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Effects of Different Antifreeze Protective Agents on Hu Sheep Semen Storage at 4 ˚C

Research Article

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ABSTRACT

The present study aimed to investigate the effects of different antifreeze protectants on the preservation of Hu sheep semen at 4 ˚C. The semen was diluted with Tris extender at room temperature, supplemented with dimethyl sulfoxide (DMSO) (0, 0.1%, 0.2%, 0.3%, 0.4% and 0.5%), glycerol (1.5%, 3.0%, 4.5% and 6.0%), glycol (2.50%, 5.00%, 7.50% and 10.00%), skimmed milk powder (SMP) (0.50%, 1.50%, 2.50% and 3.00%), soy lecithin (SL, 0.15%, 0.30%, 0.45% and 0.60%) and five optimal concentrations of antifreeze protectants (0.4% DMSO, 4.5% glycerol, 7.5% glycol, 1.5% SMP and 0.15% SL), and stored at 4 ˚C refrigerator. Spermatozoa motility parameters (spermatozoa viability, spermatozoa progressive motility, straight line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP)) were evaluated during the preservation of semen. The addition of antifreeze protectants, especially the 0.4% DMSO, 4.5% glycerol, 7.50% glycol, 1.50% SMP and 0.15% SL exerted the best effects on spermatozoa viability and progressive motility compared to the spermatozoa without antifreeze protectants (control group). In the experiment of antifreeze protective agents with the optimal concentration, the spermatozoa viability and progressive motility of 1.5% SMP were significantly higher than those of other antifreeze groups during storage. In conclusion, the addition of 1.5% SMP to semen spermatozoa preserved at 4 ˚C refrigerator can most effectively enhance the semen preservation quality compared to the other optimal concentration of antifreeze protectant groups.

KEY WORDS computer-assisted spermatozoa analysis, DMSO, glycerol, glycol, SMP, soy lecithin.

INTRODUCTION

Assisted reproductive technologies (ART) including artificial insemination (AI), *in vitro* fertilization (IVF) and embryo transfer (ET), which are used to collect and process oocytes, spermatozoa and embryos *in vitro* to improve the chances of conception (Esteves *et al.* 2019). In the field of livestock, ART can quickly spread and preserve the genetic material of cherished species and avoid the extinction of endangered species (Daly *et al.* 2020; Figueiredo *et al.* 2020). At the same time, ART can also improve breeding efficiency, reduce breeding costs and reduce the risk of disease transmission (Rodriguez, 2012). The core of ART is to prolong the preservation time of semen and to improve the quality of semen preservation by adding various substances, such as antioxidants (taurine (Zhang *et al.* 2021)), antibacterial agents (nisin (Shin *et al.* 2016)), antifreeze protection agents (yolk (Garde *et al.* 2008)), etc. In the process of semen preservation, with the influence of spermatozoa metabolic activity and external environment, it will inevitably be affected by reactive oxygen species (ROS) and bacterial microorganisms (Rezaie *et al.* 2021; Tvrdá *et al.* 2021). Therefore, it is very important to add antioxidants that can remove ROS and antimicrobial agents

that inhibit the proliferation of microorganisms. Semen is easily affected by low temperature and cold during cryopreservation which seriously affects the quality of semen preservation (Gloria *et al.* 2020). Therefore, the addition of antifreeze protectant is very important to improve the preservation quality of semen.

Antifreeze protectant is divided into permeable antifreeze protectant and non-permeable antifreeze protectant (Diaz *et al.* 2019). Dimethyl sulfoxide (DMSO), glycerol, glycol and soy lecithin (SL) are permeable antifreeze protectants. Skimmed milk powder (SMP) and bovine serum albumin are non-permeable antifreeze protectants. The permeable antifreeze protectant can pass through the spermatozoa cell membrane and enter into the spermatozoa, rearrange the fat and protein on the spermatozoa plasma membrane, increase the fluidity, reduce the formation of intracellular ice crystals, and then improve the survival rate after freezing and thawing (Blanco *et al.* 2011).

Non-permeable antifreeze protectant cannot pass through the spermatozoa cell membrane, but can only play a role in the outside. It can change the semen osmotic pressure, make the cells dehydrated, reduce the possibility of the formation of intracellular ice crystals, and then improve the spermatozoa survival rate after thawing (Rosato and Iaffaldano, 2013).

The most commonly used antifreeze protectant is egg yolk, but egg yolk is an animal-derived additive that possess a risk of biological infection as it may contain toxins, viruses, etc (Yildiz *et al.* 2013; Mehdipour *et al.* 2016). On the other hand, the properties of egg yolk may be variable depending on the breeds and feeding conditions. Therefore, it is necessary to find a safe, consistent and effective antifreeze protectant that can replace yolk. It is reported that different concentrations of DMSO, glycerol, glycol and SL play different roles in different species, such as Australian flat oyster (Hassan *et al.* 2017), bison (Hussain *et al.* 2013), stallion (Gonzalez *et al.* 2019), dog (Rota *et al.* 2006), Buffalo (Swelum *et al.* 2011), goat (Küçük *et al.* 2014), human (Reed *et al.* 2009) and Brown-bear (Alvarez *et al.* 2013).

However, there are few reports on DMSO, Glycerol, Glycol and SL to be used as antifreeze protectants for preservation of Hu sheep semen at 4 ˚C. Some researches reported that the addition of 6% glycerol could improve the cryopreservation of Hu sheep semen.

Also reported that the addition of 6% glycerol and some egg yolk could improve the cryopreservation of Hu sheep semen. Therefore, the present study aimed to determine the effect and the optimum added concentration of DMSO, Glycerol, Glycol, SL and SMP for preservation of Hu sheep semen of 4 ˚C.

MATERIALS AND METHODS

All experimental procedures were approved bythe Yangzhou University for protection of experimental animals. (SYXK [Su] 2017-0044).

Experimental design

Effect of dimethyl sulfoxide (DMSO) (0, 0.1%, 0.2%, 0.3%, 0.4% and 0.5%), glycerol (1.5%, 3.0%, 4.5% and 6.0%), glycol (2.50%, 5.00%, 7.50% and 10.00%), SMP (0.50%, 1.50%, 2.50% and 3.00%), SL (0.15%, 0.30%, 0.45% and 0.60%) and five optimal concentrations of antifreeze protectants (0.4% DMSO, 4.5% glycerol, 7.5% Glycol, 1.5% SMP and 0.15% SL) on spermatozoa motility parameters [Spermatozoa viability, spermatozoa progressive motility, straight line velocity (VSL), curvilinear velocity (VCL) and average path velocity (VAP)] of stored Hu sheep semen at 4 ˚C were evaluated every day during preservation and compared with spermatozoa motility parameters without antifreeze protectants (control group).

Animals and semen collection

Five sexually mature Hu rams, aged 2-3 years and body weighted about 75 kg, were used in this study from October to December 2021. Hu sheep were fed by stallfeeding. The rams were fed 0.2 kg concentrate/every time, twice a day, and *ad libitum* hay and water. Alfalfa was supplemented every day and feeding of corn kernels was increased during semen collection. The sheep shed is equipped with suitable types of licking bricks to supplement various trace elements. These rams were in good condition and free from any disease. A total number of 90 ejaculates were collected from the rams twice weekly with the artificial vagina. The semen volume of each ram was from 0.5 mL to 1.0 mL, which had normal smell and color. The quality assessment was carried for the motility (>80%), the spermatozoa deformity (<15%) and the spermatozoa concentration (2.5×10^9/mL). The qualified semen was diluted for conducting the experiments.

