include the need to house the mare and stallion in close proximity to the sorting facility and the ability to coordinate sorting so that an insemination dose is available within a few hours of ovulation; an event which is extremely difficult to predict accurately in the mare [4]. In addition, this requires the semen sex-sorting equipment to be available at any time; an unlikely scenario in a busy sorting facility. The ability to

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successfully cryopreserve sex-sorted stallion sperm would effectively remove the current limitations associated with the technology.

Flow cytometric sex sorting [5] and cryopreservation [6] have been shown to induce capacitation-like changes to stallion sperm, resulting in a significant decline in postthaw longevity [7]. Whereas fresh sex-sorted [8] and frozen-thawed nonsorted [9] stallion sperm have produced acceptable pregnancy rates after AI, the compounded effects of sex sorting and cryopreservation result in a considerable decline in motility [10] and fertility [1,2] in horses.

Although glycerol (GLY) is the most common cryoprotectant used in stallion semen cryodiluents [9,11–14], it has been shown to be toxic to equid sperm, causing a reduction in fertility [15,16]. For this reason there have been numerous attempts to find an alternative cryoprotectant. Although the cryoprotective ability of sugars [17] and lectins [18] have been evaluated without success, dimethyl formamide (DMF) has been identified as a potential alternative to GLY [19–22]. The improved motility and viability observed when GLY is substituted with DMF [23] was attributed to the better ability of the smaller molecule to penetrate the plasma membrane, thereby inducing less osmotic stress than GLY [24].

The optimum interval that sperm are allowed to equilibrate with cryoprotectants before cryopreservation varies markedly between species [25–27]. Despite this, there is a distinct lack of information regarding optimal cryoprotectant equilibration time for stallion sperm, and there are no reports outlining an effective protocol for the cryopreservation of liquid stored, sex-sorted stallion sperm. Because it is understood that sex-sorted sperm membranes are somewhat compromised by the sorting process, it is possible that cryoprotectants will penetrate these cells more rapidly than nonsorted sperm, thus affecting optimal equilibration time.

The objectives of this study were to assess the effects of cryoprotectant type (DMF and GLY), cryoprotectant concentration and equilibration time on postthaw parameters of stallion sperm after liquid storage and sex sorting. We hypothesized that the use of a lower molecular weight cryoprotectant, such as DMF, results in less damage to the sperm membranes and DNA of stored, sex-sorted stallion sperm.

2. Materials and methods

2.1. Chemicals and diluents

Fatty acid-free, low endotoxin BSA was purchased from ICPbio (I-BSA; Auckland, New Zealand) and SYTO-16 was purchased from Molecular Probes Inc. (Eugene, OR, USA). All other chemicals were purchased from Sigma Aldrich unless otherwise stated.

2.2. Semen collection

Semen was collected from three pony stallions (three ejaculates from each stallion) using either a Missouri artificial vagina or a Hannover artificial vagina (Minitube Australia, Ballarat, Victoria, Australia). Immediately after collection, semen was slowly diluted at a ratio of 2:1 (v:v) with Kenney's Modified Tyrode's media [28] at approximately 35 °C, placed in an Equitainer (Hamilton Thorne Research, Danvers, MA, USA) with one frozen can beneath one room temperature can and stored for 12 to 18 hours at approximately 10 °C.

