Sucrose and bovine serum albumin in association with dimethylformamide improved sperm parameters of frozen stallion semen

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Abstract

Postthaw stallion sperm integrity was determined after freezing semen in an extender supplemented with several sucrose concentrations, bovine serum albumin (BSA), and with and without dimethylformamide (DMF). Two ejaculates from 6 stallions (n =12) were diluted and aliquots (n = 7) were made for treatment groups (25 mM sucrose + BSA [S25], S25 + DMF [S25DMF], 50 mM sucrose + BSA [S50], S50 + DMF [S50DMF], 100 mM sucrose + BSA [S100], S100 + DMF [S100DMF], and Control (only DMF). Semen was frozen in a computer-controlled freezer. Sperm postthaw motility (total and progressive) and kinetics were assessed using CASA. Postthaw sperm plasma membrane and acrosomal integrity were evaluated using SYBR-14/PI and FITC-PNA, respectively. Sperm motility was higher in S100DMF and S50DMF. Plasma membrane integrity was higher in S100DMF, S50DMF, and S100. As sucrose concentration increased, plasma membrane integrity increased. Treatment groups with sucrose and BSA, regardless of DMF, had higher acrosome integrity than Control. Sucrose and BSA in association with DMF in a freezing extender protected sperm integrity during freezing and thawing.

Keywords: Stallion sperm, freezing, sucrose, dimethylformamide, plasma membrane

Introduction

Successful semen cryopreservation enhances benefits (long-distance semen transport, international trade, and extended use of superior genetic material) of artificial insemination (AI) over natural breeding.[1,2] Glycerol (GLY) is commonly used as a permeable cryoprotectant for stallion semen cryopreservation; however, GLY reduced sperm fertility.[3] Dimethylformamide (DMF) was as effective as GLY in protecting stallion sperm from cryodamage.[4,5] Furthermore, DMF improved postthaw sperm motility and plasma membrane integrity.

Nonpermeable cryoprotectants (e.g., sucrose and trehalose) had beneficial effects during freezing in several species (rodents,[7,8] rabbits,[9] cattle,[10] sheep,[11,12] and pigs[13]). In stallions, sucrose[14-16] and trehalose[16,17] were used. A skim-milk egg yolk (SMEY) extender with sucrose and bovine serum albumin (BSA) instead of GLY[14] improved stallion sperm postthaw sperm motility and plasma membrane integrity compared to sperm frozen in a SMEY-GLY freezing extender. Furthermore, donkey semen frozen in a SMEY freezing extender with sucrose and BSA without GLY had similar sperm postthaw motility and higher plasma membrane integrity compared to semen frozen with a SMEY-GLY freezing extender.[15] Improved sperm postthaw motility and plasma membrane were attributed to sucrose effects in reducing osmotic stress[9] and BSA preventing lipid peroxidation.[18]

Cryopreservation of stallion semen with sucrose and BSA in combination with DMF has not been reported. We investigated sperm postthaw integrity of semen frozen: a) in extenders containing DMF, several sucrose concentrations, and 1% bovine serum albumin (BSA); b) in extenders without DMF, several sucrose concentrations, and 1% bovine serum albumin (BSA); and c) extender with only DMF.

Materials and methods

Animals

During summer in the Southern Hemisphere, semen was collected from stallions (n = 6) of known fertility, ranging in age from 3 to 16 (10.8 ± 3.5) years. Except for 1 stallion (semen was frozen earlier using DMF) semen freezing capability was not known. Animal use and handling procedures were approved (University of Adelaide Office of Research Ethics, Compliance, and Integrity).

Semen collection

Stallions that were not actively breeding had 3 semen collections prior to use.[19] Two days after the last semen collection, stallions had their semen collected for freezing, a day apart between the first and second semen collection. Semen was collected using a Hannover or Missouri-model artificial vagina with an inline gel filter (Minitube Australia, Australia). Stallion had their penis washed with warm water and collection was performed over a phantom mount.