Semen extender

The base extender consisted of 3.07 g Tris, 2.0 g fructose, 1.64 g citric acid, 31.18 mg penicillin and 69.44 mg streptomycin in 100 mL distilled water. DMSO was added to the base extender at concentrations of 0.10%, 0.20%, 0.30%, 0.40% and 0.50%, while the control was the base extender without DMSO. Glycerol was added to the base extender at concentrations of 1.50%, 3.00%, 4.50% and 6.00%, while the control was the base extender without glycerol. Glycol was added to the base extender at concentrations of 2.50%,

5.00%, 7.50% and 10.00%, while the control was the base extender without glycol. SMP was added to the base extender at concentrations of 0.50%, 1.50%, 2.50% and 3.00%, while the control was the base extender without SMP. Soy lecithin was added to the base extender at concentrations of 0.15%, 0.30%, 0.45% and 0.60%, while the control was the base extender without SL. When preparing SL extender, it is necessary to dissolve 30 min in a water bath at 60 ˚C. Finally, all extenders were fully oscillated so that they are completely dissolved. The pH of each extender was between 7.4 and 7.5.

Dilution and evaluation of semen

The semen was diluted at 1:9 ratio with the extender preheated at 37 ˚C and preserved in 2 mL centrifuge tube. The diluted semen was kept at room temperature for 1 h. Then wrap it with 8 layers of cotton and put it in the refrigerator at 4 ˚C after wrapping. During semen preservation, the centrifuge tube for semen preservation is slowly reversed every day to prevent spermatozoa deposition. The semen preserved by 20 µL was diluted at 1:4 ratio and placed in a 37 ˚C water bath for 3min. Then 1.8 µL was dropped on a special computer spermatozoa counting board and placed on a 37 ˚C constant temperature stage. Computer-assisted spermatozoa analyse (ML-608JZ II Mailang, Nanning, China) (CASA) was used to analyse spermatozoa motility parameters such as spermatozoa viability (%), spermatozoa progressive motility (%), VSL (µm/s), VCL (µm/s) and VAP (µm/s).

Statistical analysis

The experiment was repeated for six times. Data were analyzed using SPSS 25.0 statistical software (SPSS, 2011). The Shapiro–Wilk test was performed to detect whether the data conform to the normal distribution. One-way ANOVA tests were performed to assess the difference in these parameters. Significance was set at $P \leq 0.05$ unless otherwise specified. The results are expressed as the mean ± SEM.

RESULTS AND DISCUSSION

The effects of different concentrations of DMSO on Hu sheep spermatozoa motility parameters during storage at 4 ˚C are shown in Table1. The spermatozoa viability in the 0.4% DMSO supplemented semen was the highest and significantly (P≤0.05) higher than that of the 0.5% group within 1 to 3 days. The spermatozoa viability of the 0.4% group was significantly (P≤0.05) higher than that of the other groups within 4 to 6 days. The spermatozoa progressive motility of the 0.4% group was the highest and significantly (P≤0.05) higher than that of the 0.5% group on the 1st and 3rd day.

The spermatozoa progressive motility of the 0.4% group was significantly (P≤0.05) higher than that of the other groups within 4 to 6 days. The spermatozoa VSL of the 0.4% group was not significantly (P>0.05) different from the other groups on the 4th and 6th day. The spermatozoa VCL and VAP of the 0.4% group were higher (P>0.05) than that of the other groups within 4 to 6 days.

The effects of different concentrations of glycerol on Hu sheep spermatozoa motility parameters during storage at 4 ˚C are shown in Table 2. The spermatozoa viability of the 4.5% group was the highest and significantly (P≤0.05) higher than that of the control group within 1 to 4 days. The spermatozoa viability of the 4.5% group was significantly (P≤0.05) higher than that of the other groups within 5 to 6 days. The spermatozoa progressive motility of the 4.5% group was the highest and significantly (P≤0.05) higher than that of the control and 1.5% groups within 3 to 6 days. The spermatozoa VSL of the 4.5% group was higher (P>0.05) than that of the control group within 1 to 5 days and significantly (P≤0.05) higher than that of the control group on the 2nd, 4th and 5th day. The spermatozoa VCL and VAP of the 4.5% group were significantly (P≤0.05) higher than that of the control group within 2 to 5 days.

The effects of different concentrations of glycol on Hu sheep spermatozoa motility parameters during storage at 4 ˚C are shown in Table 3.

The spermatozoa viability and progressive motility of the 7.5% group were significantly (P≤0.05) higher than that of the control group within 1 to 6 days and significantly (P≤0.05) higher than that of the 2.5% group within 1 to 4 days. The spermatozoa VCL and VAP of the 7.5% group were significantly (P≤0.05) higher than that of the control group on the 1st day. The spermatozoa VSL, VCL and VAP of the 7.5% group were significantly (P≤0.05) higher than that of the other groups on the 3rd day.

The effects of different concentrations of SMP on Hu sheep spermatozoa motility parameters during storage at 4 ˚C are shown in Table 4. The spermatozoa viability of the 1.5% group was the highest and significantly (P≤0.05) higher than that of the control group on the 1st, 2nd, 4th and 6th day. The spermatozoa viability of the 1.5% group was significantly (P≤0.05) higher than that of the other groups on the 3rd and 5th day. The spermatozoa progressive motility of the 1.5% group was significantly (P≤0.05) higher than that of the control group within 1 to 5 days. The spermatozoa progressive motility of the 1.5% group was significantly (P≤0.05) higher than that of the other groups on the 6th day. The spermatozoa VSL of the 1.5% group was significantly (P≤0.05) higher than that of the control group on the 3rd day. The spermatozoa VSL of the 1.5% group was higher (P>0.05) than that of the control group within 4 to 6 days.

Table 1 Effects of different concentrations of DMSO on Hu ram spermatozoa motility parameters stored at 4 ˚C