2.3. Sex sorting

Sex sorting was undertaken using a modified protocol described by Lindsey et al. [2]. Sperm were centrifuged at 350× *g* for 12 minutes, the supernatant was removed, and the sperm pellet resuspended to a concentration of 111 × 10^6 sperm per mL in I-BSA staining media [29] containing 10 mg/mL I-BSA, 177 mM glucose, 5.8 mM sodium lactate, 350 μM sodium pyruvate, 3.5 mM HEPES, 146 μM sodium dihydrogen phosphate, 8.75 mM sodium bicarbonate, 915 μM calcium chloride, 8.78 mM potassium chloride, 294 μM magnesium chloride, 25.2 mM sodium chloride, 0.52 mg/mL penicillin, and 0.65 mg/mL streptomycin sulfate with volume made up using Milli-Q (Millipore) water. The pH was adjusted to 7.2 and osmolarity was adjusted to 315 mOsm via addition of 60% sodium lactate syrup. Hoechst 33342 (H33342) staining was undertaken by adding 20 μL of 9 mM H33342 (Sigma Aldrich, Sydney, Australia) to 180 μL of I-BSA staining media, which was then added drop-wise to 1.8 mL of resuspended sperm, making a total volume of 2 mL for incubation (final H33342 concentration of 90 μM and sperm concentration of 100 × 10^6/mL). Stained samples were incubated at 34 °C for 45 minutes, after which 667 μL of I-BSA media containing 2 μL/mL red food dye (FD&C #40, Warner Jenkinson Company Inc., St. Louis, MO, USA) was added to quench H33342 fluorescence in membrane-damaged or dead sperm cells [30], resulting in a final sorting concentration of 75 × 10^6 sperm per mL. Samples were then sorted to separate putative X- and Y-chromosome-bearing sperm on a high speed modified flow cytometer (SX MoFlo, Dako Colorado Inc., Fort Collins, CO, USA) operating with a sheath pressure of 40 psi and an argon laser at 350 mW resulting in flow rates of 20,000 to 25,000 events per second. The sorted sperm were collected into 10-mL conical-based tubes (at a postsort concentration of 8 × 10^5 sperm per mL in sheath fluid) containing 0.5 mL of 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino] ethanesulfonic acid (TES) and tris(hydroxymethyl)aminomethane (TRIS) buffer (TEST) supplemented with 4% chicken egg yolk [31,32].

2.4. Sperm cryopreservation

For cryopreservation, nonsorted sperm were centrifuged at 350× *g* for 12 minutes after postcollection storage for 12 to 18 hours, and tubes containing sex-sorted sperm were recovered after sorting approximately 8 × 10^6 sperm and centrifuged at 700× *g* for 15 minutes. The supernatant was discarded and the remaining pellet was resuspended to a concentration of 80 × 10^6 sperm per mL with modified lactose-EDTA cryodiluent [33] at room temperature containing 20% chicken egg yolk (vol/vol; without cryoprotectant) as the first part of a two-step dilution (Fraction A). Tubes containing the sperm suspension (approximately

200 μL in the case of nonsorted sperm and 100 μL in the case of sex-sorted sperm) were placed into a plastic bottle containing 80 mL of water at room temperature and cooled to 4 °C at a rate of approximately 0.32 °C/min over a period of 120 minutes.

At various times during the cooling period (see experimental designs in section 2.7.), cryodiluent containing cryoprotectant (Fraction B; cooled to the same temperature as the sperm suspension) was slowly added to the sperm suspensions to attain a final sperm concentration of 40×10^6/mL. Extended sperm were loaded into precooled 0.25-mL straws (IMV, L'Aigle, France), sealed with polyvinyl alcohol powder (Minitube Australia, Ballarat, Victoria, Australia), and placed on a rack 3 cm above liquid nitrogen in a styrofoam box (42 × 28 × 12.5 cm) for 10 minutes. The straws were then plunged into and stored in liquid nitrogen. All cooling and freezing procedures took place in a cold room at 4 °C.

Frozen semen straws were thawed in a 37 °C water bath for 30 seconds and slowly diluted at a ratio of 4:1 (v:v) with sperm tyrode albumin lactate pyruvate medium [34] consisting of 100 mM NaCl, 3.1 mM KCL, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM sodium lactate, 2.0 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1.0 mM pyruvate, 6 mg/mL BSA, and 50 μg/mL gentamycin. Diluted sperm were incubated at 37 °C for the duration of the experiments (see experimental designs in section 2.7.).

2.5. Evaluation of sperm motility and viability

Sperm motility was determined objectively using computer-assisted sperm analysis (IVOS; Hamilton Thorne). In each sample, a minimum of 500 sperm in a minimum of five fields were assessed for percentage total motility.