Semen preparation

Immediately after collection, total volume of gel-free semen was determined using a 50 ml conical tube (Corning®, MA), and sperm concentration (10^6/ml) was determined using an automated cell counter (NucleoCounter SP-100, ChemoMetec, Denmark). Sperm motility and membrane integrity were assessed (described later). Semen from ejaculates (n = 12) was extended 1:1 with Equiplus (Minutube Australia) and centrifuged for 10 minutes at 1,000 x g. Supernatant was aspirated with a vacuum, sperm pellet was resuspended and diluted with Equiplus to a concentration of 400 x 10^6 sperm/ml. Resuspended semen was transferred to semen freezing room (18°C) and divided into 7 equal aliquots.

Experimental design

Treatment groups (Table 1) were: S25 (Equiplus, 1% [w/v] BSA and 25 mM of sucrose [Sigma-Aldrich, Australia]); S25DMF (S25 with 2% DMF); S50 (Equiplus, 1% BSA and 50 mM of sucrose); S50DMF (S50 with 2% DMF); S100 (Equiplus, 1% BSA and 100 mM of sucrose); S100DMF (S100 with 2% DMF); and Control (Equiplus with 2% (v/v) DMF (Sigma-Aldrich). Freezing extenders were prepared in advance, divided into aliquots and stored at – 80°C until used. Because semen samples were diluted (1:1) with treatment groups before freezing, freezing extenders were prepared at double the cryoprotectants concentration to reach the final concentration (200 x 10^6 sperm/ml) of treatment groups after dilution. After dilution, samples' osmolality was assessed using an osmometer (Advanced™ Micro-Osmometer, Model 3 MO Plus, ThermoFisher Scientific, Waltham, MA) that ranged from 379 to 769 mOsm kg^{-1} (Table 1).

Table 1. Freezing extenders and osmolality (mOsm kg^{-1}) of each extender

Treatment	Freezing extender	mOsm kg^{-1}
Control	Equiplus + DMF	635
S25	Equiplus + sucrose (25 mM) + BSA	379
S25DMF	Equiplus + sucrose (25 mM) + BSA + DMF	556
S50	Equiplus + sucrose (50 mM) + BSA	401
S50DMF	Equiplus + sucrose (50 mM) + BSA + DMF	657
S100	Equiplus + sucrose (100 mM) + BSA	447
S100DMF	Equiplus + sucrose (100 mM) + BSA + DMF	769

DMF = 2% v/v (0.26 M) dimethylformamide
BSA = 1% w/v bovine serum albumin

Semen freezing

Semen was frozen using a computer-controlled rate freezer (IceCube 14 S-A 230V, Minitube Australia). Semen samples were then drawn into 0.5 ml plastic straws (Minitube Australia) and sealed with a sealing ball. Straws were placed horizontally into the computer-controlled rate freezer. Cooling rate from 18°C to 4°C was 0.3°C/minute and from 4°C to –160°C was 60°C/minute. Straws were then plunged into liquid nitrogen (–196°C) and stored in liquid nitrogen tanks. After at least a week after freezing, 2 straws from each treatment group were thawed in a water bath at 37°C for 30 seconds.

Sperm motility

After collection, an aliquot (1 ml) of fresh semen was extended with warm (37°C) Equiplus to a concentration of 50 x 10^6 sperm/ml before motility assessment. Postthaw sperm motility was assessed immediately after thawing at (0,15, and 30) minutes.

Before sperm motility assessment of frozen-thawed semen and to maintain osmolality consistency among treatments, each sample was diluted with its corresponding treatment to a concentration of 50 x 10^6 sperm/ml. Five µl of fresh and/or frozen-thawed semen were placed over a warm glass slide (25 x 75 mm), covered with a coverslip (22 x 22 mm) and 3 fields were evaluated at 200 x magnification using CASA (AndroVision®, Minitube Australia). Analysis was performed using an image capture of 60 frames/second. Sperm motility and kinematics assessed were: total motility (TM %, VCL < 35 µm/s and VSL < 15 µm/s), progressive motility (PM %, VCL ≥ 35 µm/s and VSL ≥ 15 µm/s), sperm curvilinear velocity (VCL, µm/s), sperm linear velocity (VSL, µm/s), average path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), and beat-cross frequency (BCF, Hz/s).

