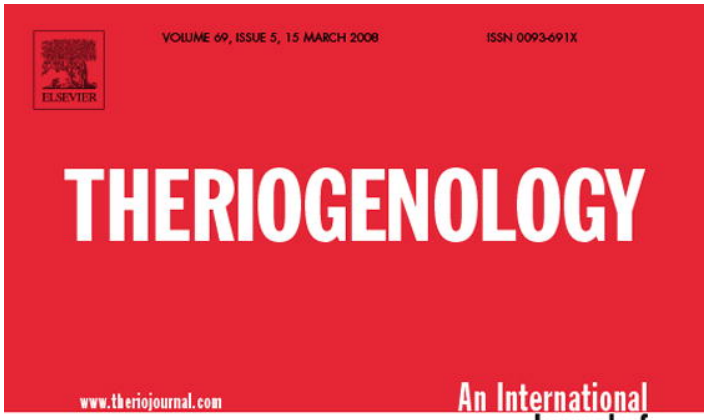

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Evaluation of amides and centrifugation temperature in boar semen cryopreservation

I. Bianchi [a], K. Calderam [a], É.F. Maschio [a], E.M. Madeira [a], R. da Rosa Ulguim [a], C.D. Corcini [a], D.C. Bongalhardo [b], É.K. Corrêa [a], T. Lucia Jr.[a], J.C. Deschamps [a], M.N. Corrêa [c,*]

[a] *PIGPEL, Centro de Biotecnologia, Brazil*
[b] *Instituto de Biologia, Brazil*
[c] *NUPEEC (Núcleo de Pesquisa, Ensino e Extensão em Pecuária), Departamento de Clínicas Veterinária, Faculdade de Veterinária, Campus Universitário s/n°, Universidade Federal de Pelotas, Pelotas, CEP 96010-900, RS, Brazil*[1]

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Abstract

Two experiments were conducted to evaluate the use of amides as cryoprotectants and two centrifugation temperatures (15 or 24 °C) in boar semen cryopreservation protocols. Semen was diluted in BTS, cooled centrifuged, added to cooling extenders, followed by the addition of various cryoprotectants. In experiment 1, mean (±S.E.M.) sperm motility for 5% dimethylformamide (DMF; 50.6 ± 1.9%) and 5% dimethylacetamide (DMA; 53.8 ± 1.7%) were superior ($P < 0.05$) to 5% methylformamide (MF; 43.2 ± 2.4%) and 3% glycerol (GLY; 38.1 ± 2.3%), with no significant difference between MF and GLY. Sperm membrane integrity was higher ($P < 0.05$) for DMA than for MF or GLY (50.9 ± 1.9, 43.3 ± 2.5, and 34.5 ± 2.8%, respectively). Sperm membrane integrity was higher in DMF (47.9 ± 2.1%) than in glycerol (34.5 ± 2.8%, $P < 0.05$), but was similar to other treatments ($P > 0.05$). In experiment 2, we tested MF, DMF, and DMA at 3, 5, and 7%. Sperm motility and membrane integrity were higher for 5% DMA (53.8 ± 1.7 and 50.9 ± 1.9%) and 5% DMF (50.6 ± 1.9 and 47.9 ± 2.1%), in comparison with 7% DMF and all MF concentrations ($P < 0.05$). For sperm motility and membrane integrity, 5% DMA exceeded ($P < 0.05$) 3% DM, with greater membrane integrity than 3% DMF ($P < 0.05$). In both experiments, sperm motility and membrane integrity were superior at 15 °C versus 24 °C ($P < 0.05$), with no interaction between centrifugation temperature and treatments ($P > 0.05$). In conclusion, boar semen was successfully cryopreserved by replacement of glycerol with amides (especially 5% DMA) and centrifugation at 15 °C, with benefits for post-thaw sperm motility and membrane integrity.