Viability was determined by dual staining sperm with propidium iodide using SYTO-16 as a counter stain. Briefly, sperm were incubated at 37 °C for 5 minutes with 12 μM propidium iodide and 0.1 μM SYTO-16, after which they were assessed via flow cytometry [35] using a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). In each sample, at least 5000 sperm were analyzed and classified as either viable or nonviable.

2.6. Sperm chromatin structure assay

The sperm chromatin structure assay (SCSA) was performed as previously described by Evenson and Jost [36]. Briefly, after thawing and dilution in sperm tyrode albumin lactate pyruvate medium [34], aliquots of sperm were further diluted to a concentration of 10×10^6 sperm per mL, snap-frozen in liquid nitrogen and stored at −80 °C until assessment. Before assessment, samples were thawed at 37 °C for 1 minute, 400 μL of acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton-X 100, pH 1.2) was added to 200 μL of sperm suspension, and exactly 30 seconds later, 1200 μL of acridine orange staining solution (0.1 M citric acid, 0.2 M Na_2PO_4, 1 mM EDTA, 0.15 M NaCl, 22.6 μM acridine orange, pH 6.0) was added. At least 5000 sperm were analyzed per sample using a FACScan flow cytometer (BD Biosciences) with a standard argon ion laser (488 nm) and CellQuest software (BD Biosciences, version 5.2.1.). Debris was gated out using a forward scatter/side scatter dot plot with a region drawn around sperm cells. Green fluorescence was detected in fluorescence detector 1 and red fluorescence was detected in fluorescence detector 3. The mean DNA fragmentation index (DFI), was obtained using SCSAsoft software (version 1.0., SCSA Diagnostics, Volga, SD, USA). The percentage of cells outside the main population (detectable DFI) was calculated from the output of CellQuest software (BD Biosciences) as described [36].

2.7. Experiments

2.7.1. Experiment 1: Effect of cryoprotectant equilibration time on nonsorted sperm

This experiment was conducted using only nonsorted sperm. After centrifugation and extension with Fraction A cryodiluent, sperm samples were split into 10 aliquots. At 0, 30, 60, 90, or 120 minutes into the cooling period, an equal volume of Fraction B cryodiluent (at the same temperature as the sperm suspension) containing 8% (vol/vol) of either GLY or DMF was slowly added to the sperm suspensions before freezing; the final cryoprotectant concentration was 4%. Sperm motility and viability were assessed at 0, 90, and 180 minutes after thawing and samples for SCSA were snap-frozen in liquid nitrogen immediately after thawing and stored at −80 °C until assessment.

2.7.2. Experiment 2: Effect of cryoprotectant combination

After semen collection and storage, extended ejaculates were split into two aliquots which were sex-sorted and cryopreserved (sex-sorted samples) or cryopreserved immediately (nonsorted samples). After processing, centrifugation and extension with Fraction A cryodiluent, an equal volume of Fraction B cryodiluent (at the same temperature as the sperm suspension) containing either 8% GLY (vol/vol), a combination of 4% GLY and 4% DMF (vol/vol; total cryoprotectant concentration of 8%; GLY:DMF) or 8% DMF (vol/vol) was slowly added at 30 minutes into the 120-minute cooling period to attain a final cryoprotectant concentration of 4% and a cryoprotectant equilibration time of 90 minutes. Sperm motility and viability were assessed at 0, 30, 60, and 90 minutes after thawing and samples for SCSA were snap-frozen in liquid nitrogen immediately after thawing and stored at −80 °C until assessment.