Plasma and acrosomal membrane integrity

Fresh and thawed semen were diluted to a 1 x 10^6 sperm/ml with phosphate buffered saline (osmolality adjusted to match treatment groups). For sperm plasma membrane integrity (PMI), diluted semen was mixed with 1 µl of the SYBR-14 and propidium iodide (SYBR-14/PI) solution (Reference [15407/0001], Minitube Australia) and incubated in dark for 10 minutes. Fluorescent probes were excited with 488 nm (SYBR-14) and 561 nm (PI) lasers. Sperm emitting in red wavelength were considered plasma membrane 'damaged' and sperm emitting in green wavelength were considered 'intact'.

For acrosome membrane integrity (ACR), an aliquot of semen was diluted to a 1 x 10^6 sperm/ml, mixed with 1 µl of the Fluorescein isothiocyanate (FITC) – peanut agglutinin (PNA) stain (Sigma-Aldrich) and incubated in the dark for 10 minutes. Fluorescent probe was excited with a 488 nm laser. Sperm emitting in green wavelength were considered 'damaged' and unstained sperm were considered 'intact'.

For plasma and acrosomal membrane, assessment was made in duplicates from each sample using an Attune NxT Flow Cytometer (Thermofisher, Australia). At least 10,000 sperm were evaluated before and after cryopreservation.

Data analyses

Equality of variance was assessed using Levene's test. Square root of VCL (0 and 30 minutes) and ALH (0 minute) were used for analysis. Effect of treatment on the mean (± SEM) postthaw TM (%), PM (%), VCL, VSL, VAP, ALH, and BCF at 0, 15, and 30 minutes were evaluated using analysis of variance (ANOVA; IBM SPSS statistics 26, US). Effect of treatment on the mean (± SEM) percentage postthaw PMI and ACR was also assessed using ANOVA. Level of significance was set at $p < 0.05$. When significance was observed, a pairwise comparison using Tukey's method was performed to assess differences among treatments.

Results

Ejaculates (n = 12) had the following (mean ± SEM) characteristics before freezing: gel-free volume = 58.9 ± 7.8 ml, concentration = 205.1 ± 30.3 x 10^6/ml, TM = 73.4 ± 2.3%, PM = 72.2 ± 2.3%, intact PMI = 71.1 ± 2.3%, and intact ACR = 91.3 ± 0.7%. There was no difference ($p < 0.05$) among ejaculates for the parameters evaluated.

There was an effect ($p < 0.05$) of treatment on postthaw sperm motility and kinematic parameters. Postthaw sperm motility (TM and PM) and sperm kinematic parameters (VCL, VSL, VAP, BCF, and ALH) at 0, 15, and 30 minutes in treatment groups S50DMF, S100DMF, and Control were the highest (Table 2 and 3). Stallion sperm frozen in treatment groups S25 and S100 had the lowest postthaw sperm motility (TM and PM) and kinematics in most of the measured time points (Figures 1 and 2).

