Research Note: Evaluation of two methods for adding cryoprotectant to semen and effects of bovine serum albumin on quality characteristics of cryopreserved rooster spermatozoa

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ABSTRACT Chicken semen cryopreservation is a tool for programs of genetic diversity management and endangered breeds conservation. Due to physiological features, the fertility rates of cryopreserved poultry sperm are lower than mammal species. Thus, improvement of the semen cryopreservation methods is required. A first study was performed by a 2 × 2 factorial design consisting of 2 methods of adding the cryoprotectant [Direct or Diluted (mixed with extender medium)] and 2 cryoprotectants (glycerol and dimethylacetamide). Then sperm quality indicators were evaluated after freezing. A second study with a 2 × 2 design was conducted to evaluate the effectiveness of bovine serum albumin (**BSA**) on the optimization of 2 different extenders (Lake and Animal Sciences Group [**ASG**]). Viability and motility variables were evaluated before and after freezing. There was no significant difference in sperm viability and motility variables between Direct or Diluted methods. Supplementation of extenders with BSA improved most of the sperm motility variables in both extenders before and after freezing. Progressive sperm, non-progressive sperm before freezing, and all post-thaw sperm motility parameters, except amplitude of lateral head displacement and beat-cross frequency, were increased in BSA-supplemented extenders ($P < 0.05$), and BSA improved sperm viability in ASG extender after thawing ($P < 0.05$). After thawing, the interaction between extender and BSA ($P < 0.05$), eliminated the differences between the 2 BSA-supplemented media in curvilinear velocity, straight-line velocity, average path velocity, and amplitude of lateral head displacement which were higher in non-supplemented ASG extender than nonsupplemented Lake medium. In conclusion, the direct or diluted methods of adding glycerol or dimethylacetamide, did not significantly affect the post-thaw sperm characteristics. BSA positively affected most of the post-thaw sperm motility indicators regardless of the type of extender and resulted in significantly higher post-thaw sperm viability in ASG medium.

Key words: sperm motility, sperm viability, dimethylacetamide, glycerol, BSA

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INTRODUCTION

The degree of cryodamage can be affected by the composition of the extender, the type and concentration of cryoprotectant, and the cooling rate (Woelders et al., 2006). Cryoprotectants in the freezing medium mitigate the adverse effects of freezing. Notably, they prevent excessively high intra- and extracellular concentrations of electrolytes and excessive shrinking of the cells during freezing (Mazur and Rigopoulos, 1983). The addition of cryoprotectants straight from the bottle could lead to brief exposure of cells to very high local cryoprotectant concentrations, which could aggravate osmotic events and lead to chemical toxicity effects. The latter seems especially relevant for dimethylacetamide (**DMA**), which is not tolerated very well by poultry sperm at very high concentrations. Thus optimizing the cryoprotectant addition method may reduce the potential stress

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of cryoprotectant addition and may overall reduce sperm injury during the cryopreservation protocols.

Serum albumin, the most or one of the most abundant proteins of the chicken seminal plasma, stimulates chicken sperm motility at the normal concentrations found in seminal plasma (Santiago-Moreno and Blesbois, 2020). It has been reported that its addition after thawing of poultry semen is also shown to give higher motility values (Woelders, 2021).

The objectives of this study were to 1) compare 2 different methods of adding the cryoprotectant (direct vs. diluted) to extended rooster semen on *in vitro* post-thaw sperm quality and 2) to test bovine serum albumin (**BSA**) as a supplement to poultry extenders.

MATERIAL AND METHODS

Chemicals, Extenders

All Chemicals used in this study were obtained from Merck (Darmstadt, Germany) and Sigma Co. (St. Louis, MO, USA). Extenders used in this study were Lake and Animal Sciences Group (ASG). Lake extender contains 1.92g sodium L-glutamate monohydrate, 0.5 g potassium acetate, 0.08 g magnesium acetate tetrahydrate, 0.8 g glucose, 0.3 g polyvinylpyrrolidone (Mr 10000), and 100 mL of water (343 mOsm/kg, pH 7.08). ASG medium contains 1.211 g sodium L-glutamate monohydrate, 0.102 g tripotassium citrate monohydrate, 0.064 g magnesium acetate tetrahydrate, 0.526 g glucose, 2.43 g BES (N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), 0.185 g sodium hydroxide, and water to make a total volume of 100 mL (325 mOsm/kg, pH 7.1).