Semen characteristics	Time (day)	Concentrations of dimethyl sulfoxide (DMSO)					
		Control (0)	0.1%	0.2%	0.3%	0.4%	0.5%
Spermatozoa viability (%)	0	86.22±1.54	86.11±1.61	86.40±1.91	85.71±1.37	86.35±0.32	85.67±2.21
	1	81.32±0.90^{ab}	80.47±1.58^{ab}	80.41±0.36^{ab}	80.77±1.68^{ab}	84.32±1.19^{a}	79.83±0.75^{b}
	2	71.60±0.50^{b}	73.54±1.50^{b}	73.38±2.30^{b}	76.44±3.40^{a}	77.25±4.20^{a}	72.10±5.00^{b}
	3	71.36±0.41^{b}	72.03±0.96^{b}	72.66±0.50^{b}	76.45±0.11^{a}	77.36±0.41^{a}	68.20±0.65^{c}
	4	51.70±1.28^{c}	61.19±1.12^{b}	63.60±0.78^{b}	64.73±1.22^{b}	74.90±1.50^{a}	64.52±0.74^{b}
	5	45.34±1.20^{d}	48.82±0.17^{c}	52.25±0.93^{b}	53.31±1.18^{b}	59.73±0.63^{a}	50.72±1.26^{bc}
	6	29.85±0.56^{d}	31.92±0.22^{d}	37.06±1.34^{c}	37.65±1.17^{c}	45.84±1.24^{a}	42.52±0.95^{b}
Spermatozoa progressive motility (%)	0	84.16±1.82	84.12±1.33	83.84±2.95	81.29±0.39	81.31±1.55	81.34±0.83
	1	72.65±2.11^{b}	74.88±1.34^{ab}	74.48±0.4^{ab}	74.01±1.21^{ab}	78.20±0.95^{a}	71.94±1.86^{b}
	2	63.51±0.50^{c}	63.36±1.30^{c}	65.54±2.50^{bc}	66.88±3.80^{b}	69.64±4.60^{a}	64.48±5.40^{bc}
	3	63.48±0.86^{abc}	63.66±1.36^{abc}	62.81±0.25^{bc}	68.11±0.83^{a}	67.34±2.46^{ab}	59.93±1.61^{c}
	4	42.75±0.22^{c}	49.77±0.56^{b}	52.56±2.20^{b}	52.73±3.13^{b}	66.16±1.74^{a}	53.45±1.26^{b}
	5	36.37±1.26^{c}	42.04±0.99^{b}	44.64±1.47^{b}	44.68±1.76^{b}	52.19±0.23^{a}	42.06±0.96^{b}
	6	22.87±0.44^{d}	23.78±1.37^{d}	27.41±0.45^{c}	28.48±0.70^{bc}	36.11±1.13^{a}	31.41±1.17^{b}
VSL (um/s)	0	41.80±2.51	39.07±0.58	40.50±2.39	43.86±0.56	43.89±1.32	43.53±1.70
	1	39.13±0.75^{bc}	39.62±0.93^{b}	37.64±0.16^{cd}	42.66±0.66^{a}	34.92±0.15^{e}	36.46±0.04^{de}
	2	33.42±0.37	33.34±0.56	32.70±0.20	32.54±0.05	32.72±1.27	32.92±0.18
	3	34.04±1.07^{ab}	33.64±0.67^{ab}	29.94±0.71^{c}	34.39±0.12^{a}	29.77±0.92^{c}	31.96±0.23^{bc}
	4	30.40±0.42	30.20±0.58	29.69±0.38	31.29±0.50	30.67±0.35	29.81±1.00
	5	29.57±1.1^{ab}	26.99±1.07^{b}	30.21±0.17^{a}	30.57±1.15^{a}	29.61±0.12^{ab}	28.89±1.28^{ab}
	6	27.77±0.54	28.83±0.35	28.80±1.56	28.59±0.07	27.92±0.43	30.48±1.23
VCL (um/s)	0	71.24±1.94	69.28±0.27	67.78±2.00	70.2±1.17	68.82±0.5	68.71±2.37
	1	75.15±1.85^{ab}	74.28±1.03^{ab}	73.17±0.35^{b}	76.64±0.31^{a}	64.39±0.61^{c}	66.46±0.54^{c}
	2	64.34±0.72^{a}	62.74±3.33^{ab}	63.56±0.81^{ab}	62.17^{ab}	63.99±1.85^{a}	57.15±2.78^{b}
	3	63.73±2.10^{ab}	64.08±1.00^{ab}	56.93±2.77^{bc}	63.19±2.53^{ab}	55.17±2.59^{c}	65.17±2.76^{a}
	4	57.93±1.37	57.68±0.12	57.88±2.84	60.79±2.72	61.33±0.84	55.84±0.65
	5	54.02±4.24	51.35±1.69	56.10±1.10	56.93±3.16	57.46±2.80	50.19±1.87
	6	53.36±3.96	57.37±0.47	55.79±4.00	51.18±1.48	54.25±1.37	52.20±0.97
VAP (um/s)	0	50.37±1.37	48.99±0.19	47.93±1.42	49.65±0.83	48.66±0.36	48.59±1.68
	1	53.14±1.31^{ab}	52.53±0.72^{ab}	51.74±0.24^{b}	54.19±0.21^{a}	45.53±0.43^{c}	47.00±0.38^{c}
	2	45.49±0.51^{a}	44.36±2.36^{ab}	44.95±0.57^{ab}	43.96^{ab}	45.25±1.31^{a}	40.41±1.96^{b}
	3	45.06±1.48^{ab}	45.31±0.71^{ab}	40.26±1.96^{bc}	44.68±1.79^{ab}	39.01±1.83^{c}	46.08±1.95^{a}
	4	40.96±0.96	40.79±0.09	40.92±2.01	42.99±1.92	43.37±0.60	39.48±0.46
	5	38.20±3.00	36.31±1.19	39.66±0.78	40.26±2.24	40.63±1.98	35.49±1.33
	6	37.73±2.80	40.57±0.33	39.45±2.83	36.19±1.04	38.36±0.97	36.91±0.69

VSL: straight line velocity; VCL: curvilinear velocity and VAP: average path velocity.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

The spermatozoa VCL and VAP of the 1.5% group were higher (P>0.05) than that of the control group within 1 to 2 days. The spermatozoa VCL and VAP of the 1.5% group were significantly (P≤0.05) higher than that of the control group within 3 to 5 days.

The effects of different concentrations of SL on Hu sheep spermatozoa motility parameters during storage at 4 ˚C are shown in Table 5. The spermatozoa viability and progressive motility of the 0.15% group were significantly (P≤0.05) higher than that of the other groups within 1 to 6 days. The spermatozoa VSL of the 0.15% group were significantly (P≤0.05) higher than that of the control group on the 1st day. The spermatozoa VSL of the 0.15% group were higher (P>0.05) than that of the control group on the 2nd and 6th day.

The spermatozoa VCL and VAP of the 0.15% group were the highest and significantly (P≤0.05) higher than that of the control and 0.6% groups within 1 to 6 days.

The effects of different antifreeze protective agents on Hu sheep spermatozoa motility parameters during storage at 4 ˚C are shown in Table 6. The spermatozoa viability and progressive motility of the 1.5% SMP group were the highest within 1 to 6 days and significantly (P≤0.05) higher than that of the other groups within 1 to 2 days and 4 to 5 days. The spermatozoa viability of the 0.15% SL group was significantly (P≤0.05) higher than that of the 0.4% DMSO, 4.5% glycerol and 7.5% glycol groups within 1 to 3 days. The spermatozoa viability of the 0.4% DMSO group was significantly (P≤0.05) lower than that of the other groups within 4 to 5 days.

Table 2 Effects of different concentrations of glycerol on Hu ram spermatozoa motility parameters stored at 4 ˚C

Semen characteristics	Time (day)	Concentrations of glycerol				
		Control (0)	1.5%	3%	4.5%	6%
Spermatozoa viability (%)	0	90.51±1.12	91.32±1.30	91.19±1.79	91.56±1.09	90.05±2.10
	1	82.55±0.65^{b}	84.87±1.24^{ab}	85.56±1.26^{ab}	85.96±0.80^{a}	84.27±0.59^{ab}
	2	84.77±0.55^{bc}	85.71±0.70^{bc}	86.55±0.38^{b}	89.96±0.47^{a}	84.55±0.73^{c}
	3	79.52±0.78^{b}	80.28±0.83^{b}	85.11±0.89^{a}	86.10±1.11^{a}	83.82±0.58^{a}
	4	68.55±1.15^{c}	74.13±0.29^{b}	76.28±0.72^{ab}	79.21±1.52^{a}	76.29±1.10^{ab}
	5	42.09±0.99^{d}	47.03±0.44^{c}	54.15±0.87^{b}	59.44±0.55^{a}	55.89±0.84^{b}
	6	26.99±0.82^{d}	32.12±1.39^{c}	31.95±1.03^{c}	46.02±0.61^{a}	41.66±0.84^{b}
Spermatozoa progressive motility (%)	0	85.91±1.09	88.31±0.85	85.30±2.10	89.78±0.11	85.3±3.63
	1	77.82±0.67	77.82±2.31	80.58±1.34	80.10±1.24	78.39±0.84
	2	77.26±0.47^{b}	79.36±2.28^{ab}	78.32±0.35^{b}	83.15±1.21^{a}	76.98±1.36^{b}
	3	68.33±0.75^{c}	72.57±0.83^{b}	77.40±1.40^{a}	79.28±1.81^{a}	78.25±0.31^{a}
	4	59.03±1.30^{c}	65.35±0.24^{b}	68.12±0.99^{b}	71.81±0.89^{a}	67.16±0.75^{b}
	5	33.41±1.35^{c}	36.94±0.98^{c}	43.48±0.89^{b}	49.27±0.98^{a}	46.75±1.71^{ab}
	6	19.95±1.06^{c}	24.49±0.53^{b}	26.01±1.27^{b}	35.64±1.28^{a}	32.83±0.48^{a}
VSL (um/s)	0	42.60±2.19	43.40±0.69	43.80±0.61	43.72±0.65	41.84±1.00
	1	44.23±0.78	44.30±1.31	45.98±0.95	45.20±0.52	44.28±1.06
	2	36.98±0.79^{c}	38.59±0.46^{abc}	40.38±0.83^{ab}	41.56±1.20^{a}	37.83±1.21^{bc}
	3	37.14±1.04^{ab}	35.10±0.40^{b}	38.78±0.94^{a}	39.11±0.72^{a}	38.48±1.09^{a}
	4	32.56±0.79^{d}	34.85±0.21^{c}	35.95±0.63^{bc}	37.08±0.56^{ab}	37.88±0.38^{a}
	5	26.47±0.33^{b}	26.83±0.79^{b}	29.33±0.72^{a}	29.49±0.58^{a}	28.84±0.43^{a}
	6	26.36±0.81^{ab}	23.60±0.42^{b}	24.61±0.74^{ab}	26.53±1.25^{ab}	27.82±2.08^{a}
VCL (um/s)	0	85.23±0.43	85.13±0.16	84.45±0.48	85.08±0.68	84.78±0.52
	1	79.35±1.87^{ab}	80.94±2.11^{ab}	83.00±0.04^{a}	80.89±0.92^{ab}	76.62±2.60^{b}
	2	68.04±0.41^{b}	71.70±1.76^{b}	77.65±1.12^{a}	80.75±2.11^{a}	70.08±2.95^{b}
	3	66.54±0.92^{c}	65.71±0.72^{c}	71.77±2.73^{b}	76.88±0.42^{a}	69.98±1.66^{bc}
	4	58.15±1.22^{b}	65.53±1.69^{a}	67.28±2.39^{a}	71.21±2.50^{a}	70.90±1.97^{a}
	5	43.64±0.79^{c}	46.06±2.07^{bc}	50.57±1.57^{abc}	55.09±4.09^{a}	52.80±1.34^{ab}
	6	40.36±1.25^{b}	40.00±0.60^{b}	45.19±1.07^{ab}	47.52±1.69^{a}	49.66±3.43^{a}
VAP (um/s)	0	49.00±2.45^{b}	50.99±0.47^{ab}	51.67±0.28^{ab}	53.54±0.80^{a}	51.77±0.48^{ab}
	1	56.11±1.32^{ab}	57.23±1.50^{ab}	58.69±0.03^{a}	57.20±0.65^{ab}	54.17±1.84^{b}
	2	48.11±0.29^{b}	50.70±1.24^{b}	54.91±0.79^{a}	57.10±1.50^{a}	49.56±2.09^{b}
	3	47.05±0.65^{c}	46.46±0.51^{c}	50.75±1.93^{b}	54.36±0.30^{a}	49.49±1.17^{bc}
	4	41.12±0.86^{b}	46.34±1.20^{a}	47.58±1.69^{a}	50.35±1.77^{a}	50.13±1.40^{a}
	5	30.86±0.56^{c}	32.57±1.46^{bc}	35.76±1.11^{abc}	38.96±2.90^{a}	37.34±0.95^{ab}
	6	28.54±0.88^{b}	28.28±0.42^{b}	31.95±0.75^{ab}	33.60±1.20^{a}	35.11±2.43^{a}