Table 2. Postthaw (mean ± SEM) TM and PM at 0, 15, and 30 minutes of stallion sperm frozen with different sucrose concentrations +/- DMF (2%) and BSA (1%)

Treatment	Time (minutes)	TM (%)	PM (%)
DMF	0	32.1 ± 4.3^{ab}	31.0 ± 4.2^{ab}
	15	29.1 ± 4.0^{ab}	29.5 ± 4.0^{abc}
	30	24.6 ± 4.6^{ab}	23.6 ± 4.5^{ab}
S25	0	14.7 ± 4.3^{c}	14.0 ± 4.2^{c}
	15	16.7 ± 4.0^{c}	15.8 ± 4.0^{d}
	30	15.1 ± 4.6^{c}	14.5 ± 4.5^{b}
S25DMF	0	23.0 ± 4.3^{abc}	22.3 ± 4.2^{abc}
	15	27.2 ± 4.0^{abc}	25.9 ± 4.0^{abcd}
	30	22.5 ± 4.6^{ab}	21.7 ± 4.5^{ab}
S50	0	19.3 ± 4.3^{bc}	18.4 ± 4.2^{bc}
	15	21.3 ± 4.0^{bc}	20.5 ± 4.0^{bcd}
	30	23.2 ± 4.6^{ab}	22.2 ± 4.5^{ab}
S50DMF	0	33.3 ± 4.3^{a}	32.1 ± 4.2^{a}
	15	36.1 ± 4.0^{a}	34.8 ± 4.0^{a}
	30	33.8 ± 4.6^{a}	32.8 ± 4.5^{a}
S100	0	16.8 ± 4.3^{c}	16.0 ± 4.2^{c}
	15	19.8 ± 4.0^{c}	18.8 ± 4.0^{cd}
	30	19.2 ± 4.6^{b}	18.4 ± 4.5^{b}
S100DMF	0	33.5 ± 4.3^{a}	32.0 ± 4.2^{a}
	15	34.4 ± 4.0^{a}	32.1 ± 4.2^{ab}
	30	35.2 ± 4.6^{a}	33.9 ± 4.5^{a}

Rows and columns without common superscripts differed ($p < 0.05$)

Table 3. Postthaw (mean ± SEM) VCL, VSL, VAP um/s, ALH, BCG and HAC at 0, 15, and 30 minutes of stallion sperm frozen with several sucrose concentrations +/- DMF (2%) and BSA (1%)

