Effect of glycerol concentration, glycerol removal method, and straw type on the quality and fertility of frozen chicken semen

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ABSTRACT The long-term semen cryopreservation is increasingly crucial for conservation of endangered livestock and poultry species. Glycerol is the most widely used cryoprotectant for freezing chicken semen. Continuous improvement in details with glycerol may help increase the fertility of post-thawed semen. Two experiments were performed in the present study to investigate the effects of glycerol concentration, removal method, and straw type on the quality of post-thawed sperm. In experiment 1, glycerol concentration (3%, 5%, 7%, 9%, 11%, and 13%) and glycerol removal method (final dilution ratio 1:1, 1:2, 1:4, 1:8, 1:16, and 1:20) combination groups were investigated for post-thawed sperm quality, residual glycerol concentration, and fertility to find the best combinations. Experiment 2 was performed to evaluate the effects of straw type (0.25 and 0.5 mL) and glycerol concentration (3%, 5%, 7%, 9%, 11%, and 13%) on the post-thawed sperm quality. Results showed that post-thawed sperm motility of 6 glycerol concentration groups were different ($P < 0.01$). Sperm motility of 5%, 7%, 9%, 11% and 13% was higher than that of 3% ($P < 0.01$). There was no difference among different concentrations of glycerol in VSL, VCL, VAP, ALH, WOB, BCF, LIN, or STR ($P > 0.05$). As for the glycerol removal method, sperm motility of 1:8 dilution was the highest, followed by 1:1 and 1:2, while the difference among groups was not statistically significant ($P = 0.11$). Glycerol concentration and removal method had no interaction effect on sperm motion parameters ($P > 0.05$). The highest fertility (48.70%) was found for the 5% and 1:2 combination. There was no difference for sperm motility between 0.25 and 0.5 mL straws ($P > 0.05$). Glycerol concentration and straw type had no interaction effect on the sperm motion parameters ($P > 0.05$). It can be concluded from these observations that the combination of 5% glycerol and 1:2 dilution rendered higher fertility should be suggested in practice, and that both 0.25 and 0.50 mL straws fit the present procedure.

Key words: chicken, semen cryopreservation, glycerol, straw, fertility

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INTRODUCTION

Since the 1940s, many studies have proved that semen cryopreservation is the most practical method for long-term preservation of animal genetic resources. However, poultry sperm is highly sensitive to the freezing and thawing process due to the special structure. The process is accompanied with temperature shock resulting in disruption of the sperm plasma membrane and a reduction in sperm motility and viability which can potentially decrease the fertility potential (Masoudi et al., 2016; Lotfi et al., 2017). Researchers have been trying to define the best freezing conditions such as cryoprotectants used and its concentration, equilibration time, and freezing and thawing rates to avoid freezing damage and improve fertility. Semen cryoprotectant is the key factor in semen freezing procedure, including intracellular cryoprotectants and extracellular cryoprotectants. At present, glycerol, dimethylsulfoxide (**DMSO**), dimethylformamide (**DMF**), dimethylacetamide (**DMA**), and ethylene glycol (**EG**)

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are the most studied cryoprotectants (Seigneurin and Blesbois, 1995; Partyka et al., 2012; Miranda et al., 2017).