VSL: straight line velocity; VCL: curvilinear velocity and VAP: average path velocity.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

However, there was no significant difference among other groups (except 1.5% SMP group). The spermatozoa viability and progressive motility of the 4.5% glycerol group were significantly (P≤0.05) higher than that of the 0.4% DMSO, 7.5% Glycol and 0.15% SL groups on the 6th day. The spermatozoa progressive motility of the 0.15% SL group was significantly (P≤0.05) higher than that of the 0.4% DMSO, 4.5% glycerol and 7.5% glycol groups within 2 to 3 days.

The spermatozoa progressive motility of the 0.4% DMSO group was significantly (P≤0.05) lower than that of the other groups on the 5th day. The spermatozoa VSL, VCL and VAP of the 1.5% SMP group were significantly (P≤0.05) higher than that of the 0.4% DMSO and 7.5% glycol groups on the 4th day.

The spermatozoa VSL, VCL and VAP of the 1.5% SMP group were significantly (P≤0.05) higher than that of the 0.4% DMSO group but was no significantly different from the other groups on the 5th day.

In this study, the effects of DMSO, glycerol, glycol, SMP and SL on the preservation of Hu sheep semen at 4 ˚C were analyzed. It was showed that adding appropriate concentration of the above five cryoprotectants can improve spermatozoa viability, progressive motility, and movement rate. It is reported that DMSO can penetrate the spermatozoa cell membrane and reduce the concentration of salt at a certain temperature, thus reducing the damage of spermatozoa at this temperature (Lovelock, 1953). In rabbits, Vicente and Viudes (1996) reported that the addition of DMSO could improve the cryopreservation of rabbit semen.

Table 3 Effects of different concentrations of glycol on Hu ram spermatozoa motility parameters stored at 4 ˚C

Semen characteristics	Time (day)	Concentrations of glycol				
		Control (0)	2.5%	5%	7.5%	10%
Spermatozoa motility (%)	0	83.97±1.30	83.25±1.51	84.03±1.76	83.39±1.25	82.56±1.31
	1	72.93±0.62^{c}	73.81±0.15^{c}	81.88±0.29^{a}	82.08±0.39^{a}	80.45±0.08^{b}
	2	73.77±1.11^{c}	77.24±0.85^{b}	78.49±0.37^{ab}	80.63±0.65^{a}	79.05±0.53^{ab}
	3	63.31±1.00^{d}	71.12±1.13^{b}	71.63±0.37^{ab}	73.72±0.54^{a}	66.50±0.45^{c}
	4	42.89±1.46^{d}	55.05±0.03^{b}	58.15±0.61^{a}	58.61±0.24^{a}	49.94±0.12^{c}
	5	38.39±1.30^{c}	53.86±0.03^{a}	47.39±0.69^{b}	54.92±0.69^{a}	40.70±0.81^{c}
	6	34.40±1.12^{b}	43.91±0.12^{a}	43.76±0.20^{a}	45.47±1.15^{a}	35.66±1.70^{b}
Spermatozoa progressive motility (%)	0	79.34±0.23	77.73±2.60	79.27±1.78	77.44±0.95	78.39±1.61
	1	65.47±0.87^{b}	66.91±0.93^{b}	74.64±0.61^{a}	75.31±0.31^{a}	75.06±0.56^{a}
	2	66.32±1.81^{b}	68.38±1.21^{ab}	69.86±0.22^{ab}	72.38±1.27^{a}	71.26±1.65^{a}
	3	52.79±0.77^{d}	61.34±0.29^{b}	64.19±0.82^{a}	64.45±0.48^{a}	55.04±0.55^{c}
	4	34.90±1.98^{c}	48.57±0.41^{a}	48.62±0.31^{a}	49.16±1.73^{a}	40.78±1.27^{b}
	5	31.70±1.33^{c}	45.51±0.26^{a}	40.35±0.37^{b}	47.00±0.28^{a}	33.08±0.85^{c}
	6	27.82±1.24^{b}	34.35±0.68^{a}	35.67±0.52^{a}	36.44±0.79^{a}	29.55±2.01^{b}
VSL (um/s)	0	43.81±3.62	42.11±3.83	42.40±3.14	39.97±4.62	41.15±5.38
	1	36.46±0.38^{bc}	39.45±0.31^{a}	37.49±0.56^{b}	37.90±0.94^{ab}	34.95±0.25^{c}
	2	38.18±1.44	37.53±0.66	38.04±1.10	35.21±0.08	36.73±0.78
	3	32.49±1.26^{c}	34.89±0.04^{b}	33.18±0.45^{bc}	37.35±0.12^{a}	33.29±0.41^{bc}
	4	27.32±0.68^{c}	33.62±1.49^{a}	34.05±0.94^{a}	30.96±0.46^{ab}	29.77±0.97^{bc}
	5	34.11±0.32^{a}	33.23±0.58^{a}	33.91±0.74^{a}	31.09±0.42^{b}	30.02±1.05^{b}
	6	34.48±0.29^{a}	30.47±0.50^{c}	32.88±0.22^{ab}	30.19±0.33^{c}	31.84±0.89^{bc}
VCL (um/s)	0	78.20±3.40	79.34±3.41	76.05±4.94	73.05±9.86	75.48±9.40
	1	70.12±0.94^{c}	73.76±0.20^{b}	75.89±0.75^{ab}	76.51±0.98^{a}	70.9±0.02^{c}
	2	73.48±1.56	73.26±1.26	74.21±1.99	71.41±0.71	72.81±2.46
	3	62.28±1.82^{c}	64.87±0.27^{bc}	67.94±1.65^{b}	73.58±0.30^{a}	63.00±2.73^{bc}
	4	57.04±1.06^{b}	60.08±3.56^{b}	67.14±1.99^{a}	62.17±0.53^{ab}	50.57±1.72^{c}
	5	56.52±1.95	56.30±3.43	64.94±1.58	64.15±0.83	59.25±3.86
	6	56.23±0.53^{ab}	50.40±0.18^{b}	62.27±1.33^{a}	62.37±1.20^{a}	55.11±4.01^{b}
VAP (um/s)	0	55.29±2.40	56.10±2.41	53.78±3.49	51.65±6.97	53.37±6.65
	1	49.58±0.66^{c}	52.16±0.14^{b}	53.66±0.53^{ab}	54.10±0.70^{a}	50.13±0.01^{c}
	2	51.96±1.10	51.80±0.89	52.47±1.40	50.50±0.50	51.49±1.74
	3	44.04±1.29^{c}	45.87±0.19^{bc}	48.05±1.17^{b}	52.03±0.21^{a}	44.55±1.93^{bc}
	4	40.34±0.75^{b}	42.49±2.52^{b}	47.48±1.41^{a}	43.96±0.37^{ab}	35.75±1.22^{c}
	5	39.97±1.37	39.81±2.42	45.92±1.12	45.36±0.59	41.90±2.73
	6	39.76±0.38^{ab}	35.64±0.13^{b}	44.04±0.94^{a}	44.10±0.85^{a}	38.97±2.83^{b}