Treatment	Time (minutes)	VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	ALH um	BCF (Hz)	HAC (rad)
DMF	0	27.3 ± 3.2^{a}	20.0 ± 2.3^{a}	20.7 ± 2.4^{a}	0.7 ± 0.1^{a}	1.6 ± 0.2	0.2 ± 0.1^{a}
	15	23.3 ± 3.0^{bc}	17.4 ± 2.5^{bc}	18.0 ± 2.6^{abc}	0.6 ± 0.1^{ab}	1.6 ± 0.2^{ab}	0.2 ± 0.0^{ab}
	30	22 ± 3.3^{ab}	17.0 ± 2.7^{abc}	17.5 ± 2.7^{ab}	0.5 ± 0.1^{ab}	1.6 ± 0.2	0.1 ± 0.0^{abc}
S25	0	13.9 ± 3.2^{b}	10.4 ± 2.3^{b}	10.7 ± 2.4^{b}	0.4 ± 0.1^{b}	1.5 ± 0.2	0.1 ± 0.1^{b}
	15	13.9 ± 3.0^{d}	10.2 ± 2.5^{c}	10.6 ± 2.6^{c}	0.4 ± 0.1^{c}	1.1 ± 0.2^{b}	0.1 ± 0.0^{c}
	30	14.2 ± 3.3^{c}	11.1 ± 2.7^{c}	11.3 ± 2.7^{c}	0.4 ± 0.1^{b}	1.4 ± 0.2	0.1 ± 0.0^{c}
S25DMF	0	20.9 ± 3.2^{ab}	15.5 ± 2.3^{ab}	16.0 ± 2.4^{ab}	0.5 ± 0.1^{ab}	1.6 ± 0.2	0.1 ± 0.1^{ab}
	15	23.6 ± 3.0^{abc}	17.3 ± 2.5^{bc}	18.0 ± 2.6^{abc}	0.6 ± 0.1^{ab}	1.5 ± 0.2^{ab}	0.2 ± 0.0^{abc}
	30	18.3 ± 3.3^{ab}	11.1 ± 2.7^{abc}	14.5 ± 2.7^{bc}	0.5 ± 0.1^{ab}	1.6 ± 0.2	0.1 ± 0.0^{bc}
S50	0	14.0 ± 3.2^{b}	10.1 ± 2.3^{b}	10.5 ± 2.4^{b}	0.4 ± 0.1^{b}	1.3 ± 0.2	0.1 ± 0.1^{b}
	15	19.4 ± 3.0^{bcd}	15.5 ± 2.5^{bc}	15.8 ± 2.6^{bc}	0.5 ± 0.1^{bc}	1.5 ± 0.2^{ab}	0.1 ± 0.0^{bc}
	30	27.3 ± 3.3^{a}	20.7 ± 2.7^{ab}	21.0 ± 2.7^{a}	0.6 ± 0.1^{a}	1.7 ± 0.2	0.2 ± 0.0^{ab}
S50DMF	0	27.1 ± 3.2^{a}	19.8 ± 2.3^{a}	20.5 ± 2.4^{a}	0.7 ± 0.1^{a}	1.7 ± 0.2	0.2 ± 0.1^{a}
	15	32.4 ± 3.0^{a}	24.9 ± 2.5^{a}	25.6 ± 2.6^{a}	0.7 ± 0.1^{a}	1.8 ± 0.2^{a}	0.2 ± 0.0^{a}
	30	16.6 ± 3.3^{c}	12.7 ± 2.7^{bc}	12.4 ± 2.7^{c}	0.5 ± 0.1^{ab}	1.4 ± 0.2	0.1 ± 0.0^{c}
S100	0	13.8 ± 3.2^{b}	9.3 ± 2.3^{b}	9.4 ± 2.4^{b}	0.4 ± 0.1^{b}	1.2 ± 0.2	0.1 ± 0.1^{b}
	15	16.4 ± 3.0^{cd}	11.7 ± 2.5^{c}	11.2 ± 2.6^{c}	0.5 ± 0.1^{bc}	1.4 ± 0.2^{ab}	0.1 ± 0.0^{bc}
	30	16 ± 3.3^{c}	11.2 ± 2.7^{c}	11.6 ± 2.7^{c}	0.4 ± 0.1^{ab}	1.3 ± 0.2	0.1 ± 0.0^{c}
S100DMF	0	27.8 ± 3.2^{a}	18.0 ± 2.3^{a}	19.0 ± 2.4^{a}	0.8 ± 0.1^{a}	1.6 ± 0.2	0.2 ± 0.1^{a}
	15	27.6 ± 3.0^{ab}	20.2 ± 2.5^{ab}	20.8 ± 2.5^{ab}	0.7 ± 0.1^{a}	1.7 ± 0.2^{a}	0.2 ± 0.0^{a}
	30	29 ± 3.3^{a}	21.4 ± 2.7^{a}	22.1 ± 2.7^{a}	0.6 ± 0.1^{a}	1.6 ± 0.2	0.2 ± 0.0^{a}

Rows and columns without common superscripts differed ($p < 0.05$)

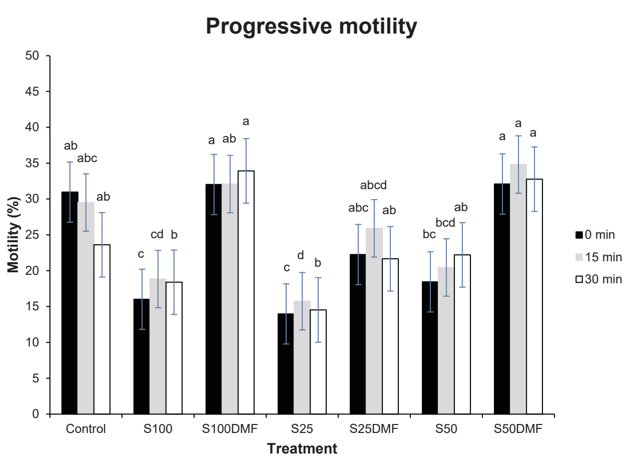

Figure 1. Postthaw (mean ± SEM) TM at 0, 15, and 30 minutes of stallion sperm frozen with several sucrose concentrations, +/- DMF and BSA; bars without common letters within treatment groups differed ($p < 0.05$).