Glycerol is widely used in germplasm cryopreservation. It has been proved firstly that glycerol is capable of maintaining rooster sperm frozen-thawed motility and improve the freezing resistance of sperm (Polge et al., 1949). During freezing, glycerol penetrates the sperm, concentrates intracellular water to reduce the formation of intracellular ice crystals and the damage caused by high concentration of solute to protect sperm. The appropriate concentration of glycerol (v/v) that can have the best protective effect on sperm has been widely studied. Glycerol has been included at concentration of 4% to 11% in poultry semen extenders with different effects depending on the other constituents of diluents (Mohammad et al., 2016). Although some used very low concentration as 3% (Siari et al., 2021), it is generally exceeding 8%, and 11% is the most used (Mocé et al., 2010). However, no studies have conclusively proved that 11% is the optimal glycerol concentration. Various levels of glycerol (0, 2%, 4%, 7%, 10%, and 15%) were used to investigate freeze-thaw damage and found that no significant differences existed among semen samples diluted in 7%, 10%, and 15% glycerol (Terada et al., 1983). The researchers compared 2% and 8% glycerol as cryoprotectant and obtained the fertility of 34.8% and 45.1%, respectively (Mehdipour et al., 2020). Meanwhile, glycerol has contraceptive action. It can lead to a significant reduction in fertility, which requires the removal of glycerol after thawing before artificial insemination (Tang et al., 2021). Initially, glycerol removal method involves a systematic stepwise dilution and centrifugation for reducing the glycerol concentration gradually after thawing. The procedure was thought to be associated with minimizing cell membrane damage (Abouelezz et al., 2017). Then the method of removing glycerol from fresh and cryopreserved rooster sperm by discontinuous Accudenz column centrifugation was evaluated (Long and Kulkarni, 2004). Compared with Accudenz method, the stepwise dilution produced more complete plasma membrane, which is crucial for the maintaining of fertility potential (Purdy et al., 2009). The glycerol of 8% and 11% were removed by stepwise dilution to a final dilution of 1:4 v/v, and the fertility was 28.8% and 2.1%, respectively (Abouelezz et al., 2015). This suggested that the glycerol concentration and dilution rate should be screened and optimized simultaneously to find the best combination. In addition, it is necessary to determine the residual glycerol content of sperm after removal with different dilution ratios. Few studies have been reported on the determination of glycerol residual concentration in thawing sperm before artificial insemination.

The effectiveness of sperm cryopreservation may also depend on the interaction between the cryoprotectant used and the freezing semen packaging method employed, that is the use of pellets or straws (Tselutin et al., 1999). It has been suggested that the best results are obtained when glycerol is used with the in-straw method (Blesbois et al., 2007). The commonly used frozen semen straw are mini straw (0.25 mL) and medium straw (0.5 mL), which are the same length and different diameters (Masoudi et al., 2018; Thélie et al., 2019). Their different surface-to-volume ratios may exert effect on post-thaw recovery of sperm motility and thus fertility. There have been some studies on the effects in boar (Eriksson and Rodriguez-Martinez, 2000), ram (Nordstoga et al., 2010), and bovine (Lone et al., 2020), but comparative studies in chickens are quite rare.

Therefore, we evaluated the effect of different glycerol concentration and glycerol removal method combination groups on residual glycerol concentration, post-thawed sperm motion parameters, and fertility to screen the best glycerol concentration and glycerol removal method combinations. Furthermore, we evaluated the effect of different glycerol concentration and straw types on post-thawed sperm motion parameters to screen better straw types. The aim of the present study was to provide reference for the establishment of efficient and stable frozen semen technology of chickens.

MATERIALS AND METHODS

Ethics Statement

The present study was approved by the Animal Care and Use Committee of Institute of Animal Sciences, Chinese Academy of Agricultural Sciences (**IAS-CAAS**, No. IAS2021-117) and was performed in accordance with the relevant guidelines and regulations set by Ministry of Agriculture and Rural Affairs of the People's Republic of China.

Experimental Design

The study consists of 2 experiments. In experiment 1, we adopted 6 × 6 two-factor experimental design to estimate the main effect of glycerol concentration and glycerol removal method (final dilution ratio), and their interaction effect on post-thawed semen quality. Residual glycerol concentration of each combination was determined and fertility of better post-thawed semen quality combination was further verified to screen the best glycerol concentration and glycerol removal method combinations. In experiment 2, we adopted 2 (straw types) × 6 (glycerol concentrations) two-factor experimental design to investigate the effects of straw type on the quality of post-thawed sperm motion parameters.

