NOTE *Theriogenology*

Evaluation of Different Cryoprotectants (CPAs) in Boar Semen Cryopreservation

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ABSTRACT. This study was performed to evaluate the use of dimethylacetamide (DMA) and dimethyl sulfoxide (DMSO) in boar sperm cryopreservation. Semen from eight boars was cryopreserved following treatment with 3, 5, and 7% DMA and DMSO, and 3% glycerol (control). After thawing, sperm conventional parameters and membrane integrities were evaluated. There were no significant differences among different DMA concentrations in all evaluations. Membrane intactness were higher in 5% and 7% DMSO than 3% DMSO (P<0.05). Sperm motility of 5% DMSO was lower than that of 3% glycerol (P<0.005), and membrane intactness were lower in 5% DMA and DMSO than 3% glycerol (P<0.05). DMA and DMSO didn't improve sperm quality and glycerol remains the most useful for boar sperm cryopreservation.

KEY WORDS: boar sperm, cryopreservation, DMA, DMSO, glycerol.

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Glycerol (at a concentration of 3%) is the most commonly used cryoprotectant (CPA) for boar sperm [3], but it has toxic effects as well as contraceptive effects on sperm [8]. Its detrimental effects have stimulated the study of alternative CPAs such as N,N-dimethylacetamide (DMA) or dimethyl sulfoxide (DMSO) [2, 10]. Due to its highly hydrophilic nature and low molecular weight, DMA reduces the formation of intracellular ice crystals and increases membrane permeability, thus decreasing osmotic damage [2]. The penetration of DMSO is also rapid, due to its lower molecular weight relative to glycerol [10] and DMSO has been used to successfully cryopreserve sperm [9]. However, there are few studies on the uses of DMA and DMSO for freezing boar semen. Therefore, the objective of this study was to evaluate the effectiveness of DMA and DMSO as possible replacement for glycerol in boar semen cryopreservation.

Three CPAs were used in this study: glycerol, DMA, and DMSO (all from Sigma-Aldrich, St. Louis, MO, U.S.A.). Glycerol was used at a final concentration of 3% as a control, and the others at final concentrations of 3, 5, and 7%. We first compared the three concentrations of DMA and DMSO (n=5), and the concentrations showing the best result for each CPA were then compared with 3% glycerol (n=8).

Ejaculate from 8 Duroc boars was collected and processed according to the straw freezing procedure [1] with some modification. Sperm-rich fractions were extended in Beltsville Thawing Solution (BTS) and were slowly cooled to 15°C for 3 hr. After centrifuging, the semen pellet was resuspended in lactose-egg yolk (LEY) extender. After further cooling to 5°C for 90 min, LEY-extended semen was aliquot for treatment with each CPAs and then two parts LEY-extended semen were mixed with one part freezing extenders (LEY extender containing 1.5% Equex STM [Nova Chemical Sales, Scituate Inc., MA, U.S.A.] and the CPAs described above, v/v). The semen was loaded into 0.5-m*l* straws (IMV, L'Aigle, France), and held in liquid nitrogen vapor 3 cm above the liquid for 20 min. The straws were then plunged into the liquid nitrogen. After thawing the straws at 37°C for 20 sec [5], thawed semen was evaluated for sperm motility [13], morphological defect, viability by eosin-nigrosin staining [4], and plasma- and acrosomal-membrane integrities. Sperm plasma-membrane integrity was assessed using 6-carboxyfluoresceindiacetate (6-CFDA; Sigma-Aldrich)/propidium iodide (PI; Sigma-Aldrich) fluorescent staining [12] and analyzed by flow cytometry. CFDA+/PI– stained sperm were classified as having an intact plasma-membrane. Sperm acrosomal-membrane integrity was evaluated using a FITC-peanut agglutinin (FITC-PNA; Sigma-Aldrich)/PI fluorescent staining following a modification [6, 11] and analyzed using flow cytometry. Analyzed sperm were classified into the following categories: viable acrosome-intact (PNA–/PI–); viable acrosome-reacted (PNA+/PI–); dead acrosome-reacted (PNA+/PI+) and total acrosome-reacted (PNA+). Flow cytometric analyses were performed using a FACScalibur flow cytometer (Becton Dickinson, San José, CA, USA) and Cell Quest Pro software (Becton Dickinson). Statistical analysis of the data was performed using SPSS software (version 15.0 for Windows; SPSS Inc., Chicago, IL, U.S.A.). One-way repeated-measures analysis of variance and Bonferroni's test were used for data with a normal distribution. Friedman test and Wilcoxon signed ranks test were used in the case of a violation of a normal distribution. Statistical significance was set at P<0.05 and all data were presented as the mean ± standard error of the mean (SEM).