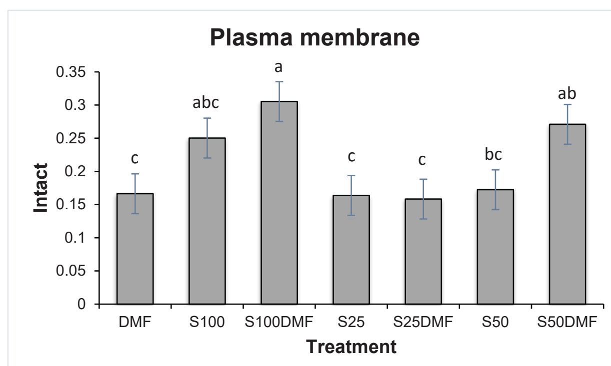

Figure 2. Postthaw (mean ± SEM) PM at 0, 15, and 30 minutes of stallion sperm frozen with several sucrose concentrations, +/- DMF and BSA; bars without common letters differed ($p < 0.05$) among treatment groups.

There was an effect ($p < 0.05$) of treatment on postthaw sperm PMI and ACR integrity. Postthaw PMI integrity of sperm frozen in treatment groups S100DMF, S50DMF, and S100 were higher than Control, S25, S25DMF, and S50 (Figure 3). As sucrose concentrations increased, postthaw intact PMI integrity increased, regardless of DMF presence (Figure 3). Postthaw sperm ACR integrity in treatment groups S100, S50, S25, S100DMF, and S50DMF was higher than sperm frozen in Control (Figure 4).

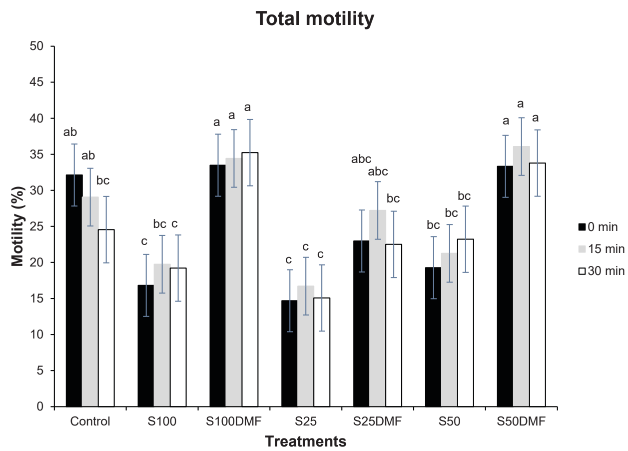

Figure 3. Mean (± SEM) postthaw intact plasma membrane (PMI) of stallion sperm frozen with different sucrose concentrations +/- DMF (2%) and BSA (1%); bars without common letters within treatments groups differed ($p < 0.05$).

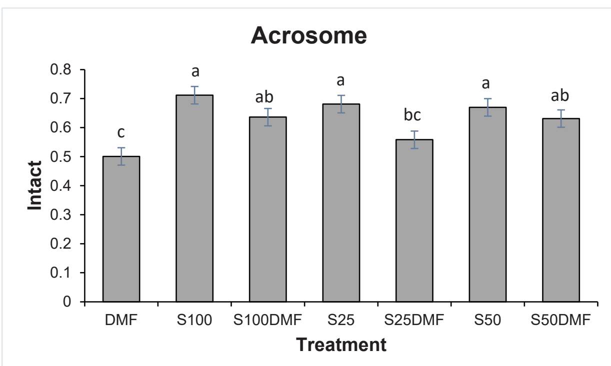

Figure 4. Mean (± SEM) postthaw intact acrosome (ACR) of equine sperm frozen with different sucrose concentrations +/- DMF and BSA; bars without common letters differed ($p < 0.05$) among treatment groups

