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ANIMAL REPRODUCTION

Use of a one-step freezing protocol for boar sperm with distinct cryoprotectants

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ABSTRACT: The present study evaluated the cryoprotectant efficacy of dimethylacetamide (DMA) and ethylene glycol in a one-step protocol to freeze boar sperm. The sperm-rich portion of the ejaculates from two boars were collected once a week, for 10 weeks. After collection, the ejaculates were diluted (1:1; v/v) in the cooling extender. After determining their spermatozoa concentration, the ejaculates were pooled with the same number of spermatozoa from each boar and stabilized at 20 °C for 120 min. Distinct cryoprotectants were added to the cooling extender at 20 °C, at different concentrations, composing six treatments: 1.25% and 2.5% glycerol (control); 1.25% and 2.5% ethylene glycol; 2.5% and 5.0% DMA. The samples were stored in 0.25 mL straws, containing 35 × 10^6 spermatozoa. After 90 min at 20 °C, the straws were submitted to a cooling curve until 5 °C (0.3 to 0.5 °C/min) and kept at 5°C for 60 min. Freezing was conducted by placing the straws horizontally 5 cm above the liquid nitrogen for 10 min, followed by immersion on liquid nitrogen. After thawing at 37 °C for 30 seconds, sperm quality was evaluated through a computer-assisted semen analysis system and flow cytometry. Sperm motility was greater ($P < 0.05$) in treatments with 5.0% and 2.5% DMA (22.2 ± 2.6% and 20.0 ± 2.8%, respectively) than in treatment with 2.5% ethylene glycol (8.2 ± 1.0%). The integrity of the plasma membrane ($P = 0.08$) and mitochondrial membrane potential ($P = 0.27$) was similar among the treatments. The treatment with 2.5% ethylene glycol was the least efficient to maintain intact acrosome membrane ($P < 0.01$). Some kinetics parameters (DAP, DCL, DSL, VAP, VCL, VSL e ALH) were positively affected by 5.0% DMA. The one-step freezing protocol resulted in unsatisfactory boar sperm motility after thawing, regardless of the cryoprotectant.
Key words: glycerol, ethylene glycol, dimethylacetamide, cryopreservation, swine.

Protocolo de congelamento one-step para sêmen suíno utilizando diferentes crioprotetores

RESUMO: O presente estudo objetivou avaliar a eficácia de um protocolo one-step de congelamento do sêmen suíno utilizando dimetilacetamida (DMA) e etilenoglicol como crioprotetores. Durante 10 semanas, a fração rica dos ejaculados de dois machos suínos foram coletados, uma vez por semana. Após a coleta, os ejaculados foram diluídos (1:1; v/v) no diluidor de resfriamento. Após a avaliação da concentração espermática, os ejaculados foram agrupados em um pool com o mesmo número de espermatozoides de cada macho e estabilizados a 20 °C por 120 min. Os crioprotetores foram adicionados ao diluidor de congelamento a 20 °C, em diferentes concentrações, compondo seis tratamentos: glicerol (controle), 1,25% e 2,5%; etilenoglicol, 1,5% e 2,5%; e DMA, 2,5% e 5,0%. As amostras foram armazendadas em palhetas de 0,25 mL contendo 35 x 10^6 espermatozoides. Após 90 min a 20 °C as palhetas foram submetidas a uma curva de resfriamento até 5 °C (0,3 a 0,5 °C/mim) e mantidas a 5 °C por 60 min. O congelamento foi realizado a partir da colocação das palhetas horizontalmente a 5 cm acima do nitrogênio líquido por 10 min, com sua posterior imersão no nitrogênio líquido. Após o descongelamento a 37 °C por 30 segundos a qualidade espermática foi avaliada através de um sistema computadorizado e por citometria de fluxo. A motilidade espermática foi maior ($P < 0,05$) nos tratamentos com 5,0% e 2,5% DMA (22,2 ± 2,6% e 20,0 ± 2,8%, respectivamente) do que no tratamento com 2,5% etilenoglicol (8,2 ± 1,0%). A integridade da membrana plasmática ($P = 0,08$) e potencial de membrana mitocondrial ($P = 0,27$) foi similar entre os tratamentos. O tratamento com 2,5% de etilenoglicol foi menos eficiente em manter membrana acrossomal intacta ($P < 0,01$). Alguns parâmetros de cinética espermática (DAP, DCL, DSL, VAP, VCL, VSL e ALH) foram afetados positivamente pelo uso de DMA a 5.0%. O protocolo simplificado para congelamento de sêmen suíno resultou em motilidade espermática insatifatória após o descongelamento, independente do crioprotetor utilizado.
Palavras-chave: glicerol, etilenoglicol, dimetilacetamida, criopreservação, suíno.