Experimental Birds

Animals were handled according to procedures approved by the INIA Ethics Committee (Reference number PROEX 046/16) and were performed in accordance with the Spanish law of animal protection RD53/2013 which is in accordance with the European Union Directive 2010/63/UE.

Sperm Collection, Processing, Freezing, and Thawing

We used 10 males from 2 Spanish breeds of chickens (5 Birchen Leonesa roosters and 5 Red Villafranquina roosters). Semen was collected using the abdominal massage method 2 to 3 times a wk from the 10 roosters. The semen was pooled directly during semen collection for 10 times (ten replicates). In order to eliminate the effect of the breed, the semen of both breeds were mixed in the pooled samples. Samples were transferred immediately to the laboratory and kept warm in the hand to prevent cold shock. In the laboratory, the pooled semen was diluted 1:1 (v/v) at field temperature using the respective extender as described below for experiments 1 and 2. The extended semen samples were transferred to a refrigerator at 5 °C. Cooling down to 5 °C lasted about 1 h. During this period the evaluation of the fresh sperm variables were performed. Afterwards, extender and cryoprotectant were added, and were allowed to equilibrate for 10 min at 5°C. After equilibration, the samples were loaded into 0.25-mL straws (Minitüb, Germany) and then frozen using a Computer Freezer-Icetube 1810 freezer unit (Minitüb, Tiefenbach, Germany) with a medium freezing rate (from 5°C to −35°C at 7°C/min and then from −35°C to −140°C at 60°C/min). Then the frozen straws were submerged into and maintained in liquid nitrogen (at -196°C). For thawing, the straws containing glycerol and DMA were warmed for 3 min and 30 s, respectively, in a water bath at 5°C with continuous stirring. Then, post-thaw motility and viability were evaluated.

Experiment 1 (Methods of adding cryoprotectant). In this experiment 2 methods of addition of the cryoprotectant were compared, with glycerol or DMA as cryoprotectant. Each pool was extended 1:1 (v:v) with Lake extender and cooled to 5°C. To evaluate whether the samples were of sufficient quality to be frozen, evaluation of the fresh sperm variables was performed before division of the pool. Four equal aliquots of the semen were placed in tubes. The first aliquot was mixed 1:1 (v/v) with Lake medium containing 16% of glycerol (Diluted-glycerol). The second aliquot was first mixed 1:0.84 (v/v) with Lake medium, and then glycerol (0.16v) was added directly (Direct-glycerol). As a result, both aliquots eventually contained 8% glycerol that was added by 2 methods: diluted (Diluted-Method) and directly (Direct-Method). The third aliquot was mixed 1:1 (v/v) with Lake medium containing 8% of DMA (Diluted-DMA); and the fourth aliquot was first extended 1:0.92 (v/v) with Lake medium, and then DMA (0.08v) was added directly (Direct-DMA). Therefore, both aliquots contained 4% DMA that was added by two methods: diluted (Diluted-Method) or directly (Direct-Method). Thus, the 4 treatments were: Diluted-glycerol, Direct-glycerol, Diluted-DMA, and Direct-DMA. After equilibration time, aliquots were frozen in the morning and were thawed in the afternoon on the same day.

Experiment 2 (Supplementation with BSA). In this experiment, semen was frozen with 8% (v/v) glycerol as cryoprotectant. After measuring sperm concentration, the pooled semen was split into 4 aliquots. Each aliquot was extended 1:1 (v/v) with either Lake or ASG extender with or without BSA (10 mg/mL), that is, Lake, Lake-BSA, ASG, ASG-BSA and cooled to 5 °C for 1h, while the sperm parameters of each aliquot were determined (Table 2, first section). Afterward, each aliquot was extended 1:1 (v/v) with their specific extender (with which it had been previously extended) containing 16% glycerol. Finally, all aliquots contained 8% glycerol. After equilibration time, the semen samples were frozen in the morning and were thawed in the afternoon on the same day for assessment of post-thaw sperm motility and viability. For motility evaluations, frozen and/or

thawed semen was diluted with the same extender as was used before freezing.