2.7.3. Experiment 3: Effect of cryoprotectant concentration

After semen collection and storage, extended ejaculates were split into two aliquots which were sex-sorted and cryopreserved (sex-sorted samples) or cryopreserved immediately (nonsorted samples). After processing, centrifugation and extension with Fraction A cryodiluent, an equal volume of Fraction B cryodiluent (at the same temperature as the sperm suspension) containing 4%, 6%, or 8% (vol/vol) of either GLY or DMF was slowly added at 30 minutes into the 120-minute cooling period to attain a final cryoprotectant concentration of 2%, 3%, or 4% and a cryoprotectant equilibration time of 90 minutes. Sperm motility and viabiity were assessed at 0, 45, and 90 minutes after thawing and samples for SCSA were snap-frozen in liquid nitrogen immediately after thawing and stored at −80 °C until assessment.

2.8. Statistical analysis

Data were analyzed using REML variance components analysis in Genstat 12th Edition software (Lawes Agricultural Trust). Main effects of individual treatments and effects of interactions between treatments were assessed with stallion and ejaculate number used as blocking terms. Values were determined to be significantly different when $P < 0.05$. When no significant interactions between groups or time points were observed, data were combined for analysis. Results are shown as means ± SEM.

3. Results

3.1. Experiment 1: Effect of cryoprotectant equilibration time

There was an effect of cryoprotectant on total motility, and an effect of equilibration time on total motility and viability. Because no significant interactions between any factors (stallion, postthaw time, cryoprotectant, or equilibration time) on total motility or viability were observed, data were averaged over postthaw time and stallion. The total motility of sperm cryopreserved with DMF (33.9 ± 1.9%) was greater than that of sperm cryopreserved with GLY (27.5 ± 1.9%; $P < 0.05$). An equilibration time of 90 minutes resulted in higher total motility (33.8 ± 3.3%) than equilibration for 0 (28.5 ± 2.8%) or 120 minutes (29.8 ± 3.2%; $P < 0.05$), with 30 (30.6 ± 3.2%) and 60 minutes (31.4 ± 3.2%) being intermediate irrespective of cryoprotectant treatment. The viability of sperm equilibrated with the cryoprotectant for 120 minutes was greater than sperm equilibrated for 0 or 30 minutes (33.1% compared with 30.5% and 31.0% respectively; $P < 0.01$), with the viability of sperm after 60- and 90-minute equilibration being intermediate (32.3% and 31.9%, respectively).

There was no effect of cryoprotectant or equilibration time on detectable DFI (range: 9.4%–9.5%), or mean DFI (range: 217.6–219.2).

3.2. Experiment 2: Effect of cryoprotectant

There were statistical interactions between cryoprotectant and sex sorting on total motility ($P < 0.01$) and viability ($P < 0.001$). Although there was no effect of cryoprotectant on the total motility of sex-sorted sperm, the total motility of nonsorted sperm cryopreserved with DMF alone was greater than that of sperm cryopreserved with either GLY alone or GLY:DMF ($P < 0.001$; Table 1). Conversely, whereas cryopreservation of sex-sorted sperm with either GLY:DMF or DMF alone resulted in significantly higher viability than GLY alone ($P < 0.05$), the viability of nonsorted sperm was not affected by cryoprotectant type (Table 1).

Because there were no significant interactions between any factors (stallion, cryoprotectant, and sex sorting) on SCSA parameters, data were pooled to observe main effects. There were main effects of sex sorting on detectable DFI and mean DFI such that sex-sorted sperm had higher detectable DFI ($P < 0.001$) and mean DFI ($P < 0.001$) than nonsorted sperm. Cryopreservation with GLY alone or GLY:DMF resulted in a higher mean DFI than DMF alone for nonsorted and sex-sorted sperm (Table 1).

3.3. Experiment 3: Effect of concentration of cryoprotectant

An interaction between sex sorting and cryoprotectant on total motility ($P < 0.001$) showed that though there was no effect of cryoprotectant on the total motility of sex-sorted sperm, total motility of nonsorted sperm was significantly improved by DMF compared with GLY (37.9% vs. 31.3%, respectively). There was an interaction between sex sorting and postthaw time on total motility ($P < 0.001$); although there was no difference between the total motilities of nonsorted and sex-sorted sperm immediately after thawing, the motility of sex-sorted sperm declined rapidly after thawing and by 45 minutes, the total motility of sex-sorted sperm was significantly lower than that of nonsorted sperm (Fig. 1).