In comparisons among CPA concentrations, there was no

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Table 1. Evaluation of frozen-thawed sperm following different concentrations of each CPA

CPAs	Motility (%)		Viability (%)	Morphological defect (%)	Intact plasma membrane (%)	Acrosomal membrane integrity (%)			
	Total motilty	Progressive motility				Viable acrosome-intact	Viable acrosome-reacted	Dead acrosome-reacted	Total acrosome-reacted
3% DMA	40.00±4.18	35.00±4.18	58.90±3.17	34.30±1.69	30.29±3.95	30.98±3.57	2.63±0.65	26.23±2.97	28.86±3.00
5% DMA	44.00±2.92	39.00±2.92	58.96±1.81	31.50 ±2.67	35.45±3.46	35.90±4.69	2.57±0.77	28.71±2.33	31.27±2.68
7% DMA	36.00±6.96	31.00±6.96	49.80±4.33	36.53±2.71	29.76±6.04	30.31±6.98	1.98±0.46	38.36±3.74	40.34±3.71
3% DMSO	29.00±6.20	24.00± 6.20	57.04±4.09	31.10±2.92	23.87±1.85[a)]	24.33±2.44[a)]	2.42±0.23	23.17±2.74	25.58±2.93
5% DMSO	28.00±5.61	23.00± 5.61	60.82±9.24	36.10±1.03	30.19±2.98[b)]	30.28±3.47[b)]	2.52±0.25	23.47±3.28	25.99±3.48
7% DMSO	28.00±4.64	23.00 ± 4.64	59.02±7.22	39.52±3.88	29.86±2.89[b)]	29.89±1.75[b)]	2.42 ±0.50	25.12±2.76	27.54±2.90

Different superscripts indicate significant differences among concentrations within each CPAs: a,b) $P<0.05$.

Table 2. Evaluation of frozen-thawed sperm treated by different CPAs

CPAs	Motility (%)		Viability (%)	Morphological defect (%)	Intact plasma membrane (%)	Acrosomal membrane integrity (%)			
	Total motilty	Progressive motility				Viable acrosome-intact	Viable acrosome-reacted	Dead acrosome-reacted	Total acrosome-reacted
3% Glycerol	48.13±3.65[a)]	41.88±3.40[a)]	65.72±1.67	28.13±2.42	51.80±2.61[c)]	51.54±2.72[e)]	4.82±1.03[e)]	18.5±1.56[e)]	23.31±1.78[c)]
5% DMA	45.63±1.99[a)]	40.63±2.20[a)]	59.85±1.63	32.38 ±3.30	38.01±3.06[d)]	39.03±3.83[f)]	3.25± 0.69[e)]	28.04±1.81[f)]	31.28±2.19[d)]
5% DMSO	26.88±3.53[b)]	21.25±3.50[b)]	59.01±2.65	35.56±2.91	30.89 ±1.98[d)]	30.49±2.24[f)]	3.37±0.67[f)]	24.05±2.11[f)]	27.42±2.47[c)]

Within a column, different superscripts indicate significant differences: a,b) $P<0.005$; c,d) $P<0.01$; e,f) $P<0.05$.

difference among samples treated with the three DMA concentrations in all evaluations ($P>0.05$; Table 1). Samples treated with 5% and 7% DMSO had a higher proportion of plasma membrane-intact and viable acrosome-intact sperm after freeze-thawing than samples treated with 3% DMSO ($P<0.05$), but the other parameters were not significantly different among the three DMSO concentrations. Although the differences were not significant, 5% DMA and 5% DMSO treatments showed better results than the other CPA concentrations and were selected for comparison with 3% glycerol. In comparisons among different CPAs, sperm total and progressive motility for DMA treatment were not different from those for glycerol ($P>0.05$), and those for DMSO were decreased compared with glycerol (Table 2; $P<0.005$). The proportion of plasma membrane-intact and viable acrosome-intact sperm decreased in DMA and DMSO compared with glycerol ($P<0.05$).

These results were different from previous reports that 5% DMA can successfully replace glycerol for boar semen cryopreservation [3]. Contrary to finding that DMA or DMSO could be used as an alternative CPA for freeze-thawing in stallions [14, 15], DMA and DMSO decreased sperm quality during freeze-thawing compared with glycerol in boar sperm. There appears to be considerable interspecies variation in the relative permeability of CPAs. Interestingly, DMSO gave a good sperm viability but low sperm motility as compared to other CPA. Sperm can be non-motile but viable. DMSO might induce sperm having these characteristic much more than other CPA. These sperm might indicate a transitional phase beginning to be dead. Such moribund sperm should be considered part of the dead sperm population because they are, in fact, dead on the basis of their functional capacity [7]. The proportion of viable acrosome-reacted sperm decreased in DMSO ($P<0.05$), but the number of viable acrosome-reacted sperm was very low for all CPAs. Likewise, the proportion of dead acrosome-reacted sperm increased in DMA and DMSO ($P<0.05$) with an increase in the proportion of total acrosome-reacted sperm compared with glycerol. This might indicate that DMA or DMSO cause death of viable acrosome-reacted sperm or deterioration of the acrosome after death of viable acrosome-intact sperm, rather than prevention of acrosomal exocytosis. In conclusion, this study demonstrated that DMA and DMSO have no beneficial effects on boar sperm cryopreservation compared with glycerol. Further study is required to develop suitable CPAs as glycerol replacements in frozen-thawed boar semen.

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