VSL: straight line velocity; VCL: curvilinear velocity and VAP: average path velocity.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

In goats, Kundu *et al*. (2000) reported that the addition of DMSO could improve the cryopreservation of goat semen. In this study, the addition of DMSO improved the preservation effect of semen, of which 0.4% DMSO had the best effect on spermatozoa motility, progressive motility and movement rate, and 0.5% DMSO also played a certain protective effect, but the effect was not as good as 0.4% DMSO. The molecular weight of DMSO is relatively low and its permeation rate in spermatozoa is faster, so the high concentration of DMSO does not further improve the preservation effect of semen, which may be due to the toxicity of DMSO rather than its osmotic effect. Studies have reported that glycerol can rearrange membrane lipids and proteins, increase membrane fluidity, and bind to metal ions to dehydrate cells and prevent the fracture in the frozen solutions by reducing the total ice volume expansion during water solidification (Lohmann *et al.* 1964; Maxwell and Salamon, 1979; Gao *et al.* 1995). In boars, Pursel and Johnson (1975) reported that the addition of glycerol could improve the cryopreservation of porcine semen. In bulls, Polge (1953) reported that the addition of glycerol could improve the cryopreservation of bovine semen. In this study, the addition of glycerol improved the preservation effect of semen, of which 4.5% glycerol had the best effect on the kinematic performance of spermatozoa, and 6% glycerol also had a certain protective effect, but the effect was not as good as 4.5% glycerol. This may be because the relative molecular weight of glycerol is large, which will have a certain penetration and toxic pressure on spermatozoa, resulting in spermatozoa damage. On the other hand, it may be that higher concentration of glycerol can promote programmed death of spermatozoa (Wündrich *et al.* 2006).

Table 4 Effects of different concentrations of skimmed milk powder (SMP) on Hu ram spermatozoa motility parameters stored at 4 ˚C

Semen characteristics	Time (day)	Concentrations of SMP				
		Control (0)	0.5%	1.5%	2.5%	3%
Spermatozoa viability (%)	0	83.50±1.04	83.14±1.10	85.21±0.95	82.75±1.38	84.78±0.83
	1	78.00±0.24^{c}	80.58±0.73^{ab}	82.13±0.31^{a}	80.01±0.56^{b}	79.09±0.71^{bc}
	2	73.82±1.37^{b}	74.28±0.33^{b}	81.03±0.95^{a}	79.44±0.59^{a}	79.27±0.58^{a}
	3	60.22±0.81^{c}	68.02±0.93^{b}	71.34±0.75^{a}	68.60±0.39^{b}	68.18±0.42^{b}
	4	45.95±1.21^{c}	53.55±1.32^{b}	60.33±0.57^{a}	60.25±1.00^{a}	52.81±0.14^{b}
	5	24.43±0.80^{d}	37.91±0.90^{b}	45.27±0.89^{a}	34.26±0.03^{c}	25.16±1.21^{d}
	6	8.94±0.26^{c}	11.22±0.60^{bc}	13.71±0.45^{a}	12.19±1.10^{ab}	9.03±0.83^{c}
Spermatozoa progressive motility (%)	0	81.41±0.95^{ab}	81.94±1.20^{ab}	82.13±0.62^{ab}	79.15±0.33^{b}	83.70±1.50^{a}
	1	71.48±0.78^{b}	74.79±1.29^{a}	75.22±0.65^{a}	72.01±0.50^{b}	68.81±0.48^{c}
	2	65.93±2.34^{bc}	65.34±1.04^{c}	73.44±2.00^{a}	70.56±1.23^{ab}	71.68±0.11^{a}
	3	49.49±1.63^{c}	57.72±2.21^{ab}	60.49±0.59^{a}	56.44±1.09^{ab}	55.29±0.24^{b}
	4	36.49±1.38^{d}	44.89±1.95^{bc}	47.71±0.65^{ab}	50.35±0.20^{a}	42.44±0.85^{c}
	5	19.32±1.42^{c}	30.76±1.24^{a}	33.72±0.49^{a}	25.95±0.78^{b}	18.80±2.06^{c}
	6	5.33±0.25^{cd}	7.17±0.84^{bc}	10.21±0.42^{a}	7.92±0.49^{b}	5.12±0.78^{d}
VSL (um/s)	0	41.80±2.51	41.01±0.69	39.28±0.68	39.34±0.25	41.70±1.57
	1	41.09±1.59	39.32±0.66	40.93±0.17	40.72±1.02	40.35±0.16
	2	37.75±0.48^{b}	38.49±0.47^{ab}	39.44±0.69^{ab}	40.41±1.15^{a}	40.01±0.61^{ab}
	3	33.36±1.88^{b}	35.27±1.22^{ab}	38.25±1.22^{a}	38.78±0.08^{a}	35.84±0.53^{ab}
	4	31.54±0.45^{c}	35.52±1.06^{ab}	33.22±1.02^{bc}	35.11±0.67^{ab}	36.09±0.05^{a}
	5	27.29±0.63^{b}	33.21±2.04^{a}	30.48±1.23^{ab}	28.32±2.12^{ab}	29.32±0.88^{ab}
	6	23.22±1.64	20.66±0.41	24.58±1.70	22.17±1.36	23.09±0.34
VCL (um/s)	0	69.88±2.72	68.92±0.35	65.99±2.43	66.40±3.57	68.3±0.93
	1	75.87±2.40^{a}	75.98±0.98^{a}	76.79±1.10^{a}	70.36±1.40^{b}	68.83±0.36^{b}
	2	69.18±1.40	72.75±1.14	71.94±0.27	74.54±2.50	74.33±2.68
	3	60.40±1.61^{c}	65.16±2.58^{bc}	69.93±1.79^{ab}	72.08±0.04^{a}	65.43±1.21^{bc}
	4	51.99±3.08^{c}	57.30±2.33^{bc}	61.45±2.33^{ab}	67.15±1.15^{a}	66.10±0.56^{a}
	5	40.04±1.78^{b}	57.38±1.81^{a}	58.81±2.85^{a}	58.91±3.44^{a}	54.57±5.82^{a}
	6	39.47±3.55^{b}	46.64±0.35^{ab}	47.41±3.82^{ab}	53.40±2.76^{a}	55.81±3.74^{a}
VAP (um/s)	0	49.41±1.92	48.73±0.25	46.66±1.72	46.95±2.53	48.29±0.66
	1	53.65±1.70^{a}	53.73±0.69^{a}	54.30±0.78^{a}	49.75±0.99^{b}	48.67±0.25^{b}
	2	48.92±0.99	51.44±0.81	50.87±0.19	52.71±1.77	52.56±1.90
	3	42.71±1.14^{c}	46.07±1.83^{bc}	49.45±1.26^{ab}	50.97±0.03^{a}	46.27±0.85^{bc}
	4	36.76±2.18^{c}	40.52±1.64^{bc}	43.45±1.65^{ab}	47.48±0.81^{a}	46.74±0.40^{a}
	5	28.31±1.26^{b}	40.57±1.28^{a}	41.59±2.02^{a}	41.65±2.44^{a}	38.59±4.12^{a}
	6	27.91±2.51^{b}	32.97±0.25^{ab}	33.53±2.7^{ab}	37.76±1.96^{a}	39.47±2.65^{a}