Assessment of Sperm Variables

Concentration and objective kinetic sperm parameters were evaluated by a computer-aided sperm analysis system coupled to a phase-contrast microscope (Nikon Eclipse model 50i; negative contrast, Nikon Instruments Europe B.V., Izasa S.A., Barcelona, Spain). Sperm concentration was measured loading the extended semen onto a warmed (37 °C) 20 μm Leja 8-chamber slide (Leja Products B.V., Nieuw-Vennep, The Netherlands). The percentage of motile sperm, progressive motile sperm and kinematic values were recorded with the Sperm Class Analyzer SCA v.4.0. Software (Microptic S.L., Barcelona, Spain). A minimum of 3 fields and 200 sperm tracks were evaluated at a magnification of 100× for each sample (image acquisition rate 25 frames/s).

Staining with propidium iodide and SYBR-14 were used to measure sperm viability, a total of 200 spermatozoa were counted by a fluorescence microscope (Eclipse E200, Nikon, Tokyo, Japan) at 400× (wavelength: 450 −490 nm).

Statistical Analysis

To assess whether the data can be modeled by a normal distribution, Kolmogorov−Smirnov, Lilliefors, and Shapiro−Wilk tests were performed. If needed, the arcsin transformation, log-transformation, or the box-cox transformation of data was used. A first study was conducted as a 2×2 factorial arrangement in completely randomized design, including 2 factors: 2 methods of adding the cryoprotectant (Direct or Diluted) and 2 cryoprotectants (glycerol and DMA). A second study was randomly assigned according to a 2×2 factorial arrangement to effect of BSA on 2 different extenders (Lake and ASG). Data were analyzed by GLM-ANOVA using the GLM procedure of SAS (Statistical Analysis System) 9.1 software (SAS Institute Inc., Cary, NC, USA). The level of significance was set at $P<0.05$, and Tukey's test was performed for the mean comparison. When interaction effects were significant, the SLICE command in SAS was accomplished and significant letters were capitalized.

RESULTS AND DISCUSSION

Experiment 1

Post-Thaw Sperm Variables in 2 Different Methods of Adding Cryoprotectants. There were no significant differences in post-thaw sperm viability and sperm motility variables between the 2 addition methods (Direct vs. Diluted) for either glycerol or DMA (Table 1). The interaction between the methods of adding and type of cryoprotectant were not significant for any of the measured parameters. Overall, glycerol showed significantly better results that DMA for all measured parameters ($P < 0.05$). This indicates that addition of straight, undiluted cryoprotectants does not appreciably aggravates the (potential) chemical and osmotic stress associated with addition of cryoprotectants. In fact, post-thaw results for both addition methods obtained with glycerol seem almost identical. However, for DMA, diluted addition resulted in numerically higher values for all post-thaw sperm variables compared with direct addition. While these differences were not significant, the observations are in line with the notion that DMA is a more damaging compound for poultry sperm than glycerol, as just the addition of DMA before freezing resulted in significantly more sperm damage, compared with glycerol (Tselutin et al., 1999). We speculate that this may be related to the higher partition coefficient for octanol/

Table 1. Effect of adding diluted or direct cryoprotectant on frozen-thawed semen characteristics.