INTRODUCTION

The lower fertility with frozen boar semen is caused by the cryodamages of the spermatozoa during freezing and thawing, which may lead to premature membrane destabilization, which impair their subsequent survival in the female reproductive tract (PARKS & LYNCH, 1992; WATSON, 2000).

Traditional freezing protocols for boar sperm are based on the descriptions of WESTENDORF et al. (1975), with removal of the seminal plasma when the cooling curve reaches 15 °C, and of PAQUIGNON et al. (1974), with immediate removal of the seminal

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plasma after collection through centrifugation. As such approaches require many processing processes, they are both considered labor intensive. Therefore, current studies about boar semen cryopreservation are focused on developing protocols to improve membrane protection (BIANCHI et al., 2008a; PINHO et al., 2014; KNOX et al., 2015; PASSARELLI et al., 2020). Glycerol is the most common cryoprotectant used to cryopreserve boar semen (HOLT, 2000; RODRIGUEZ-MARTINEZ & WALLGREN, 2011). However, replacing glycerol with alternative compounds with lower molecular weight may help to improve the protection of spermatozoa membrane.

Dimethylacetamide (DMA) has been tested as an alternative to glycerol for boar semen cryopreservation, with conflicting results. Although, some studies reported promising results (BIANCHI et al., 2008b; PINHO et al., 2014), other studies did not observe any advantage of using DMA compared to glycerol (KIM et al., 2011; WU et al., 2012; YANG et al., 2016). Ethylene glycol has been tested as a cryoprotectant in bull (GORORO et al., 2020), ram (NAJAFI et al., 2017) and stallion (OLDENHOF et al., 2017) sperm, since it has lower molecular weight than glycerol. Nevertheless, there is scarce information about the use of ethylene glycol as a cryoprotectant for frozen boar semen.

Additionally, besides testing alternative cryoprotectant substances, improvements in cryopreservation protocols should also test distinct concentrations to be included in extenders, since post-thawing sperm quality may be impaired by cytotoxic effects related to excessive concentration of cryoprotectants (BUHR et al., 2001). Therefore, this study evaluated a one-step protocol to boar semen cryopreservation with different concentrations of DMA, ethylene glycol and glycerol in post-thawing quality parameters.

MATERIALS AND METHODS

The experimental procedures involving animals performed in the study were approved by the Ethics in Use and Animals Committee (CEUA) of the Instituto Federal Catarinense Campus Araquari (protocol 348/2020).

Two boars (Landrace × Large White; > 24 months old) were used. The boars were housed in individual pens, were fed a corn-soybean diet and had *ad libitum* access to water. For ten weeks, the sperm-rich fraction was collected once a week from both boars, through the gloved hand method, in a pre-warmed collection cup (37 °C) equipped with a filter for gel fraction removal. Immediately after collection, sperm motility was evaluated using phase-contrast optical microscopy, under 200 × magnification (Primo Star, Zeiss, Germany). Only ejaculates with sperm motility equal or greater than 80% were processed.

From each ejaculate, a 10 mL-aliquot of the sperm-rich fraction was diluted (1:1; v/v) in the cooling extender, containing 11% lactose (79.2%, v/v); Orvus Ex Paste (0.8%; v/v; Minitub, Germany) and egg yolk (20% v/v). After the evaluation of the individual sperm concentration in a hemocytometric chamber (Neubauer Improved chamber; Optik Labor, Germany), a specific volume of the diluted ejaculate from each animal was mixed, so that, each boar contributed with the same number of cells in the final pool. Then, 120 min after collection, distinct cryoprotectants were added to the cooling extender at 20 °C, at different concentrations, composing six treatments: 1.25% glycerol; 2.5% glycerol; 1.25% ethylene glycol; 2.5% ethylene glycol; 5.0% DMA; and 2.5 % DMA. The samples were stored in 0.25 mL straws (Minitub, Germany), containing 35×10^6 spermatozoa.

After 90 min at 20 °C, the straws were submitted to a cooling curve until 5 °C (0.3 to 0.5 °C/min) and kept at 5 °C for 60 min. Freezing was conducted by placing the straws horizontally 5 cm above the liquid nitrogen for 10 min, followed by immersion on liquid nitrogen. Thawing was performed at 37 °C for 30 seconds, by resuspending the samples in Beltsville Thawing Solution (PURSEL & JOHNSON, 1975) (1:20, v/v), previously warmed at 37 °C.