VSL: straight line velocity; VCL: curvilinear velocity and VAP: average path velocity.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

The study reported that glycolis less likely to cause osmotic shock to the spermatozoa (Moore *et al.* 2006). In this study, 7.5% glycol had the best protective effect on cryopreserved Hu ram spermatozoa. Rota *et al.* (2006) reported that the addition of 5% glycol could improve the cryopreservation of dog semen. The addition of 5% of the report is different from that of 7.5% in this study, which may be due to different species. In this study, all concentrations of SMP improved the preservation effect of semen, but the higher concentration of SMP did not have the best preservation effect. This may be due to the addition of too much SMP, which increases the viscosity of the solution, makes spermatozoa more prone to agglutination and reduces its preservation effect.

Skimmed milk powder may contain antioxidants, membrane stabilizers and carbohydrate nutrients, so it can improve the preservation effect of semen in many ways (Vernet *et al.* 2001; Chen *et al.* 2002). It is reported that SL contains low-density lipoprotein components similar to yolk, which can protect the integrity of phospholipid membrane at low temperature and may also be an antioxidant (Dalmazzo *et al.* 2018). Zhao *et al.* (2021) reported that the addition of SL had a good effect on the preservation of Duolangrams semen at 0 ˚C. In this study, the optimum concentration of SL was 0.15%. Although the addition of higher concentration could also have a beneficial effect on semen preservation, it did not further improve its preservation effect.

Table 5 Effects of different concentrations of soy lecithin on Hu ram spermatozoa motility parameters stored at 4 ˚C

Semen characteristics	Time (day)	Concentrations of soy lecithin				
		Control (0)	0.15%	0.3%	0.45%	0.6%
Spermatozoa viability (%)	0	82.77±0.56	83.21±1.61	83.06±1.30	83.60±1.51	82.98±1.32
	1	62.84±1.31^{d}	80.70±0.51^{a}	78.17±0.45^{ab}	77.36±0.66^{b}	69.65±0.81^{c}
	2	61.14±0.88^{d}	80.09±0.99^{a}	77.20±0.33^{b}	75.06±0.58^{b}	63.57±0.47^{c}
	3	53.04±0.32^{d}	72.23±0.90^{a}	61.12±0.77^{b}	56.45±0.57^{c}	38.89±0.19^{e}
	4	45.75±0.58^{b}	65.11±1.07^{a}	45.93±0.92^{b}	42.60±0.94^{c}	24.84±0.23^{d}
	5	24.32±0.62^{c}	55.10±1.01^{a}	27.23±0.64^{b}	20.81±0.60^{d}	11.11±0.53^{e}
	6	11.03±0.85^{b}	30.36±1.75^{a}	9.47±0.60^{b}	9.21±0.81^{b}	1.52±0.38^{c}
Spermatozoa progressive motility (%)	0	80.43±0.30	80.55±1.00	77.04±3.32	79.81±0.50	79.60±0.54
	1	54.62±1.53^{d}	75.39±0.45^{a}	70.29±1.06^{b}	71.83±1.22^{b}	60.49±0.73^{c}
	2	53.25±1.24^{d}	75.72±1.19^{a}	71.51±0.94^{b}	68.33±0.89^{c}	55.73±0.02^{d}
	3	43.98±1.24^{d}	66.00±1.50^{a}	53.53±0.05^{b}	48.70±1.30^{c}	30.25±0.18^{e}
	4	37.41±1.36^{b}	56.07±1.94^{a}	36.49±1.14^{bc}	32.98±0.40^{c}	18.72±0.42^{d}
	5	17.73±1.19^{b}	44.73±1.95^{a}	21.34±0.93^{b}	13.83±1.04^{c}	8.09±0.41^{d}
	6	7.31±1.10^{b}	23.94±0.22^{a}	5.70±0.25^{b}	6.09±0.38^{b}	0.93±0.27^{c}
VSL (um/s)	0	40.86±3.29	38.79±0.39	38.84±0.88	40.54±2.13	41.50±1.73
	1	34.79±0.26^{c}	40.11±0.95^{a}	38.81±0.16^{ab}	35.94±0.60^{c}	37.84±0.23^{b}
	2	35.26±0.76^{abc}	37.51±0.27^{a}	34.89±0.46^{bc}	33.77±0.65^{c}	37.07±1.2^{ab}
	3	33.07±0.54^{a}	29.69±0.42^{b}	24.18±0.22^{d}	24.95±0.68^{cd}	26.42±0.90^{c}
	4	37.22±1.49^{a}	34.70±0.55^{a}	23.70±1.08^{b}	23.40±1.55^{b}	19.34±0.39^{c}
	5	28.86±1.19^{a}	24.45±0.71^{b}	20.23±0.14^{c}	20.37±0.73^{c}	18.44±1.76^{c}
	6	24.20±0.73^{ab}	25.02±1.86^{a}	19.55±0.78^{c}	20.81±1.59^{bc}	8.75±0.35^{d}
VCL (um/s)	0	68.17±3.78	65.58±2.82	66.01±3.47	69.63±2.82	66.47±3.67
	1	63.09±0.29^{c}	78.42±1.73^{a}	76.78±0.35^{a}	71.95±1.27^{b}	73.46±0.42^{b}
	2	66.84±1.65^{b}	79.24±2.56^{a}	70.29±0.61^{b}	69.65±0.06^{b}	70.40±1.90^{b}
	3	60.27±1.05^{b}	72.05±1.66^{a}	60.38±0.58^{b}	60.17±1.14^{b}	56.73±1.67^{b}
	4	63.68±1.4^{b}	71.77±1.48^{a}	62.53±1.77^{b}	57.18±1.74^{c}	42.79±1.25^{d}
	5	50.09±3.11^{b}	64.83±1.87^{a}	52.50±0.12^{b}	50.56±2.06^{b}	42.48±2.16^{c}
	6	43.49±1.61^{c}	65.33±3.27^{a}	51.82±3.66^{bc}	56.86±2.79^{ab}	26.90±2.73^{d}
VAP (um/s)	0	48.20±2.67	46.37±1.99	46.68±2.45	49.24±1.99	47.00±2.59
	1	44.61±0.21^{c}	55.45±1.22^{a}	54.29±0.25^{a}	50.87±0.89^{b}	51.94±0.30^{b}
	2	47.26±1.17^{b}	56.03±1.81^{a}	49.70±0.43^{b}	49.25±0.04^{b}	49.78±1.35^{b}
	3	42.62±0.75^{b}	50.95±1.17^{a}	42.69±0.41^{b}	42.55±0.81^{b}	40.11±1.18^{b}
	4	45.03±0.99^{b}	50.75±1.05^{a}	44.21±1.24^{b}	40.43±1.23^{c}	30.25±0.88^{d}
	5	35.42±2.20^{b}	45.84±1.32^{a}	37.13±0.08^{b}	35.76±1.45^{b}	30.04±1.53^{c}
	6	30.75±1.14^{c}	46.20±2.32^{a}	36.64±2.59^{bc}	40.20±1.98^{ab}	19.03±1.93^{d}

VSL: straight line velocity; VCL: curvilinear velocity and VAP: average path velocity.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

It could be that the decreased spermatozoa motility in samples with increased extender concentrations could be related both to the high viscosity of SL preventing spermatozoa movement and to the formation of extender debris (Forouzanfar *et al.* 2010). Another hypothesis is that excessive addition of SL impairs the function of spermatozoa mitochondria (Del *et al.* 2012).