Method:	Diluted		Direct		Method		Cryoprotectant		SEM	Significance		
Cryoprotectant:	GLY	DMA	GLY	DMA	Diluted	Direct	GLY	DMA		Method	CR	Method×CR
Viable spermatozoa (%)	40.20	17.40	40.25	13.20	28.80	26.72	40.22[a]	15.30[b]	1.84	NS	*	NS
Motility quality score (1-5)[1]	3.10	0.95	3.07	0.85	2.02	1.96	3.08[a]	0.90[b]	0.14	NS	*	NS
Non-progressive sperm (%)	31.26	13.20	33.28	11.04	22.23	22.16	32.27[a]	12.12[b]	1.59	NS	*	NS
Progressive sperm (%)	18.90	2.44	20.90	1.45	10.67	11.17	19.90[a]	1.95[b]	1.27	NS	*	NS
Total motility (%)	50.16	15.65	54.19	12.50	32.91	33.34	52.17[a]	14.08[b]	1.55	NS	*	NS
VCL (μm/s)	63.03	34.80	62.94	30.61	48.91	46.77	62.98[a]	32.70[b]	2.97	NS	*	NS
VSL (μm/s)	40.34	19.52	40.76	13.58	29.93	27.17	40.55[a]	16.55[b]	2.63	NS	*	NS
VAP (μm/s)	48.72	24.06	49.13	20.20	36.39	34.66	48.93[a]	22.13[b]	2.74	NS	*	NS
LIN (%)	62.49	53.07	63.45	44.29	57.78	53.87	62.97[a]	48.68[b]	2.76	NS	*	NS
STR (%)	81.59	78.60	81.48	68.33	80.09	74.91	81.54[a]	73.46[b]	2.75	NS	*	NS
WOB (%)	76.21	66.68	77.25	65.17	71.45	71.21	76.73[a]	65.92[b]	1.78	NS	*	NS
ALH (μm)	2.80	1.96	2.80	1.76	2.38	2.28	2.80[a]	1.86[b]	0.20	NS	*	NS
BCF (Hz)	8.17	6.28	8.27	6.20	7.23	7.24	8.22[a]	6.24[b]	0.69	NS	*	NS

Abbreviations: GLY, glycerol; DMA, dimethylacetamide; CR, cryoprotectant; NS, non-significant differences; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency.

[a,b]Means within a row with no common superscript differ significantly ($*P < 0.05$)

[1]Subjective measurement: The quality of motility was scored on a scale of 0 (lowest) to 5 (highest); 0 = no movement, 1 = tail movements but no sperm progression, 2 = only circular sperm movements, 3 = a large percentage of spermatozoa showed progressive but no rectilinear movement, 4 = a large percentage of spermatozoa showed rectilinear but not very vigorous movement, and 5 = a large percentage of spermatozoa showed vigorous, rectilinear, progressive movement.

water (K_{ow}), with Log (K_{ow}) values being −0.77 and −1.76 for DMA and glycerol, respectively (https://pubchem.ncbi.nlm.nih.gov). These values indicate that a substantial fraction of DMA partitions in octane ([DMA] in octane = 17% of [DMA] in water), that is, DMA is quite lipophilic. If we assume that partitioning of DMA into lipid is similar, a medium DMA concentration of 4% (v/v) would mean that the sperm membranes contain approximately 0.7 % (v/v) of DMA, which could affect membrane stability. It seems not unlikely that this would be aggravated by brief exposure to locally elevated concentrations of DMA in the direct (undiluted) addition method.

Experiment 2

Pre-Freeze Sperm Variables in BSA-Supplemented Extenders. Semen extended in media without BSA supplementation showed better pre-freeze sperm viability and VSL in Lake than in ASG medium (Table 2; $P < 0.05$). However, supplementation of BSA and the interaction effect between BSA and ASG extender improved sperm viability in ASG extender and made no difference between 2 BSA-supplemented extenders. BSA improved most sperm motility parameters in both extenders and reduced non-progressive sperm in fresh semen ($P < 0.05$).

Post-thaw Sperm Variables in BSA-Supplemented Extenders. Supplementation with BSA resulted in better values in post-thaw semen for most sperm motility parameters (Table 2) in both extenders after thawing ($P < 0.05$) and it only had no effect on ALH and BCF. The interaction between extender type and treatments (with or without BSA) was significant ($P < 0.05$) for sperm viability, curvilinear velocity (**VCL**), straight-line velocity (**VSL**), average path velocity (**VAP**), and amplitude of lateral head displacement (**ALH**). While the % viable sperm was not different in the 2 non-supplemented extenders, % viable sperm was higher in ASG-BSA compared with Lake-BSA ($P < 0.05$). Also, supplementation of the extenders with BSA annulled the

Table 2. Effect of supplementing BSA to Lake and ASG extenders on rooster semen quality.