Immediately after thawing, a 3 µL sample was loaded into a chamber slide (Leja® 20 µm, The Netherlands). Sperm kinetics parameters were evaluated using a computer-assisted semen analysis system (SpermVision®, Minitub, Germany), at 200 × magnification under a phase-contrast microscope equipped with a warm stage at 37 °C, evaluating 5 fields per sample. The evaluated kinetics parameters were: total and progressive motility; average path distance (DAP); distance in a curvilinear (DCL) and in a straight line (DSL); average path velocity (VAP); velocity in a curvilinear (VCL) and in straight line (VSL); straightness (STR); linearity (LIN); wobble (WOB); amplitude of lateral head displacement (ALH); and beat-cross frequency (BCF). Spermatozoa were defined motile when showing an amplitude of lateral head displacement (ALH) > 1.0 µm and a velocity curved line (VCL) > 24 µm/s, according to the manufacturer's recommendation. The progressive motility was defined when spermatozoa presented VSL > 24 µm/s and VCL > 28 µm/s.

Flow cytometry analyses were performed using the Attune Acoustic Focusing Cytometer® (Life Technologies, USA), and the Attune Cytometric Software. The debris were eliminated based on scatter plots, and on the Hoechst 33342 staining. Ten thousand events were analyzed per sample at a flow rate of 200 events/s.

A volume of thawed sperm containing approximately 2×10^6 spermatozoa was added to 300 µL of a PBS-based solution containing 0.001 mg/mL of PI, 0.001 mg/mL of FITC-PNA, and 0.0025 mg/mL of Hoechst 33342. Samples were incubated at room temperature for 15 min prior to analysis (MARTINS et al., 2016). Sperm cells negative for PI and FITC-PNA were considered as having intact plasma and acrosomal membrane.

The mitochondrial membrane potential was evaluated using the JC-1 dye (Life Technologies, Eugene, OR, USA). A sample containing approximately 2×10^6 spermatozoa was added to 300 µL of a PBS-based solution containing 0.0019 mg/mL of JC-1 and incubated at 37 °C for 30 min prior to analysis (MARTINS et al., 2016). Spermatozoa with high mitochondrial membrane potential presented high orange fluorescence and those with low potential presented high green fluorescence.

The Hoechst 33342 fluorescence was detected by the filter 450/40, while the green fluorescence of FITC-PNA and JC-1 was read by the filter 530/30. Filters 603/48 and 640 (long pass filter) were used to read the orange and the red fluorescence of JC-1 and PI, respectively.

Data were analyzed with SAS® (SAS® 9.4 Institute Inc., Cary, NC, USA). Data were tested for normal distribution through the Shapiro-Wilk test. Total and progressive motility, integrity of plasma and acrosome membranes, and the kinetic parameters VAP, STR, ALH and WOB were analyzed using the Kruskal-Wallis analyses of variance, followed by Wilcoxon rank sum test adjusted by Bonferroni correction. The GLIMMIX procedure was used to analyze the mitochondrial functionality and the others kinetic, through an analysis of variance with subsequent comparison of means values using the Tukey test. For this model, the week of collection was considered as a random effect and the treatment as fixed effect.

RESULTS AND DISCUSSION

Progressive sperm motility was greater ($P < 0.05$) in treatments with 5.0% and 2.5% DMA (16.5±2.5 and 14.5 ± 2.2, respectively) than in the treatment with 2.5% ethylene glycol (4.8 ± 0.9) (Table 1). Although the motility results resemble those

Table 1 - Sperm motility and kinetic parameters in post-thawing boar semen frozen with different cryoprotectants, at distinct concentrations, using the one-step freezing protocol (mean ± standard error of mean).