In this study, the protective effect of 7.5% glycol was better than 4.5% glycerol, and 4.5% glycerol was better than 0.4% DMSO. Gilmore *et al.* (2000) reported that the protective effect of adding glycol was better than that of glycerol in the freezing of human semen. Guthrie *et al.* (2002) reported that the protective effect of glycol was also better than that of glycerol in the cryopreservation of bovine semen.

Najafi *et al.* (2017) reported the least protective effect of DMSO in the cryopreservation of Ghezel ram semen. The results of this study are consistent with those reported above. However, Silva *et al.* (2012) also reported that the protective effect of glycerol was stronger than that of glycol in Morada Nova ram semen cryopreservation, which may be related to sheep breed, preservation method and added concentration. In this study, 1.5% SMP had the best effect on cryopreservation of Hu ram spermatozoa, which was better than 0.15% SL group.

Küçük *et al.* (2014) reported that the protective effect of SMP was better than that of egg yolk in cryopreservation of goat semen. The main substance of egg yolk to protect spermatozoa is lecithin, SL is considered as a substitute for egg yolk (Kmenta *et al.* 2011).

Table 6 Effects of different antifreeze protection agents on Hu ram spermatozoa motility parameters stored at 4 ˚C

Semen characteristics	Time (day)	Different antifreeze protection agents				
		0.4% DMSO	4.5% glycerol	7.5% glycol	1.5% SMP	0.15% soy lecithin
Spermatozoa viability (%)	0	84.36±0.50	84.33±0.60	84.67±0.75	84.40±0.46	85.00±0.72
	1	76.88±0.64^{c}	77.33±0.32^{c}	77.11±0.50^{c}	81.66±0.36^{a}	79.38±0.34^{b}
	2	69.87±0.41^{d}	72.67±0.15^{c}	72.27±0.93^{c}	79.56±0.75^{a}	76.94±0.90^{b}
	3	68.83±0.20^{b}	66.52±0.81^{c}	68.70±0.61^{b}	75.40±0.90^{a}	73.74±0.60^{a}
	4	60.12±1.09^{c}	64.14±0.56^{b}	66.69±0.64^{b}	70.82±1.16^{a}	65.43±1.40^{b}
	5	48.05±0.66^{c}	52.61±0.43^{b}	54.43±0.34^{b}	59.50±2.14^{a}	55.85±0.86^{b}
	6	33.66±0.76^{cd}	43.16±1.60^{b}	34.28±1.17^{cd}	47.68±1.24^{a}	36.37±0.61^{c}
Spermatozoa progressive motility (%)	0	81.71±1.51	80.74±1.33	81.21±0.37	78.55±1.95	80.99±0.93
	1	68.55±0.81^{b}	70.20±1.05^{b}	68.04±0.74^{b}	74.86±0.29^{a}	70.53±1.31^{b}
	2	61.78±0.90^{cd}	63.80±0.88^{c}	64.32±0.92^{c}	71.85±0.96^{a}	68.17±0.62^{b}
	3	57.87±1.41^{bc}	56.13±0.96^{c}	60.62±0.45^{b}	66.44±1.02^{a}	65.54±0.44^{a}
	4	50.23±1.05^{c}	52.98±0.97^{bc}	55.32±1.32^{b}	61.39±0.94^{a}	54.23±0.50^{b}
	5	37.87±0.53^{c}	43.83±1.39^{b}	42.97±0.67^{b}	47.73±1.25^{a}	42.55±0.47^{b}
	6	24.82±0.90^{bc}	32.50±1.33^{a}	26.94±1.43^{b}	35.85±0.41^{a}	27.49±1.10^{b}
VSL (um/s)	0	39.30±1.01	39.63±0.54	39.28±0.68	39.68±0.60	39.65±0.66
	1	36.06±1.44^{a}	38.76±1.38^{a}	35.19±0.16^{ab}	38.08±0.06^{a}	32.14±1.09^{b}
	2	36.97±0.76^{a}	35.87±0.78^{ab}	35.27±1.33^{ab}	35.61±0.29^{ab}	35.23±1.23^{ab}
	3	34.46±0.64	31.90±0.21	33.46±0.30	32.67±1.44	31.80±1.07
	4	28.84±0.23^{b}	32.57±1.58^{a}	27.67±0.45^{b}	33.94±0.23^{a}	27.50±0.18^{b}
	5	25.88±0.50^{b}	29.07±1.19^{a}	27.48±0.54^{ab}	28.57±0.32^{a}	27.71±0.95^{ab}
	6	25.53±0.27^{c}	28.30±0.84^{a}	25.95±0.78^{bc}	25.89±0.54^{bc}	27.72±0.24^{ab}
VCL (um/s)	0	68.15±0.56	65.55±2.12	65.99±2.43	69.21±2.04	67.40±1.20
	1	65.38±1.85^{b}	72.73±3.04^{a}	66.91±2.35^{ab}	68.21±0.29^{ab}	67.09±2.27^{ab}
	2	69.08±1.29^{a}	67.82±1.42^{a}	70.17±2.56^{a}	64.85±0.77^{ab}	65.98±1.44^{ab}
	3	64.17±1.27^{ab}	61.64±0.71^{b}	66.33±0.41^{a}	64.15±1.18^{ab}	66.24±1.38^{a}
	4	49.51±0.30^{c}	57.75±4.12^{b}	55.73±2.14^{b}	67.47±0.35^{a}	65.37±1.24^{a}
	5	42.38±1.88^{c}	50.69±3.83^{b}	56.58±1.21^{ab}	56.63±0.07^{ab}	60.94±0.61^{a}
	6	44.77±0.56^{d}	50.10±1.37^{c}	55.09±0.37^{b}	52.66±0.52^{bc}	62.28±1.28^{a}
VAP (um/s)	0	48.19±0.40	46.35±1.50	46.66±1.72	48.94±1.44	47.66±0.85
	1	46.23±1.31^{b}	51.43±2.15^{a}	47.31±1.66^{ab}	48.23±0.21^{ab}	47.44±1.60^{ab}
	2	48.85±0.91^{a}	47.96±1.00^{a}	49.62±1.81^{a}	45.86±0.55^{ab}	46.65±1.02^{ab}
	3	45.38±0.90^{ab}	43.58±0.50^{b}	46.90±0.29^{a}	45.36±0.84^{ab}	46.83±0.98^{a}
	4	35.01±0.21^{c}	40.83±2.92^{b}	39.41±1.52^{b}	47.71±0.25^{a}	46.23±0.87^{a}
	5	29.97±1.33^{c}	35.84±2.71^{b}	40.00±0.85^{ab}	40.04±0.05^{ab}	43.09±0.44^{a}
	6	31.65±0.39^{d}	35.42±0.97^{c}	38.95±0.26^{b}	37.24±0.37^{bc}	44.04±0.91^{a}

DMSO: dimethyl sulfoxide; SMO: skimmed milk powder; VSL: straight line velocity; VCL: curvilinear velocity and VAP: average path velocity.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

SMP may regulate osmotic pressure and reduce the concentration of electrolyte in semen. On the other hand, it can also attach to the surface of spermatozoa cell membrane, protect spermatozoa membrane and maintain the normal morphological structure and physiological function of spermatozoa. The protective ability of various antifreeze protectants depends on their permeability, solubility, the number of unpaired electrons in the compound and their effect on the membrane structure (Wu *et al.* 2015). Some authors stated that the addition of cryoprotectants modified water permeability, lowering the hydraulic conductivity and thus limiting volumetric excursion and osmotic stress (Swelum *et al.* 2011). Therefore, according to the different species, preservation conditions and experimental methods, the type and concentration of antifreeze protectants are very important.

CONCLUSION

In conclusion, when DMSO, glycerol, glycol, SMP, SL were respectively added to Hu ram semen at 4 ˚C, the most suitable concentration was 0.4%, 4.5%, 7.5%, 1.5% and 0.15%. When each optimal concentration of antifreeze protectant was added to Hu ram semen at 4 ˚C, 1.5% SMP had the best effect on the preservation of Hu ram semen at 4 ˚C.