Animals and Farm Management

All chickens used in this study were kept on the experimental farm of IAS-CAAS. Healthy 52-wk-old Beijing-You chicken roosters (n = 160) were exposed to semen quality evaluation every other day after regular training of semen collection by abdominal massage (Burrows and Quinn, 1937). Thirty males with qualified semen were selected for the following studies. A total of 360 Rhode Island Red hens (70-wk-old) were used for artificial insemination to estimate the fertility of frozen/thawed sperm. All chickens were housed in individual battery

cages and the nutrient level of the diet was designed according to the chicken feeding standard (NY/T 33-2004). The light rhythm was 16 L:8 D (16 h light: 8 h darkness), with the light intensity of 20 lx.

Chemicals and Extenders

All Chemicals used in this study were obtained from Sigma Co. (St. Louis, MO). Lake extender (Lake and Stewart, 1978) containing 1.92 g sodium L-glutamate monohydrate, 0.5 g potassium acetate, 0.08 g magnesium acetate tetrahydrate, 0.6 g fructose, 0.3 g polyvinylpyrrolidone (MW 10000), and 100 mL Milli-Q water (343 mOsm/kg, pH 7.08) was used as basic extender in this study.

Semen Collection and Dilution

Before the experiments, quality evaluation of individual semen collection (n = 160) was performed as following. A drop of 10 μL fresh semen was immedicably sent for sperm concentration and motility were determined estimation by the computer-aided semen analysis (**CASA**) system (Nanning Songjingtianlun Biotechnology Co., Ltd., Nanning, China). The specific operation was as following: 10 μL fresh semen was slowly diluted with 990 μL dulbecco's modified eagle medium (**DMEM**) preheated at 37°C. A drop of 10 μL diluted semen was added into the standard CASA analysis chamber slide (ML-CASA 20 (chamber height = 20 μm), Nanning Songjingtianlun Biotechnology Co., Ltd., Nanning, China) placed on the 37°C-microscope stage. Five fields per sample were captured by the CASA system from the microscope equipped with a negative phase-contrast lens for further analysis. The individuals were selected on the basis of criteria as follows: sperm concentration $\geq 1.6 \times 10^9$ sperm/mL, and sperm motility ≥50%.

Collection of qualified semen from 30 males was collected into preheated 2 mL Eppendorf tubes, immediately placed in a dry bath (H2O3-100C, Coyote Bioscience [Beijing] Co., Ltd., Beijing, China) at 37°C, mixed, and diluted at a ratio of 1:1 (v/v) with extender preheated at 37°C. After diluting, the semen was divided into 2 mL Eppendorf tubes to ensure the uniformity of cooling at 4°C for 30 min, during which, the temperature could reach 4°C in 5 min. Then, the semen was divided into six equal aliquots and further diluted at a ratio of 1:2 (v/v) using the extender containing different concentration of glycerol, making the final glycerol concentration of 3%, 5%, 7%, 9%, 11%, and 13%, respectively. The semen was equilibrated at 4°C for 10 min.

Experiment 1: Effects of Glycerol Concentration and Glycerol Removal Method on Post-Thawed Sperm Quality

Semen Freezing and Thawing After equilibration, semen of each glycerol concentration group was packaged with 0.5 mL French straws (IMV, L'Aigle, France) and sealed with polyvinyl alcohol powder. The straws were placed in a programmed freezer for cooling and freezing at a rate of 12°C/min from 4°C to −44°C, followed by freezing at a rate of 40°C/min from −44°C to −120°C, and finally plunged into liquid nitrogen for a storage of 1 wk. Then frozen semen was thawed in a water bath at 5°C for 3 min with continuous stirring.

Glycerol Removal and Residual Glycerol Concentration Determination The glycerol were removed by stepwise dilution method at 4°C. Six dilution ratios were designed (1:1, 1:2, 1:4, 1:8, 1:16, and 1:20). The glycerol-free extender was added in 6 times every 2 min with volume progressively increased and then centrifuged at 600 × g for 10 min. The supernatant was discarded and the pellet was resuspended with extender. Sperm motion parameters were evaluated by the CASA system including sperm motility (MOT), straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), amplitude of the lateral head displacement (ALH), wobble (WOB), beat cross frequency (BCF), linearity (LIN), and straightness (STR). The specific operation was the same as the earlier description, with the exception that 10 μL post-thawed semen was slowly mixed with 240 μL DMEM preheated at 37°C, in view of the fact that sperm concentration of the post-thawed semen was lower. Glycerol assay kit (F005-2-1, Nanjing Jiancheng Biotech Co., Ltd., Nanjing, China) was used to determine the residual glycerol content in the sperm of each concentration group following the manufacturer' instructions. The analysis of all treatments were carried out with 3 replicates.