	Treatment					
	Glycerol (%)		Ethylene glycol (%)		Dimethylacetamide (%)	
	2.5	1.25	2.5	1.25	5.0	2.5
TM	11.9 ± 2.0^{ab}	12.2 ± 2.2^{ab}	8.2 ± 1.0^{b}	14.7 ± 2.1^{ab}	22.2 ± 2.6^{a}	20.0 ± 2.8^{a}
PM	7.7 ± 1.6^{ab}	7.6 ± 1.5^{ab}	4.8 ± 0.9^{b}	10.5 ± 1.7^{ab}	16.5 ± 2.5^{a}	14.5 ± 2.2^{a}
DAP	22.1 ± 0.7^{b}	20.4 ± 1.7^{b}	22.5 ± 1.7^{b}	22.4 ± 1.2^{b}	28.2 ± 0.5^{a}	22.8 ± 1.7^{ab}
DCL	42.7 ± 1.4^{b}	38.9 ± 3.3^{b}	42.1 ± 3.6^{b}	43.2 ± 2.9^{b}	55.9 ± 1.2^{a}	44.5 ± 3.9^{ab}
DSL	13.9 ± 0.6^{b}	12.6 ± 1.1^{b}	12.8 ± 1.1^{b}	13.1 ± 0.7^{b}	18.7 ± 0.6^{a}	14.4 ± 0.9^{b}
VAP	47.5 ± 1.3^{b}	43.8 ± 3.6^{b}	47.7 ± 3.6^{b}	48.2 ± 2.7^{b}	61.2 ± 1.1^{a}	49.0 ± 3.7^{ab}
VCL	91.7 ± 2.3^{b}	82.9 ± 6.8^{b}	89.2 ± 7.5^{b}	92.9 ± 6.1^{b}	121.1 ± 2.5^{a}	95.5 ± 8.5^{b}
VSL	29.9 ± 1.0^{b}	27.2 ± 2.2^{b}	27.3 ± 2.3^{b}	28.3 ± 1.4^{b}	40.6 ± 1.1^{a}	31.1 ± 2.0^{b}
STR	0.57 ± 0.0^{ab}	0.60 ± 0.0^{ab}	0.52 ± 0.0^{b}	0.59 ± 0.0^{b}	0.64 ± 0.0^{a}	0.61 ± 0.0^{ab}
LIN	0.32 ± 0.01	0.32 ± 0.01	0.31 ± 0.01	0.30 ± 0.01	0.33 ± 0.01	0.33 ± 0.01
WOB	0.46 ± 0.01	0.52 ± 0.01	0.59 ± 0.01	0.51 ± 0.01	0.48 ± 0.01	0.50 ± 0.01
ALH	2.6 ± 0.0^{b}	2.5 ± 0.02^{b}	2.6 ± 0.02^{b}	2.7 ± 0.02^{b}	3.2 ± 0.0^{a}	2.6 ± 0.02^{b}
BCF	29.2 ± 0.9	28.2 ± 1.1	27.8 ± 1.6	27.5 ± 0.8	31.4 ± 0.5	27.5 ± 1.1

^{ab}Different letters indicate significant differences ($P < 0.05$).

TM = Total motility (%); PM = Progressive motility (%); DAP = average path distance (µm); DCL = curvilinear distance (µm); DSL = straight-line distance (µm); VAP = average path velocity (µm/s); VCL = curvilinear velocity (µm/s); VSL = straight-line velocity (µm/s); STR = straightness (VSL/VAP); LIN = linearity (VSL/VCL); WOB = wobble (VAP/VCL); ALH = amplitude of lateral head displacement (µm); BCF = beat-cross frequency (Hertz).

observed by TORRES et al. (2016), they are still not those recommended for use in artificial insemination (CBRA, 2013). For sperm frozen with 5.0% DMA, the kinetic parameters DAP, DCL, DSL, VAP, VCL, VSL and ALH were higher ($P < 0.05$). However, LIN, WOB and BCF were similar in all treatments (Table 1). As reviewed by Jung et al. (2015), there is no consensus in publications on the influence of these variables on semen fertility. RAVAGNANI et al. (2018) observed improvement in sperm kinetic parameters when using concentrations below 300 × 10^6 spermatozoa in 0.5 ml straws.

Previously, BIANCHI et al. (2008a) and PINHO et al. (2014) reported greater post-thawing motility for sperm frozen with 5.0% DMA than for sperm frozen with glycerol, different from that observed in the present study. Nonetheless, other studies reported no improvement in post-thawing motility for sperm frozen with DMA (KIM et al., 2011; WU et al., 2012; YANG et al., 2016). The greater ability of DMA to maintain sperm kinetics may be due to the lower viscosity and molecular weight of amides compared to glycerol (ALVARENGA et al., 2005). Additionally, the two methyl radicals combined with the nitrogen present in the DMA molecule enables its permeation through membranes, improving its intracellular availability (BIANCHI et al., 2008a). Therefore, DMA penetrates and is excluded from the intracellular medium faster than glycerol, which probably minimizes potential detrimental effects of cell dehydration and rehydration during freezing and thawing (GILMORE et al., 1995), preserving the integrity of spermatozoa's membranes.