Keywords: Amides; Glycerol; Cryoprotectant; Boar semen cryopreservation; Sperm

1. Introduction

Most AI in swine are performed using liquid semen stored at temperatures between 15 and 18 °C for 1–5 days, with nearly 85% of inseminations done on the day of semen collection or the following day [1]. Some extenders have been developed to store liquid semen at these temperatures for up to 7 days [2], or at lower

* Corresponding author. Tel.: +55 53 9983 9408.
E-mail address: marcio.nunescorrea@pesquisador.cnpq.br (M.N. Corrêa).
[1] www.ufpel.edu.br/nupeec.

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temperatures, e.g. 5 °C [3]. Although frozen boar semen has been commercially available for 30 years, it is typically used under specific conditions, e.g. for importing genetics to produce breeding stock [4]. Moreover, the commercial use of frozen semen is limited as it requires two to three times more sperm per dose. Frozen semen also reduces both farrowing rate and litter size, compared to AI with liquid semen [5–9].

Glycerol (at a concentration of 3%) is the most commonly used penetrating cryoprotectant for boar sperm [7,8,10,11]. Despite its benefits, glycerol is potentially cytotoxic at some concentrations [7,12–14], and has a contraceptive effect in some species, e.g. dogs [15] and poultry [18]. The low quality of frozen–thawed boar semen is generally attributed to the composition of the extender and specifically the cryoprotectant used; therefore, it is desirable to develop a suitable cryoprotectant [19–21]. Similarly, cryopreservation of equine semen is quite challenging and recently amides have been used in lieu of glycerol [22–25]. Few studies have addressed alternative cryoprotectants for freezing boar semen; the use of amides has not been reported.

In boar semen freezing protocols, semen centrifugation is generally conducted at 15 °C [11], although one study described centrifugation at 35 °C [49]. Preliminary work conducted in our laboratory (prior to the present study) indicated that the protocol described by Westendorf et al. [11] would provide acceptable sperm motility. However, as centrifugation at 15 °C needs a refrigerated centrifuge, conducting such procedures at 24 °C would be cheaper and require less sophisticated equipment.

The objective of the present study was to evaluate the effect of methylformamide (MF), dimethylformamide (DMF) and dimethylacetamide (DMA) as penetrating cryoprotectants, in various concentrations, using two different centrifugation temperatures, in comparison with 3% glycerol, in boar semen freezing protocols.

2. Materials and methods

Two experiments were conducted, both using four crossbred boars (Landrace × Large White) approximately 24 months of age and known to be fertile. The boars were housed in individual pens, under the same environmental conditions, at the Palma Experimental Station of the Universidade Federal de Pelotas (Brazil).

Semen collections were performed using the gloved-hand technique [26], using a plastic glass kept at 32 °C (protected by an isothermic vacuum bottle) and covered with gauze (to separate the gel-rich fraction). Only the sperm-rich fraction of the ejaculate was used for further processing [11,27]. After collection, sperm motility was evaluated subjectively, using a phase contrast optical microscopy, under 200× magnification [28]. Only ejaculates with ≥70% motility were processed.

2.1. Experiment 1

Ten ejaculates were collected from each male. Immediately after collection, two 20 mL aliquots from the sperm-rich fraction were placed into 50 mL conic tubes and diluted (1:1, v/v) in Beltsville Thawing Solution extender (BTS) [29]. After dilution, semen was refrigerated for 60 min at 24 °C.

Two semen centrifugation temperatures were tested: 15 and 24 °C. For centrifugation at 24 °C, semen samples were cooled to 24 °C for 60 min and then centrifuged at 800 × *g* for 10 min (SORVALL®RC6, Thermo Scientific, Orlando, FL, USA). The supernatant was discarded and the sperm pellet was resuspended in the cooling extender [80% mL of 11% (wt.:v) lactose solution and 20 mL egg yolk] up to a final concentration of 450×10^6 sperm/mL, and subsequently cooled to 15 °C for 60 min. The cooling extender was at 15 °C to prevent cold shock. For centrifugation at 15 °C, after cooling at 24 °C, also in 60 min, semen samples were kept cool for an additional 60 min (until reaching 15 °C). At that temperature, samples were centrifuged at 800 × *g* for 10 min (SORVALL®RC6). After centrifugation, all procedures were the same as described above for centrifugation at 24 °C. For both centrifugation temperatures, cooling up to 5 °C was conducted in 90 min. Therefore, the incubation period was the same for each centrifugation temperature.