Artificial Insemination and Fertility Evaluation Finally, the 12 groups (4 glycerol concentration (5%, 7%, 9%, and 11%) × 3 glycerol removal methods (1:2, 1:4, and 1:8)) with higher post-thawed sperm motility after glycerol removal were chosen to evaluate the in vivo fertility. A total of 360 hens were divided into 12 equal aliquots for artificial insemination. Each hen was inseminated with about 100×10^6 sperms each time. Artificial insemination was performed 3 times at a 2-d interval. Eggs were collected for incubation. After 7 days of incubation, the eggs were opened to check the presence of embryos. The fertility was counted for each group as follow: Fertility (%) = (number of fertilized eggs/number of setting eggs) × 100.

Experiment 2: Effects of Glycerol Concentration and Straw Type on Post-Thawed Sperm Quality

After equilibration, each concentration group was packaged with 0.25 mL and 0.5 mL straws, respectively. Following the freezing and storage procedure as described in experiment 1, sperm motion parameters including MOT, VSL, VCL, VAP, ALH, WOB, BCF, LIN, and STR were determined by the CASA system.

Statistical Analysis

All data were analyzed by 2-factor analysis of variance using SAS software (version 9.2; SAS Inst. Inc., Cary, NC), followed by Dunnett post hoc tests if significant

effect was detected. All percentage data were normalized with an arcsine transformation. For experiment 1, the main effects included glycerol concentration, glycerol removal method, and their interaction. An analysis of $\chi 2$ test was done to assess significance of fertility of different combinations of glycerol concentrations and glycerol removal methods. For experiment 2, the main effects included glycerol concentration, straw type, and their interaction. $P < 0.05$ was set as the significant level. Results are shown as non-transformed means ± SEM.

RESULTS

Experiment 1: Effects of Glycerol Concentration and Glycerol Removal Methods on Frozen Sperm Quality

Effects of Glycerol Concentration and Glycerol Removal Methods on Post-Thawed Sperm Motion Parameters As shown in Table 1, post-thawed sperm motility of different glycerol concentration groups exerted differences ($P < 0.01$). Sperm motility of 5%, 7%, 9%, 11%, and 13% was higher than that of 3% ($P < 0.01$). There were no significant differences among different concentrations of glycerol on the VSL, VCL, VAP, ALH, WOB, BCF, LIN, or STR ($P > 0.05$). In the aspect of glycerol removal method, sperm motility of 1:8 dilution was numerically the highest, followed by 1:1 and 1:2, while the difference between groups was not statistically significant ($P = 0.11$). Other sperm motion parameters did not differ among the glycerol removal method groups ($P > 0.05$). Glycerol concentration and glycerol removal method had no interaction effect on sperm motion parameters investigated ($P > 0.05$).

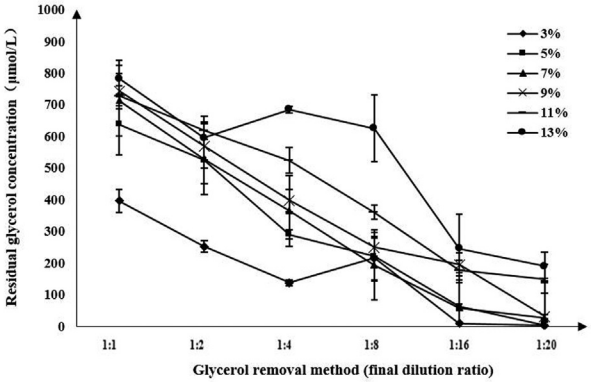

Figure 1. Residual glycerol content of the sperm of different glycerol concentration group (3%, 5%, 7%, 9%, 11% and 13%) interacted with different glycerol removal method (stepwise dilution to a final dilution ratio of 1:1, 1:2, 1:4, 1:8, 1:16, and 1:20, respectively).