An interaction between cryoprotectant concentration and sex-sorting on postthaw viability was observed ($P < 0.001$), such that the viability of nonsorted sperm decreased significantly as the concentration of cryoprotectant increased, though the concentration of cryoprotectant had no effect on the viability of sex-sorted sperm (Table 2). There was a main effect of sex sorting on sperm viability, such that the viability of sex-sorted sperm (12.4%) was lower than that of nonsorted sperm (30.0%; $P < 0.001$).

Interactions between cryoprotectant type and sex sorting on SCSA parameters were observed (detectable DFI, $P = 0.015$ and mean DFI, $P < 0.05$), such that whereas SCSA parameters of sex-sorted sperm were significantly improved by the use of DMF compared with GLY, there was no effect of cryoprotectant type on the SCSA parameters of nonsorted sperm (Table 3). There was an overall effect of sex sorting on SCSA parameters; sex-sorted sperm had significantly higher detectable DFI (63.6% vs. 11.3%, $P < 0.001$) and mean DFI (285.1% vs. 211.3%; $P < 0.001$) than nonsorted sperm.

Table 1
Interaction between cryoprotectant and sorting treatment on the total motility, viability, and mean DFI of stallion sperm.

Cryoprotectant	Total motility (%)		Viability (%)		Mean DFI	
	SS	NS	SS	NS	SS	NS
GLY	7.6 ± 1.0[a]	15.8 ± 1.6[a]	19.3 ± 1.0[a]	22.5 ± 1.5[a]	283.9 ± 7.1[a]	261.9 ± 3.6[a]
GLY:DMF	7.3 ± 1.1[a]	16.6 ± 1.6[a]	22.6 ± 1.0[b]	20.5 ± 1.3[a]	286.5 ± 4.5[a]	260.7 ± 4.6[a]
DMF	8.4 ± 1.3[a]	24.0 ± 2.0[b]	25.3 ± 1.2[b]	19.9 ± 1.3[a]	277.8 ± 5.4[b]	253.0 ± 5.1[b]

Data were pooled for stallion and postthaw time. Values are given as mean ± SEM.
Abbreviations: DFI, DNA fragmentation index; DMF, dimethyl formamide; GLY, glycerol; NS, nonsorted; SS, sex-sorted.
[a,b] Within a column, means without a common superscript differed ($P < 0.05$).

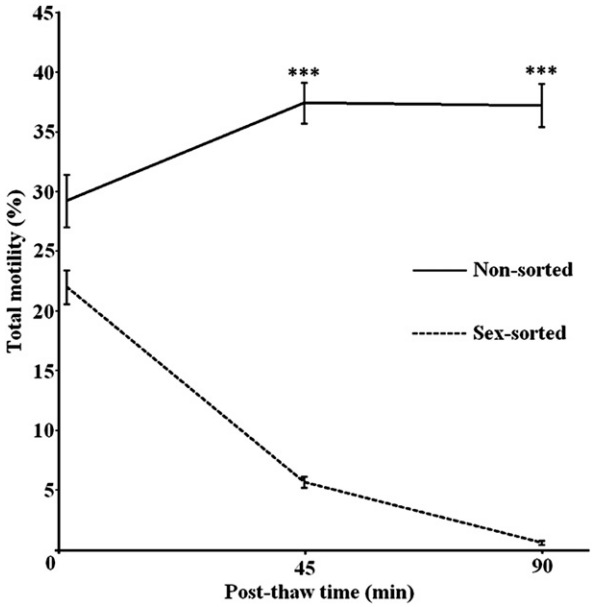

Fig. 1. Mean ± SEM postthaw motility of nonsorted and sex-sorted stallion sperm over time (data pooled for cryoprotectant type and concentration). *** $P < 0.001$.