The integrity of the plasma membrane (Figure 1A; $P = 0.08$) and mitochondrial membrane potential (Figure 1C; $P = 0.27$) was similar among the treatments. The treatment with 2.5% ethylene glycol was the least efficient to maintain intact acrosome membrane (Figure 1B; $P < 0.01$). In fact, other studies reported reduction in the integrity of plasma membrane and acrosome using extenders including 5% DMA, compared to extenders including glycerol (KIM et al., 2011; YANG et al., 2016). Such findings suggest evidence of cytotoxicity, which would compromise fertility, considering that acrosome integrity is fundamental for

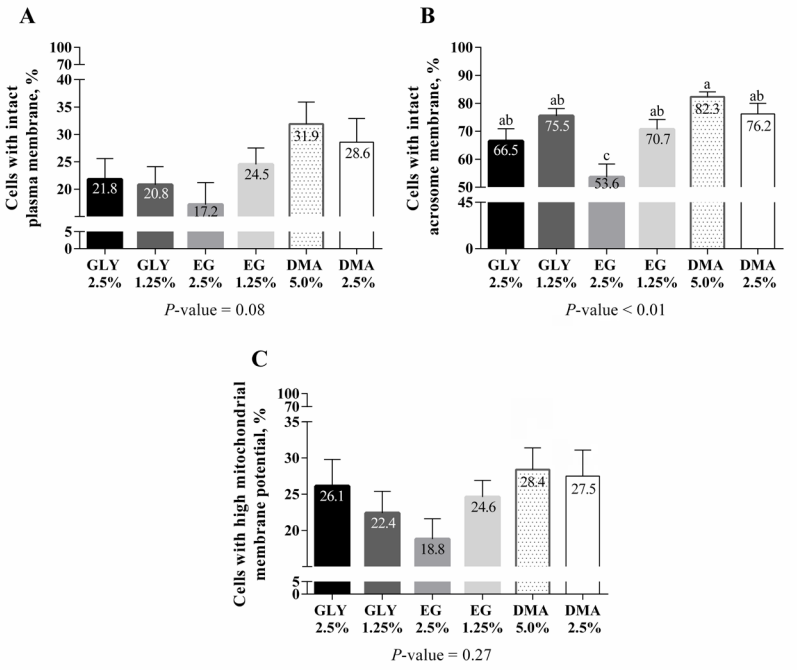

Figure 1 - Percentage of sperm cells with intact plasma membrane (A), intact acrosomal membrane (B) and high mitochondrial membrane potential (C) in boar semen frozen with different cryoprotectants, at distinct concentrations, using the one-step freezing protocol.

GLY: glycerol; EG: ethylene glycol; DMA: dimethylacetamide. Different letters indicate difference among treatments ($P < 0.05$).

the success of mammalian fertilization (TULSIANI et al., 1998).

To the best of our knowledge, this is the first study investigating the use of ethylene glycol for boar semen cryopreservation. So far, the use of ethylene glycol in swine has been limited to embryo freezing (DOBRINSKI et al., 1997; CAMERON et al., 2006). Compared to glycerol, ethylene glycol presents similar chemical structure and lower molecular weight. Therefore, it could be expected that ethylene glycol would provide cryoprotection for boar semen similar to that of glycerol. This seems to be true for stallion (MANTOVANI et al., 2002; OLDENHOF et al., 2017), bull (FORERO-GONZALEZ et al., 2012; BÜYÜKLEBLEBICI et al., 2014; SESHOKA et al., 2016; GORORO et al., 2020) and ram sperm (SILVA et al., 2012; NAJAFI et al., 2017). As observed in the present study, post-thawing sperm quality observed for the treatment with 1.25% ethylene glycol was similar to that of the treatments with glycerol. Inclusion of 2.5% ethylene glycol, although with no significant differences, resulted in the lowest values for the evaluated parameters, which may be an indicator of toxicity. Finally, we point out that the sperm motility results we found are below those recommended for use in artificial insemination (CBRA, 2013).

CONCLUSION

The one-step freezing protocol did not result in acceptable quality of boar sperm after thawing, with any of the tested cryoprotectants included in the freezing extender. Although, the kinetic parameters (DAP, DCL, DSL, VAP, VCL, VSL and ALH) indicate possible benefits of using 5.0% DMA, this needs to be evaluated in future studies with larger numbers of samples and animals, or even *in vivo*.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript. All authors critically revised the manuscript and approved of the final version.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The use of animals and the experimental procedures proposed in this study were approved by the Ethics in Use and Animals Committee (CEUA) of the Instituto Federal Catarinense Campus Araquari, protocol 348/2020.

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