Residual Glycerol Content of Sperm of Different Glycerol Concentration and Glycerol Removal Methods

As shown in Figure 1, for the same glycerol concentration, the larger the dilution ratio, the lower the residual glycerol concentration after the removal. There was some exception in the highest glycerol concentration group (13%) that the 1:2 dilution ratio resulted with lower concentration than the 1:4 and 1:8 group.

Effects of Different Glycerol Concentrations and Glycerol Removal Methods on Fertility

As shown in Table 2, 12 combinations of selected glycerol concentration (5%, 7%, 9%, and 11%) and glycerol removal methods (1:2, 1:4, and 1:8) were further

Table 1. Effects of glycerol concentration and glycerol removal method on post-thawed sperm motion parameters.

Item	MOT (%)	VSL (μm/s)	VCL (μm/s)	VAP (μm/s)	ALH (μm)	WOB (%)	BCF (Hz)	LIN (%)	STR (%)
Glycerol concentration									
3%	28.62 ± 2.24[b]	17.59 ± 0.58	49.74 ± 2.04	45.36 ± 2.76	0.49 ± 0.02	90.60 ± 2.84	3.03 ± 0.28	34.70 ± 1.16	39.70 ± 1.85
5%	37.44 ± 1.37[a]	19.42 ± 0.52	56.45 ± 1.35	52.54 ± 1.51	0.55 ± 0.01	93.00 ± 1.13	2.72 ± 0.14	34.55 ± 0.96	37.24 ± 1.47
7%	37.93 ± 2.20[a]	18.74 ± 1.21	53.83 ± 3.26	47.27 ± 4.40	0.53 ± 0.03	85.45 ± 4.63	2.50 ± 0.10	35.90 ± 1.32	42.09 ± 2.75
9%	40.33 ± 2.40[a]	16.74 ± 0.97	48.32 ± 2.24	41.15 ± 3.06	0.47 ± 0.02	82.76 ± 3.09	2.40 ± 0.17	34.50 ± 1.30	42.48 ± 2.08
11%	40.32 ± 2.87[a]	18.28 ± 0.67	53.07 ± 1.19	47.67 ± 1.64	0.52 ± 0.01	89.64 ± 1.44	3.04 ± 0.27	34.73 ± 1.18	38.70 ± 1.85
13%	36.65 ± 2.15[a]	18.23 ± 0.43	53.33 ± 1.73	47.02 ± 2.33	0.52 ± 0.02	87.35 ± 2.21	2.76 ± 0.10	34.35 ± 0.54	40.15 ± 1.90
Glycerol removal method									
1:1	39.86 ± 3.12	17.88 ± 0.75	47.85 ± 3.25	41.90 ± 4.61	0.47 ± 0.03	85.33 ± 4.22	2.68 ± 0.28	34.78 ± 1.00	42.02 ± 3.57
1:2	38.84 ± 1.68	18.06 ± 1.04	52.91 ± 2.71	47.40 ± 3.56	0.52 ± 0.03	87.43 ± 3.61	2.54 ± 0.12	33.77 ± 0.75	39.73 ± 1.85
1:4	34.87 ± 2.70	17.90 ± 1.02	53.93 ± 1.65	48.93 ± 1.83	0.53 ± 0.02	90.50 ± 1.25	2.47 ± 0.17	35.33 ± 0.92	39.03 ± 1.42
1:8	40.09 ± 2.30	17.91 ± 0.67	52.61 ± 2.11	47.21 ± 2.75	0.51 ± 0.02	88.79 ± 2.39	2.67 ± 0.13	33.64 ± 1.29	39.01 ± 1.64
1:16	34.25 ± 1.88	18.41 ± 0.48	53.61 ± 1.34	47.09 ± 1.60	0.52 ± 0.01	87.71 ± 1.72	2.86 ± 0.15	34.71 ± 0.77	39.57 ± 1.43
1:20	33.38 ± 2.11	18.09 ± 0.86	51.23 ± 2.06	44.77 ± 3.18	0.50 ± 0.02	85.57 ± 3.78	2.94 ± 0.25	36.23 ± 1.35	42.64 ± 2.76
P-value									
Glycerol concentration	<0.01	0.27	0.12	0.15	0.11	0.25	0.14	0.99	0.73
Glycerol removal method	0.11	0.99	0.67	0.74	0.67	0.84	0.42	0.65	0.71
Glycerol concentration × Glycerol removal method	0.64	0.48	0.57	0.58	0.63	0.79	0.64	0.58	0.62