During the freezing process, four penetrating cryoprotectants were used: glycerol ($C_3H_8O_3$; mol:wt. 92.09; G 4094, Merck), *N*-methylformamide (C_2H_5NO; mol:wt. 59.07; M 2769), *N*,*N*-dimethylformamide (C_3H_7NO; mol:wt. 73.10; D4254), and *N*,*N*-dimethylacetamide (C_4H_9NO; mol:wt. 87.12; D5511). Glycerol was obtained from Merck S.A. (São Paulo, SP, Brazil) and the amides were obtained from Sigma Chemical Company (St. Louis, MO, USA). Glycerol (GLY) was used at a final concentration of 3% [11] and all three amides at 5% final concentration. Therefore, the combination of cryoprotectants and centrifugation temperatures followed a 4 × 2 factorial design. The freezing extenders to be added at 5 °C were prepared with the cooling extender containing 1.5% *Orvus Es Paste*, Equex-Paste (Minitüb, Tiefenbach, Germany) and the respective cryoprotectants up to the final concentration described above (v/v).

After reaching 5 °C, 2 mL from each 50 mL tube were transferred to 15 mL conic tubes, and then 1 mL of one of the four cryoprotectant extenders was added. Semen was packaged in 0.5 mL straws (Minitüb, Germany) so that each straw contained 150×10^6 sperm. Semen was frozen in liquid nitrogen vapours by placing straws horizontally 5 cm above the level of liquid nitrogen for 20 min, and stored in liquid nitrogen until thawing.

2.2. Experiment 2

Ten semen collections were conducted from each of the four males. Semen processing and cryopreservation procedures were identical to those described for experiment 1. The same two centrifugation temperatures were tested (15 and 24 °C). However, only the three amides (MF, DMF, and DMA) were used as penetrating cryoprotectants for freezing, using three concentrations (3, 5, and 7%) of each amide ($3 \times 3 \times 2$ factorial design). The freezing extenders were added at 5 °C, including the cooling extender with the addition of 1.5% (v/v) *Orvus Es Paste* (Equex-Paste; Minitüb, Germany), up to the same final concentration as in experiment 1.

2.2.1. Thawing

In both experiments, straws were thawed at 37 °C for 20 s, and semen was resuspended in a conical tube containing 10 mL of BTS (1:20, v/v) previously warmed to 37 °C [30].

2.2.2. Sperm motility and membrane integrity analysis

After thawing, semen samples were incubated for 10 min in a water bath at 37 °C, and sperm motility was evaluated with phase contrast microscopy at 200× magnification [28,31].

After the evaluation of sperm motility, sperm membrane integrity was evaluated using fluorescence markers [32], i.e. carboxyfluorescein diacetate (CFDA; C 5041) and propidium iodide (PI; P 4170). Both markers were obtained from Sigma Chemical Company (St. Louis, MO, USA). We prepared a 1 mL of a solution containing 950 μL of sodium citrate 3%, 20 μL of PI, 20 μL of CFDA, and 10 μL of phormoldehyde (Solution 1). Subsequently, 40 μL of Solution 1 and 10 μL of the thawed semen sample were put in a 250 μL Eppendorf tube (Solution 2). The tube was kept in the dark for 5 min, then 5 μL of Solution 2 was put on a slide under a coverslip and evaluated with an epifluorescent microscope (Olympus BX 51, America Inc., Sapporo, Japan), under 400× magnification (filter wave length of 525 nm). Two hundred sperm were counted in each slide and classified as intact (green fluorescence) or damaged (red fluorescence). Both evaluations were performed by the same technician and were identical for both experiments.