BSA:	−		+		BSA		Extender			Significance		
Extender:	Lake	ASG	Lake	ASG	−	+	Lake	ASG	SEM	BSA (A)	Extender (B)	A×B
FRESH SEMEN												
Viable spermatozoa (%)	80.50$^{A(2)}$	71.50$^{B(2)}$	78.50	77.60	76.00	78.05	79.50^{a}	74.55^{b}	1.34	NS	*	*$^{(2)}$
Motility quality score (1-5)[1]	2.72	2.85	3.97	3.95	2.78^{b}	3.96^{a}	3.35	3.40	0.10	*	NS	NS
Non-progressive sperm (%)	37.62	34.69	16.74	27.92	36.15^{a}	22.33^{b}	27.18	31.30	2.47	*	NS	NS
Progressive sperm (%)	31.52	23.66	55.92	46.50	27.59^{b}	51.21^{a}	43.72	35.08	3.15	*	NS	NS
Total motility (%)	69.14	58.35	72.67	74.42	63.75	73.54	70.90	66.39	3.61	NS	NS	NS
VCL (μm/s)	75.39	68.61	116.69	100.88	72.00^{b}	108.78^{a}	96.04	84.74	4.41	*	NS	NS
VSL (μm/s)	45.20	35.25	92.59	75.80	40.23^{b}	84.20^{a}	68.89^{a}	55.53^{b}	4.39	*	*	NS
VAP (μm/s)	55.45	47.00	102.11	85.91	51.22^{b}	94.01^{a}	78.78	66.45	4.29	*	NS	NS
LIN (%)	57.27	50.73	78.80	74.24	54.00^{b}	76.52^{a}	68.03	62.48	2.37	*	NS	NS
STR (%)	78.88	73.51	90.36	87.46	76.20^{b}	88.91^{a}	84.62	80.49	1.62	*	NS	NS
WOB (%)	72.01	68.18	87.03	84.67	70.09^{b}	85.85^{a}	79.52	76.42	1.46	*	NS	NS
ALH (μm)	2.97	2.92	3.06	3.28	2.95	3.17	3.02	3.10	0.11	NS	NS	NS
BCF (Hz)	9.60	8.78	10.28	9.82	9.19^{b}	10.05^{a}	9.94	9.30	0.24	*	NS	NS
POST-THAW SEMEN												
Viable spermatozoa (%)	36.90	30.30	33.90$^{B(3)}$	43.40$^{A(3)}$	33.60^{b}	38.65^{a}	35.40	36.85	1.71	*	NS	*$^{(3)}$
Motility quality score (1-5)[1]	1.47	1.95	2.70	2.72	1.71^{b}	2.71^{a}	2.08	2.33	0.14	*	NS	NS
Non-progressive sperm (%)	12.49	14.04	15.65	20.50	13.26^{b}	18.08^{a}	14.07	17.27	1.37	*	NS	NS
Progressive sperm (%)	5.39	9.48	16.58	15.28	7.43^{b}	15.93^{a}	10.99	12.38	1.23	*	NS	NS
Total motility (%)	17.88	23.52	32.24	35.78	20.70^{b}	34.01^{a}	25.06	29.65	2.24	*	NS	NS
VCL (μm/s)	48.22$^{B(4)}$	61.76$^{A(4)}$	67.90	61.84	54.99^{b}	64.87^{a}	58.06	61.80	2.61	*	NS	*$^{(4)}$
VSL (μm/s)	28.42$^{B(5)}$	39.07$^{A(5)}$	50.19	45.83	33.74^{b}	48.01^{a}	39.30	42.45	2.45	*	NS	*$^{(5)}$
VAP (μm/s)	34.68$^{B(6)}$	47.08$^{A(6)}$	56.32	51.70	40.88^{b}	54.01^{a}	45.50	49.39	2.55	*	NS	*$^{(6)}$
LIN (%)	58.13	62.62	73.18	73.64	60.38^{b}	73.41^{a}	65.66	68.13	1.79	*	NS	NS
STR (%)	81.07	82.45	88.68	88.43	81.76^{b}	88.55^{a}	84.88	85.44	1.11	*	NS	NS
WOB (%)	71.42	75.68	82.30	83.15	73.55^{b}	82.73^{a}	76.86	79.42	1.25	*	NS	NS
ALH (μm)	2.16$^{B(7)}$	2.74$^{A(7)}$	2.59	2.39	2.45	2.49	2.38	2.57	0.09	NS	NS	*$^{(7)}$
BCF (Hz)	8.84	9.14	9.55	9.20	8.99	9.37	9.19	9.17	0.26	NS	NS	NS