Abbreviations: MOT, motility (%); VSL, straight-line velocity (μm/s); VCL, curvilinear velocity (μm/s); VAP, average path velocity (μm/s); ALH, mean amplitude of the lateral head displacement (μm); WOB, wobble (%); BCF, mean of the beat cross frequency (Hz); LIN, linearity (%); STR, straightness (%).

[a,b]Different letters in the same column within the main effect indicate significant differences ($P < 0.05$).

Table 2. Fertility of different combinations of glycerol concentrations and glycerol removal methods.

Glycerol concentration	Glycerol removal method	No. of fertilized eggs	No. of unfertilized eggs	Fertility (%)	*P*-value
5%	1:2	56	59	48.70	0.17
	1:4	48	69	41.03	
	1:8	44	76	36.67	
7%	1:2	46	70	39.66	0.40
	1:4	58	63	47.93	
	1:8	49	69	41.52	
9%	1:2	33	79	29.46[b]	0.03
	1:4	47	56	45.63[a]	
	1:8	33	68	32.67[ab]	
11%	1:2	9	109	7.63	0.54
	1:4	12	89	11.88	
	1:8	11	90	10.89	
Main effect					
Glycerol concentration	5%	148	204	42.13[a]	<0.01
	7%	153	202	43.04[a]	
	9%	113	203	35.92[a]	
	11%	32	288	10.13[b]	
Glycerol removal method	1:2	144	317	31.36	0.08
	1:4	165	277	36.62	
	1:8	137	303	30.44	

[a,b]Different letters in the same column within the main effect indicate significant differences ($P < 0.05$).

evaluated for fertility via artificial insemination. The highest fertility was found for the 5% glycerol and 1:2 combination (48.70%), and the lowest fertility of 7.63% was from the 11% glycerol and 1:2 combination. The results of $\chi 2$ test showed that the fertility of 11% glycerol concentration group was significantly lower than those of other groups ($P < 0.01$). Different glycerol removal methods had no significant effect on the fertility ($P = 0.08$), but there was a trend that the fertility was higher after low proportion dilution. The fertility of 1:4 was higher than that of 1:2 when the glycerol concentration was 9% ($P < 0.05$).

Experiment 2: Effects of Glycerol Concentration and Straw Type on Post-thawed Sperm Quality

As shown in Table 3, there were significant differences in the sperm motility among the glycerol concentration groups ($P < 0.01$). The sperm motility of 5%, 9%, and 11% was higher than others ($P < 0.01$). Sperm LIN and STR of 3% was lower than other glycerol concentration groups ($P < 0.01$). Sperm VSL of 3% was lower than 9% glycerol concentration group ($P < 0.01$). Sperm motility between 0.5 mL and 0.25 mL straw had no difference ($P > 0.05$). Glycerol concentration and straw type had no significant interaction effect on sperm motion parameters ($P > 0.05$).

DISCUSSION

The glycerol is the most used cryoprotectant in chicken semen cryopreservation. Its different concentration may result with varying effect on sperm function and therefore fertilizing ability. It has been indicated that the optimum glycerol concentration was 7% (range of 6%−9%) (Watanabe et al., 1975). In another study, the results showed that glycerol concentration of 4%, 6%, 7%, and 8% differed in sperm motility and viability, but not in the fertilizing ability (23%−30%) (Blanch et al., 2014). However, the results of our study clearly showed that the post-thawed sperm motility of

Table 3. Effects of glycerol concentration and straw type on post-thawed sperm motion parameters.