4. Discussion

During this study, the protocol for the cryopreservation of liquid stored, sex-sorted stallion sperm was optimized by comparing the cryoprotective abilities of GLY and DMF, alone and in combination (GLY:DMF), using a two-step dilution procedure. Postthaw characteristics of nonsorted sperm were significantly improved after equilibration of sperm for 90 minutes with DMF. The parameters to benefit most from cryopreservation with DMF differed between sex-sorted and nonsorted sperm. Although cryopreservation of sex-sorted sperm with DMF significantly improved viability and DNA integrity, the motility characteristics of nonsorted sperm were predominantly improved by the use of DMF.

The superior postthaw results achieved using DMF might be because of a reduction in the osmotic stress exerted on sperm. The lower molecular weight of DMF compared with GLY allows it to pass more freely across membranes, thereby reducing the amount of time that the sperm are hypotonic or hypertonic [37]. Osmotic stress not only damages sperm membranes, but also leads to the production of reactive oxygen species (ROS) which in turn induce DNA damage [38]. Because sperm membranes and DNA are destabilized during the processes of sex sorting and cryopreservation [39,40], the use of DMF to reduce the damage associated with osmotic stress and ROS is an appealing strategy to improve the postthaw function of these already susceptible cells.

Amides, including DMF, have previously been identified as effective cryoprotectants and as an alternative to GLY, particularly with respect to improving the *in vitro* quality and fertility of sperm from stallions that are classified as 'poor freezers' because of the increased susceptibility of their sperm to cryoinjury [19,20,41,42]. It is therefore not surprising that the viability of sex-sorted sperm, which are also more susceptible to cryoinjury, can be improved by the use of DMF. This finding is supported by a previous study in which DMF was shown to be the cryoprotectant of choice over GLY for the cryopreservation of thawed, sex-sorted refrozen stallion sperm [43]. Despite this, in several studies, GLY was superior to DMF for cryopreservation of nonsorted stallion sperm [11,44]. Sieme et al. [44] reported that cryopreservation of Warmblood stallion sperm with GLY produced more favorable results than cryopreservation with DMF [44]. However, it should be noted that for entry into the Warmblood stud book, a stallion's sperm must have postthaw motilities in excess of 35% for them to be approved as breeding animals, making them 'good freezers' [45], and therefore poor models for improving freezability.

The addition of the cryoprotectant at 30 minutes into a 120-minute cooling period (90-minute equilibration) resulted in superior postthaw sperm quality compared with the addition of cryoprotectants at room temperature at the start of the cooling period. This contrasts with the findings of a previous investigation in which the motility and fertility of stallion sperm were significantly higher when GLY was added at room temperature (22 °C) as opposed to 4 °C [46]. However because centrifugation was also performed at the two experimental temperatures, it is likely that centrifugation at 4 °C, and not cryoprotectant addition at 4 °C contributed to the deleterious effects, because of the reduced fluidity of the sperm membranes at lower temperatures [47]. This argument was supported by

Table 2
Interaction between cryoprotectant, cryoprotectant concentration, and sorting treatment on the viability of stallion sperm.

Cryoprotectant	Concentration (% vol/vol)	Sex-sorted (% viable)	Nonsorted (% viable)
GLY	2	15.2 ± 1.4[a]	32.7 ± 1.3[a]
	3	13.8 ± 1.5[a]	30.9 ± 1.3[b]
	4	12.7 ± 1.5[a]	25.5 ± 0.9[c]
DMF	2	11.0 ± 1.5[a]	34.1 ± 1.5[a]
	3	11.4 ± 1.6[a]	30.8 ± 1.2[b]
	4	10.2 ± 1.5[a]	26.3 ± 1.0[c]

Data were pooled for postthaw time, cryoprotectant, and stallion. Values are given as mean ± SEM.
Abbreviations: DMF, dimethyl formamide; GLY, glycerol.
[a–c] Within a column and cryoprotectant type, means without a common superscript differed ($P < 0.05$).