2.3. Statistical analyses

Analyses of variance with repeated measures were used for statistical comparisons in both experiments. In both experiments, the models consisted of treatments, centrifugation temperatures, week of collection, interactions among treatments, centrifugation temperatures and week, and the boar effect nested within the effect of treatment. The subsequent comparisons among means were conducted with Tukey's test. Additionally, Pearson's correlation coefficients were generated to evaluate the linear association between estimates of sperm motility by optical microscopy and sperm membrane integrity obtained by fluorescent markers. All analyses were performed with the same statistical program [33].

3. Results

3.1. Experiment 1

Cryoprotectant by centrifugation temperature interaction on post-thaw sperm motility and membrane integrity was not significant ($P > 0.05$); sperm motility and membrane integrity by treatment and centrifugation temperature are shown (Table 1). Post-thawing sperm motility did not differ ($P > 0.05$) between DMA ($53.8 \pm 1.7\%$) and DMF ($50.6 \pm 1.9\%$), but both were higher ($P < 0.05$) than those for MF ($43.2 \pm 2.4\%$) and GLY ($38.1 \pm 2.3\%$), which did not differ from each other ($P > 0.05$). Motility for samples centrifuged at 15 °C was higher ($P < 0.05$) than that observed for those in temperature 24 °C ($52.8 \pm 1.2\%$ vs. $40.1 \pm 1.2\%$, respectively).

Sperm membrane integrity after thawing for DMA ($50.9 \pm 1.9\%$) did not differ ($P > 0.05$) from that observed for DMF ($47.9 \pm 2.1\%$), but it was higher ($P < 0.05$) than for MF and GLY (43.3 ± 2.5 and $34.5 \pm 2.8\%$, respectively). Membrane integrity did not differ between DMF and MF, whereas GLY had the lowest proportion of sperm with an intact membrane (Table 1). Samples centrifuged at 15 °C had a higher proportion of spermatozoa with intact membranes ($P < 0.05$) than those centrifuged at 24 °C ($49.0 \pm 1.4\%$ vs. $39.3 \pm 1.5\%$, respectively). Estimates of

Table 1
Mean (±S.E.M.) post-thaw sperm motility and membrane integrity of boar semen, by cryoprotectant and centrifugation temperature (experiment 1)[a]

Cryoprotectant	Motility (%)			Membrane integrity (%)		
	Centrifugation temperature			Centrifugation temperature		
	15 °C	24 °C	Total	15 °C	24 °C	Total
3% glycerol	42.2 ± 2.4	34.0 ± 2.4	38.1 ± 2.3 a	36.5 ± 1.7	32.5 ± 1.7	34.5 ± 2.8 a
5% MF	48.7 ± 2.4	37.6 ± 2.4	43.2 ± 2.4 a	48.6 ± 1.7	38.0 ± 1.7	43.3 ± 2.5 b
5% DMF	58.7 ± 2.4	42.5 ± 2.4	50.6 ± 1.9 b	53.8 ± 1.7	42.0 ± 1.7	47.9 ± 2.1 b
5% DMA	61.5 ± 2.4	46.1 ± 2.4	53.8 ± 1.7 b	57.0 ± 1.7	44.8 ± 1.7	50.9 ± 1.9 c

MF, methylformamide; DMF, dimethylformamide; DMA, dimethylacetamide. a–c, within a column, means without a common letter differ ($P < 0.05$).
[a] n per treatment = 40.

sperm motility and membrane integrity were highly correlated ($r = 0.92$, $P < 0.0001$).

3.2. Experiment 2

There was a cryoprotectant by concentration interaction influencing both sperm motility and membrane integrity post-thawing ($P < 0.05$; Table 2). However, there was no interaction with centrifugation temperature ($P > 0.05$). Samples conditioned in DMA at 5% had higher motility and membrane integrity ($P < 0.05$) than those extended in MF at any concentration, DMF at 7% and DMA at 3%. Sperm motility was higher ($P < 0.05$) for centrifugation at 15 °C (51.8 ± 0.7%) than at 24 °C (40.3 ± 0.8%; $n = 360$/treatment). Centrifugation at 15 °C was also associated with higher ($P < 0.05$) sperm membrane integrity (49.8 ± 0.8%) than centrifugation at 24 °C (39.3 ± 1.0%; $n = 324$/treatment). There was a high correlation ($r = 0.92$, $P < 0.0001$) between estimates of sperm motility and membrane integrity.