Item	MOT (%)	VSL (μm/s)	VCL (μm/s)	VAP (μm/s)	ALH (μm)	WOB (%)	BCF (Hz)	LIN (%)	STR (%)
Glycerol concentration									
3%	43.05 ± 3.41[d]	20.79 ± 0.48[b]	58.46 ± 1.38	53.51 ± 1.46	0.57 ± 0.01	91.50 ± 0.62	2.59 ± 0.08	35.67 ± 0.67[b]	38.91 ± 0.76[b]
5%	61.40 ± 2.57[abc]	22.41 ± 0.33[ab]	58.55 ± 1.35	52.77 ± 1.94	0.57 ± 0.01	90.00 ± 1.37	2.32 ± 0.03	38.17 ± 0.70[a]	42.69 ± 1.28[a]
7%	56.28 ± 3.07[c]	21.78 ± 0.42[ab]	56.94 ± 2.02	50.66 ± 2.24	0.56 ± 0.02	88.83 ± 0.95	2.31 ± 0.08	38.50 ± 0.85[a]	43.26 ± 1.20[a]
9%	67.52 ± 4.79[a]	22.79 ± 0.32[a]	58.49 ± 1.13	52.77 ± 1.14	0.57 ± 0.01	90.20 ± 0.37	2.37 ± 0.03	39.20 ± 0.37[a]	43.22 ± 0.45[a]
11%	64.84 ± 4.67[ab]	22.33 ± 0.34[ab]	56.08 ± 1.23	50.34 ± 1.44	0.55 ± 0.01	89.71 ± 1.04	2.29 ± 0.08	40.14 ± 0.59[a]	44.52 ± 1.05[a]
13%	57.15 ± 5.29[bc]	21.15 ± 0.59[ab]	52.53 ± 1.87	46.41 ± 1.81	0.51 ± 0.02	88.33 ± 0.99	2.32 ± 0.07	40.50 ± 0.50[a]	45.69 ± 0.85[a]
Straw type									
0.5 mL	59.88 ± 3.20	21.97 ± 0.35	57.14 ± 1.03	51.64 ± 1.18	0.56 ± 0.01	90.28 ± 0.68	2.33 ± 0.05	38.67 ± 0.50	42.77 ± 0.76
0.25 mL	56.86 ± 2.59	21.75 ± 0.21	56.41 ± 0.92	50.37 ± 1.00	0.55 ± 0.01	89.22 ± 0.44	2.40 ± 0.04	38.78 ± 0.54	43.40 ± 0.73
P-value									
Glycerol concentration	<0.01	0.04	0.08	0.08	0.08	0.31	0.05	<0.01	<0.01
Straw type	0.20	0.59	0.58	0.40	0.52	0.24	0.27	0.82	0.51
Glycerol concentration × Straw type	0.26	0.67	0.23	0.26	0.27	0.59	0.67	0.10	0.23

Abbreviations: MOT: motility (%); VSL: straight-line velocity (μm/s); VCL: curvilinear velocity (μm/s); VAP: average path velocity (μm/s); ALH: mean amplitude of the lateral head displacement (μm); WOB: wobble (%); BCF: mean of the beat cross frequency (Hz); LIN: linearity (%); STR: straightness (%).
[a-d]Different letters in the same column within the main effect indicate significant differences ($P < 0.05$).

5%, 9%, and 11% glycerol concentration group was higher than those of 3%, 7%, and 13% groups. This observation was basically consistent with the rules that higher glycerol concentration within limits producing better protective effect. The extreme high glycerol concentration (15%) may have toxic effect in the form of sperm membrane irregularity and labile plasmalemma (Bakst and Howarth, 1977). High glycerol concentration increases the osmotic pressure of diluent and dehydrates sperm, resulting in protein denaturation and damage of cell membrane structure and formation of sperm, known as "Osmotic Damage." The glycerol concentration may affect the morphology and function of sperm via adjusting the osmotic pressure of diluent, making it is reasonable to find an optimal concentration where the glycerol is protective and introduces the least osmotic damage.