(-) Absence of BSA, (+) Supplementation with BSA

Abbreviations: Lake, Lake and Ravie 1984 medium; ASG, Animal Sciences Group (Wageningen University, Lelystad, The Netherlands) medium; NS, non-significant differences; VCL, curvilinear velocity; VSL, straight-line velocity; VAP, average path velocity; LIN, linearity; STR, straightness; WOB, wobble; ALH, amplitude of lateral head displacement; BCF, beat-cross frequency.

[a,b]Means within a row with no common superscript differ significantly (*$P < 0.05$)

[1]Subjective measurement: The quality of motility was scored on a scale of 0 (lowest) to 5 (highest); 0 = no movement, 1 = tail movements but no sperm progression, 2 = only circular sperm movements, 3 = a large percentage of spermatozoa showed progressive but no rectilinear movement, 4 = a large percentage of spermatozoa showed rectilinear but not very vigorous movement, and 5 = a large percentage of spermatozoa showed vigorous, rectilinear, progressive movement.

[2(A,B)]Interaction effect (BSA×Extender) for viable spermatozoa (%) in fresh semen.

[3(A,B)]Interaction effect (BSA×Extender) for viable spermatozoa (%) in post-thaw semen.

[4(A,B)]Interaction effect (BSA×Extender) for VCL (μm/s) in post-thaw semen.

[5(A,B)]Interaction effect (BSA×Extender) for VSL (μm/s) in post-thaw semen.

[6(A,B)]Interaction effect (BSA×Extender) for VAP (μm/s) in post-thaw semen.

[7(A,B)]Interaction effect (BSA×Extender) for ALH (μm) in post-thaw semen.

differences in VCL, VSL, VAP, and ALH, which were higher in non-supplemented ASG extender than non-supplemented Lake medium.

The somewhat more pronounced effect of the presence of BSA in ASG than in Lake may be due to the fact that Lake contains 0.3% polyvinylpyrrolidone. This is a macromolecule that is used as a blood plasma substitute as it shares a number of properties with BSA. Woelders (2021) reported that the addition of BSA after freezing and thawing is also positive for the motility. It is mentioned that BSA adsorb on the membranes of rabbit sperm cells and coats the surface area of sperm (Blank et al., 1976). The coating causes the sperm to be less hindered in their motility by physical obstacles (Bakst and Cecil, 1992). Chakarov and Mollova (1980) suggested that adsorption of BSA to the sperm plasma membrane rendered ram sperm more resistant to the otherwise harmful effect of a high rate of semen dilution. BSA could augment the elimination of free radicals generated by oxidative stress, and protect the sperm membrane from cold shock during freeze-thaw in canine sperm (Uysal et al., 2005). It has been reported the antioxidant property of BSA (5 mg/mL) as it is able to increase the activity of catalase and protect the sperm morphology, and preserve the fertilizing potential of bull sperm after the freeze-thawing process (Sariözkan et al., 2009). Since BSA is a mammal-derived protein and may cause immune responses in hens' reproductive system, its effectiveness in improving fertility rates by artificial insemination should be considered in future studies.

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DISCLOSURES

None of the authors have any conflict of interest to declare.

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