Discussion

Freezing extender containing sucrose (50 and 100 mM), BSA, and DMF improved postthaw sperm integrity compared to sperm frozen in a freezing extender containing DMF only (Control). Postthaw sperm total and progressive motility, and kinematics of semen frozen in extenders containing sucrose and BSA in association with DMF (S50DMF and S100DMF) were either higher or similar to Control. Osmolality of the extenders in treatment groups S50DMF (657 mOsm kg^{-1}), S100DMF (769 mOsm kg^{-1}) and DMF (635 mOsm kg^{-1}) were higher than the 500 mOsm kg^{-1} reported osmotic tolerance threshold for stallion sperm to maintain motility.[20] Nevertheless, the reported[20] osmotic tolerance threshold GLY and use of DMF in our study may have reduced the osmotic stress suffered by the sperm due to its lower molecular weight (MW = 73.10 g/mol).[6]

There was no interaction between sucrose and permeable cryoprotectants (dimethylacetamide, DMF or GLY) preserving PMI sperm integrity during freezing stallion semen,[16] possibly by the presence of egg yolk (10%) in the freezing extender. We did not use egg yolk and higher concentrations of sucrose (S100DMF and S50DMF) better preserved plasma membrane integrity. Furthermore, that S50DMF treatment group PMI integrity was higher than the S50 treatment and not different than S100DMF, suggested that sucrose positively interacted with DMF. Beneficial effects of this association may be due to the following. Sucrose associated with membrane phospholipids head, stabilized membrane bilayer during freezing[21] via electrostatic binding of saccharide hydroxyl groups to phosphate groups on the membrane lipid head,[22] reduced ice crystal formation[23-25] and provided an energy substrate.[26] Additionally, DMF stabilized the membrane bilayer,[21] modulated cell dehydration,[24] and decreased osmotic tension of the unfrozen fraction.[27] Furthermore, BSA antioxidant properties prevented lipid peroxidation that is involved in motility loss following cryopreservation.[28]

During fertilization, viable sperm undergo a morphological change known as capacitation.[1] Freezing-thawing stallion sperm induce capacitation-like changes, increasing the proportion of sperm with reacted acrosomes.[29] In our study, postthaw acrosome integrity was better preserved with sucrose. Similar to results of PMI integrity, as sucrose concentrations increased, postthaw acrosome integrity preservation improved. However, our results did not appear to suggest that sucrose and DMF interacted to improve acrosome protection during freezing-thawing of stallion sperm.

Minimum acceptable postthaw motility of frozen semen for commercial use is 30%.[2,30] Extenders S50DMF and S100DMF achieved a postthaw sperm total and progressive motility > 30% immediately and it was maintained until 30 minutes after thawing. Additionally, S50DMF, and S100DMF extenders had higher postthaw intact PMI and ACR. This suggested that freezing extenders containing sucrose and BSA in association with DMF may be an alternative for freezing stallion semen.

Our study had some limitations. Freezing extender containing sucrose (50 and 100 mM), BSA, and DMF appeared acceptable protecting stallion sperm during freezing-thawing; however, the optimal concentration of sucrose for stallion sperm cryopreservation must be determined. Although extenders with sucrose, BSA, and DMF, achieved a postthaw motility above the recommended 30%, the fertilization capacity of the thawed sperm is unknown. Further research must investigate the optimal concentration of sucrose and fertilizing capacity after cryopreservation of stallion sperm with sucrose extenders.

Combining sucrose BSA and DMF in the freezing extender may be an alternative for cryopreservation of stallion semen. As concentrations of sucrose increased in combination with DMF, the postthaw PMI and ACR integrity improved.

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University of Adelaide School of Animal and Veterinary Sciences.

Conflict of interest

None to declare.

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