Glycerol improves the freezing resistance of sperm, but it also has anti-fertilization effect, which is particularly obvious after freezing (Mphaphathi et al., 2016). It is therefore necessary to remove the glycerol before artificial insemination. The commonly used method is the stepwise dilution, by gradually changing the osmotic pressure of chicken semen dilution to remove glycerol molecules from the sperm cells (Blesbois et al., 2007). Glycerol has a great influence on the osmotic pressure of diluent. Therefore, further study on the different dilution methods was performed to screen out the best dilution ratio. Researchers have suggested that 11% glycerol and high proportion dilution (e.g., 1:19) can obtain higher fertility (Seigneurin and Blesbois, 1995; Blesbois et al., 2007; Seigneurin et al., 2013). We earlier speculated that high concentration and high final dilution ratio could protect the rooster sperm better and remove glycerol better, resulting in the least residual glycerol and the highest fertility. On the contrary, lower dilution ratio of 1:2 of the 6% glycerol obtained the highest average post-thawed semen fertility of 72.31%, and the highest single day fertility of 88.99% (Zong et al., 2020). Then, we carried out the determination of glycerol residue after glycerol removal in this study, which is rarely reported in semen freezing research. The results showed that for the same glycerol concentration, the larger dilution ratio, the lower residual glycerol concentration after the removal. This result is also consistent with the previous speculation. But the motility and fertility are the same as previous study, thawed semen treated with a low proportion of stepwise dilution method achieved higher sperm motility and fertility. Although different glycerol removal methods had no significant effect on fertility, there was still a trend of high fertility with low dilution ratio. Thus, we hypothesize that the higher the dilution ratio, the greater the change of sperm osmotic pressure. The increase in water molecules entering cells, centrifugation, and resuspension of the sperm may cause significant physical damage to sperm membranes and organelles. It may be irreversible damage to sperm, which affects fertility severely. This also can be seen from the results in Tables 1 and 3 that the process of glycerol removal may have negative effects on sperm motion parameters. In practice, the high proportion of dilution would increase the preprocessing time and dilution cost of artificial insemination. Therefore, we consider low concentration glycerol and low proportion dilution (5% and 1:2) as the most suitable combination based on this study. The fertility in this study was lower than before (Zong et al., 2020), which may be due to the decrease of egg production rate of the older hens and roosters involved here.

At present, the main common forms of frozen semen packaging include pellet and straw. Different cryoprotectants with appropriate packaging types would obtain better fertility results (Partyka et al., 2013; Zaniboni et al., 2014). The French poultry semen freezing bank mainly adopts DMA pellets/straw and glycerol straws for semen frozen (Blesbois et al., 2007). However, compared with the straw frozen semen, pellet is not convenient for labeling and easy to be contaminated when it is removed from the freezer for manipulation (Blackburn et al., 2009). Straw frozen semen is suitable for rapid freezing, uniform temperature, standard dose, distinct mark, convenient thawing and insemination etc. A study on boar semen showed that the cryopreservation effect is better with a small straw size (Hernandez et al., 2007). Another study also found that 0.25 mL straws-packaged semen resulted in the highest lambing rate, and was numerically better than 0.5 mL straws regardless of thawing procedure used (Nordstoga et al., 2010). However, in our study with chickens, straw sizes had no significant effect on the post-thawed sperm quality. There are also some literatures presented similar results (Duplaix. and Sexton, 1984). Similarly, there were no difference in boars post-thawed spermatozoa motility in 0.25 mL and 0.5 mL straws (Buranaamnuay et al., 2010). This indicated that both straw sizes fit the procedure used here.

CONCLUSIONS

It is speculated from this study that in a limited range (5%−11%), with the increase of glycerol concentration, the better the protective effect could have on chicken sperm, and that a relatively high sperm motility and fertility could be obtained by glycerol removal at a lower dilution ratio. The combination of 5% and 1:2 dilution ratio rendered the highest fertility. This combination also saves the extender to a large extent and should be suggested in practice.

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DISCLOSURES

The authors declare that they have no conflict of interest.

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