4. Discussion

This study is the first to report efficient cryoprotectant effect of low molecular weight amides on post-thawing sperm motility and membrane integrity of boar semen. The best motility results in this study seemed comparable to those obtained by others [5,8,9,34–39], and quite close to some of the best results obtained with frozen boar semen [10], but all those results were obtained using glycerol as cryoprotectant. The correlations between motility and membrane integrity observed in this study were also in agreement with those reported by others [23]. We concluded that amides, especially DMA and DMF, can successfully replace glycerol as penetrating cryoprotectants in freezing protocols for boar semen, as reported in horses [23–25], roosters [40], fish [4,41], geese [42], and rabbits [43]. Furthermore, DMA at 5% was generally more efficient than the other tested amides, especially considering the satisfactory means observed for sperm

Table 2
Mean (±S.E.M.) post-thaw sperm motility and membrane integrity of boar semen, by cryoprotectant, concentration and centrifugation temperature (experiment 2)[a]

Cryoprotectant	Motility (%)			Membrane integrity (%)		
	Centrifugation temperature			Centrifugation temperature		
	15 °C	24 °C	Total	15 °C	24 °C	Total
3% MF	50.6 ± 1.9	37.8 ± 1.9	44.3 ± 1.9 a	49.2 ± 1.9	37.5 ± 1.9	43.4 ± 2.2 ab
5% MF	48.7 ± 1.9	37.6 ± 1.9	43.2 ± 2.4 a	48.6 ± 1.9	38.0 ± 1.9	43.3 ± 2.5 ab
7% MF	39.6 ± 1.9	30.0 ± 1.9	34.8 ± 2.1 b	40.3 ± 1.9	29.2 ± 1.9	34.7 ± 2.5 c
3% DMF	53.6 ± 1.9	42.2 ± 1.9	47.9 ± 1.8 acd	51.3 ± 1.9	41.2 ± 1.9	46.3 ± 2.2 abd
5% DMF	58.7 ± 1.9	42.5 ± 1.9	50.6 ± 1.9 cd	53.8 ± 1.9	42.1 ± 1.9	47.9 ± 2.1 de
7% DMF	50.1 ± 1.9	38.1 ± 1.9	44.1 ± 2.0 a	48.7 ± 1.9	35.8 ± 1.9	42.3 ± 2.3 b
3% DMA	50.0 ± 1.9	43.1 ± 1.9	46.6 ± 2.1 ad	48.4 ± 1.9	42.0 ± 1.9	45.2 ± 2.6 abd
5% DMA	61.5 ± 1.9	46.1 ± 1.9	53.8 ± 1.7 c	57.0 ± 1.9	44.8 ± 1.9	50.9 ± 1.9 e
7% DMA	53.0 ± 1.9	44.8 ± 1.9	48.9 ± 2.3 acd	50.6 ± 1.9	42.9 ± 1.9	46.7 ± 2.7 ade

MF, methylformamide; DMF, dimethylformamide; DMA, dimethylacetamide. a–d, within a column, means without a common letter differ ($P < 0.05$).
[a] n per treatment = 40.

membrane integrity, although they were not significantly different from those observed for DMF at 5%.