Table 3
Interaction between cryoprotectant and sorting treatment on SCSA parameters of stallion sperm.

Sorting treatment	Cryoprotectant	Detectable DFI	Mean DFI
Sex-sorted	GLY	66.8 ± 5.3[a]	289.2 ± 9.7[a]
	DMF	60.2 ± 6.0[b]	280.9 ± 8.6[b]
Nonsorted	GLY	11.1 ± 0.8[a]	211.0 ± 1.9[a]
	DMF	11.4 ± 0.7[a]	211.7 ± 1.9[a]

Data were pooled over cryoprotectant concentration and stallion. Values are given as mean ± SEM.
Abbreviations: DFI, DNA fragmentation index; DMF, dimethyl formamide; GLY, glycerol; SCSA, sperm chromatin structure assay.
[a,b] Within a column and sorting treatment, means without a common superscript differed ($P < 0.05$).

a significant decline in the postthaw viability of avian sperm when cryoprotectants were added at room temperature as opposed to 4 °C [48].

The optimal prefreeze cooling rates of stallion sperm has previously been investigated [9,11], but there are no reports on the optimal length of time to equilibrate stallion sperm with the cryoprotectant before freezing. It was hypothesized that the smaller molecular mass of DMF allows it to equilibrate across the cell membrane more rapidly than GLY. Despite this, in the present study, there was no difference between the optimal equilibration times of GLY and DMF, with both being 90 minutes before cryopreservation. There is a marked difference among species in their optimal cryoprotectant equilibration times. Dehydration of bull sperm on exposure to a cryoprotectant occurs extremely quickly, with an optimal equilibration time of less than 2 minutes before cryopreservation [25]. In contrast, the optimum equilibration time for cryopreservation of monkey sperm is 75 minutes [27], and increasing cryoprotectant equilibration time increased fertility of oyster sperm [26].

During this study, sperm were stored for 12 to 18 hours at approximately 10 °C before sorting. This storage step was necessary for logistical reasons associated with the proximity of the stallions to the sorting facility. Previous studies have shown that liquid storage of stallion semen for 12 to 24 hours before cryopreservation is not detrimental to motility or fertility [12,49,50], though none of these studies extended beyond initial pregnancy detection at 15 days. It is quite likely that the combined insults of storage and sorting before cryopreservation are responsible for the high levels of DNA damage during the present study in which an increase in DNA fragmentation rates after sex-sorting was observed in all stallions. There is a distinct lack of information regarding DNA integrity of stallion sperm after flow cytometric sex sorting. Bochenek et al. [40] reported that when data were averaged across stallions, there was no detrimental effect of sex sorting on sperm chromatin. However, there was a significant stallion effect, with sperm from a particular stallion displaying rates of DNA damage as high as 56.5% after sex sorting alone, indicating that the SCSA assay should be routinely used after sorting to screen stallions and determine the effects of sorting on sperm DNA integrity.

Based on these findings, stallion sperm were significantly more vulnerable to the cumulative effects of sex-sorting and cryopreservation than sperm of other species. For example, bull sperm have a high osmotic tolerance [51], and therefore do not suffer high levels of cryoinjury involving ROS-induced DNA damage. In fact, sex-sorted, cryopreserved bull sperm had higher DNA stability than nonsorted cryopreserved sperm [52]. Stallion sperm has been shown to have a similar osmotic tolerance to that of boar sperm [53], approximately 100 times lower than that of bull sperm [51,54], predisposing stallion sperm to high levels of cryoinjury.

4.1. Conclusions

The *in vitro* postthaw characteristics of liquid stored, cryopreserved stallion sperm, sex-sorted and nonsorted, were significantly improved by a cryopreservation protocol involving a 2-hour cooling period with addition of 2% (final % vol/vol) DMF after 30 minutes of cooling and allowing a 90-minute equilibration before cryopreservation. Such incremental improvements to the cryopreservation process will contribute to advance the use of sperm sex sorting and cryopreservation technologies in the horse industry.

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