Although both glycerol and amides can protect sperm cells during cryopreservation, their cryoprotectant properties are achieved by different mechanisms. Glycerol is an alcohol that contains three functional hydroxyl groups, which can accept one hydrogen from the water molecule in six different binding sites [44]. In boar semen cryopreservation protocols, the final maximum concentration of glycerol is commonly 3% [7,8,10,11,35], because glycerol is potentially cytotoxic at higher concentrations [12–14]. Also, glycerol hydroxyl radicals may sometimes bind among themselves, decreasing the probability of binding with water molecules, which is undesirable due to the higher viscosity of the solution [45]. Conversely, amides are formed by functional groups that contain nitrogen (NH–C=O); amides interact with the water by binding their nitrogen and hydrogen content to the hydrogen present in the water molecule. The amides tested in this study are highly lipophilic due to incorporation of methyl (CH_3) into the amide molecule, which increases its permeability through the sperm cell membrane. Moreover, a highly hydrophilic nature of the amide molecule allows a higher interaction with the water, which reduces the formation of intracellular ice crystals. Furthermore, due to their lower molecular weight and viscosity in comparison to glycerol, amides have higher membrane permeability, decreasing the possibility of cellular damage caused by osmotic stress [46]. Therefore, the cryoprotectant effect of amides can be attributed to their ability to permeate the cell membrane due to their low molecular weights, which likely allows them to bind to water molecules more efficiently than glycerol [45,46].

In protocols for freezing semen from fish [41,47,48] and poultry [40], DMA is frequently used as cryoprotectant, due to benefits for the post-thawing response. In one study with poultry semen, glycerol was less likely to cause morphologic sperm abnormalities than dimethylsulfoxide and DMA [40], but samples diluted in DMA had better post-thaw fertility than those diluted in glycerol. In the present study, sperm motility and membrane integrity after thawing were superior at 5%, versus 3 or 7%. We hypothesize that, at 7%, DMA may be toxic for boar sperm (similar to glycerol at concentrations >3%). However, further studies must be conducted to clarify that. When used in cryopreservation protocols for fish semen, DMA concentrations ranged from 10 to 15% [41].

Although centrifugation temperature influenced post-thawing sperm motility and membrane integrity, there was no interaction between centrifugation temperature with either cyroprotectant or concentration. The best results for both sperm motility and membrane integrity estimates were observed with centrifugation at 15 °C, with longer exposure of sperm cells to both the seminal plasma and the extender. At 15 °C, it was necessary to use a refrigerated centrifuge to stabilize the temperature; otherwise the heat generated during centrifugation would raise the temperature to nearly 24 °C, which could reduce the efficiency of this protocol. The objective of testing centrifugation at 24 °C was to optimize the protocol, using a non-refrigerated bench centrifuge, common in most laboratories, since after centrifugation at 24 °C, the temperature would remain stable. Perhaps better results could be obtained with centrifugation at 24 °C, if samples could be kept for at least 60 additional min at the same temperature until the centrifugation was completed, so the total time from the semen collection to centrifugation would be similar to centrifugation at 15 °C.

Most boar semen freezing protocols currently used are based on modifications of two methods. In the first one, seminal plasma is removed by centrifugation, immediately after the ejaculate collection [49]. In the second one, seminal plasma is removed only when the freezing curve reaches 15 °C [11]. The influence of seminal plasma on sperm quality is controversial since its presence may be associated with higher sensitivity to cold shock [50], whereas its removal may have no effect on post-thawing viability [51]. Also, no influence of the presence of seminal plasma on semen quality was observed when seminal plasma was added to the sperm-rich fraction of the ejaculate [1]. In studies with minipigs, seminal plasma contained factors that modified the sperm cell before freezing, reducing its ability to penetrate oocytes *in vitro*, after freezing [52]. The deleterious effect of the presence of seminal plasma, were also reported for semen stored for at least 6 h before cryopreservation [53]. Conversely, beneficial effects related to the presence of seminal plasma proteins were reported in many studies [54–57]. Some authors [58] reported that bulls having different levels of fertility with frozen semen would have differences in the protein content of their seminal plasma, suggesting that such proteins could be biochemical markers for semen freezability. Such differences may be related to individual differences in semen freezability [59], as well as genetic influences, as observed in boars [60,61].

Extenders for freezing of boar semen can include amides as cryoprotectants, instead of glycerol, with centrifugation at 15 °C, with benefits for sperm motility and membrane integrity. Based on comparisons of the

tested amides at various concentrations, 5% DMA would be more efficient as a cryoprotectant.

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