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REFERENCES

Alvarez R.A., Alvarez M., Anel-López A., Martínez-Rodríguez M., Martínez-Pastor M., Borragan S., Anel L. and de Paz P. (2013). The antioxidant effects of soybean lecithin- or low-density lipoprotein-based extenders for the cryopreservation of brown-bear (*Ursus arctos*) spermatozoa. *Reprod. Fertil. Dev.* **25(8),** 1185-1193.

Blanco J.M., Long J.A., Gee G., Wildt D.E. and Donoghue A.M. (2011). Comparative cryopreservation of avian spermatozoa: benefits of non-permeating osmoprotectants and ATP on turkey and crane sperm cryosurvival. *Anim. Reprod. Sci.* **123(3),** 242-248.

Chen H., Cheung M.P., Chow P.H., Cheung A.L., Liu W. and WS O. (2002). Protection of sperm DNA against oxidative stress *in vivo* by accessory sex gland secretions in male hamsters. *Reproduction.* **124(4),** 491-499.

Dalmazzo A., Losano J.D.A., Rocha C.C., Tsunoda R.H., Angrimani D.S.R., Mendes C.M., Assumpção M.E.O.D.Á., Nichi M. and Barnabe V.H. (2018). Effects of soy lecithin extender on dog sperm cryopreservation. *Anim Biotechnol.* **29(3),** 174-182.

Daly J., Smith H., McGrice H.A., Kind K.L. and van Wettere W.H.E.J. (2020). Towards improving the outcomes of assisted reproductive technologies of cattle and sheep, with particular focus on recipient management. *Animals.* **10(2),** 293-308.

Del V., Gómez D., Holt W.V., Muiño B. and Cebrián P. (2012). Soy lecithin interferes with mitochondrial function in frozen-thawed ram spermatozoa. *J. Androl.* **33(4),** 717-725.

Diaz J., Dorado J., Pereira B., Consuegra C., Ortiz I. and Hidalgo M. (2019). Is sperm cryopreservation in absence of permeable cryoprotectants suitable for subfertile donkeys? *Reprod. Domest. Anim.* **54(4),** 102-105.

Esteves S.C., Humaidan P., Roque M. and Agarwal A. (2019). Female infertility and assisted reproductive technology. *Panminerva Med*. **61(1),** 1-2.

Figueiredo C.C., Bisinotto D.Z., Brandão G.V.R., Umaña Sedó S. and Bisinotto R.S. (2020). Impact of assisted reproduction techniques on subsequent reproductive performance of dairy heifers and lactating cows. *Theriogenology.* **158,** 97-104.

Forouzanfar M., Sharafi M., Hosseini S.M., Ostadhosseini S., Hajian M., Hosseini L., Abedi P., Nili N., Rahmani H.R. and Nasr-Esfahani M.H. (2010). *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology.* **73(4),** 480-487.

Gao D.Y., Lin S., Watson P.F. and Critser J.K. (1995). Fracture phenomena in an isotonic salt solution during freezing and their elimination using glycerol. *Cryobiology.* **32(3),** 270-284.

Garde J.J., del Olmo A., Soler A.J., Espeso G., Gomendio M. and Roldan E.R. (2008). Effect of egg yolk, cryoprotectant, and various sugars on semen cryopreservation in endangered Cuvier's gazelle (*Gazella cuvieri*). *Anim. Reprod. Sci.* **108(3),** 384-401.

Gilmore J.A., Liu J., Woods E.J., Peter A.T. and Critser J.K. (2000). Cryoprotective agent and temperature effects on human sperm membrane permeabilities: Convergence of theoretical and empirical approaches for optimal cryopreservation methods. *Hum. Reprod.* **15(2),** 335-343.

Gloria A., Zambelli D., Carluccio A., Cunto M., Ponzio P. and Contri A. (2020). Is the protective effect of egg yolk against osmotic and cryogenic damage on dog spermatozoa dose-dependent? *Anim. Reprod. Sci.* **213,** 1-11.

Gonzalez C., Trentin J.M., Carnevale E.M. and Graham J.K. (2019). Effects of extender, cryoprotectants and thawing protocol on motility of frozen-thawed stallion sperm that were re-frozen for intracytoplasmic sperm injection doses. *Theriogenology.* **136,** 36-42.

Guthrie H.D., Liu J. and Critser J.K. (2002). Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa. *Biol. Reprod.* **67(6),** 1811-1816.

Hassan M.M., Li X., Liu Y. and Qin J.G. (2017). Sperm cryopreservation in the spermcasting Australian flat oyster Ostrea angasi by a programmable freezing method. *Cryobiology.* **76,** 119-124.

Hussain S.A., Lessard C. and Anzar M. (2013). A strategy for improvement of postthaw quality of bison sperm. *Theriogenology.* **79(1),** 108-115.

Kmenta I., Strohmayer C., Müller-Schlösser F. and Schäfer-Somi S. (2011). Effects of a lecithin and catalase containing semen extender and a second dilution with different enhancing buffers on the quality of cold-stored canine spermatozoa. *Theriogenology.* **75(6),** 1095-1103.

Küçük N., Aksoy M., Uçan U., Ahmad E., Naseer Z., Ceylan A. and Serin I. (2014). Comparison of two different cryopreservation protocols for freezing goat semen. *Cryobiology.* **68(3),** 327-331.

Kundu C.N., Chakraborty J., Dutta P., Bhattacharyya D., Ghosh A. and Majumder G.C. (2000). Development of a simple sperm cryopreservation model using a chemically defined medium and goat cauda epididymal spermatozoa. *Cryobiology*. **40(2),** 117-125.

Lohmann W., Fowler C.F., Moss A.J. and Perkins W.H. (1964). Some remarks about the effect of glycerol on cells during freezing and thawing: electron-spin resonance investigations concerning this effect. *Experientia.* **20(5),** 290-301.

Lovelock J.E. (1953). The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. *Biochim. Biophys. Acta.* **11(1),** 28-36.

Maxwell W.M. and Salamon S. (1979). Fertility of frozen-thawed boar semen. *Australian J. Biol. Sci.* **32(2),** 243-249.

Mehdipour M., Daghigh Kia H., Najafi A., Vaseghi Dodaran H. and García-Álvarez O. (2016). Effect of green tea (*Camellia sinensis*) extract and pre-freezing equilibration time on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender. *Cryobiology.* **73(3),** 297-303.

Moore A.I., Squires E.L., Bruemmer J.E. and Graham J.K. (2006). Effect of cooling rate and cryoprotectant on the cryosurvival of equine spermatozoa. *J. Equine Vet. Sci.* **26,** 215-218.

Najafi A., Daghigh-Kia H., Dodaran H.V., Mehdipour M. and Alvarez-Rodriguez M. (2017). Ethylene glycol, but not DMSO, could replace glycerol inclusion in soybean lecithin-based extenders in ram sperm cryopreservation. *Anim. Reprod. Sci.* **177,** 35-41.

Polge C. (1953). The storage of bull semen at low temperatures. *Vet. Rec.* **65,** 557-569.

Pursel V.G. and Johnson LA. (1975). Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. *J. Anim. Sci.* **40(1),** 99-102.

Reed M.L., Ezeh P.C., Hamic A., Thompson D.J. and Caperton C.L. (2009). Soy lecithin replaces egg yolk for cryopreservation of human sperm without adversely affecting postthaw motility, morphology, sperm DNA integrity, or sperm binding to hyaluronate. *Fertil. Steril.* **92(5),** 1787-1790.

Rezaie F.S., Hezavehei M., Sharafi M. and Shahverdi A. (2021). Improving the post-thaw quality of rooster semen using the extender supplemented with resveratrol. *Poult. Sci.* **100(9),** 1-21.

Rodriguez M. (2012). Assisted reproductive techniques for cattle breeding in developing countries: A critical appraisal of their value and limitations. *Reprod. Domest. Anim.* **47(1),** 21-26.

Rosato M.P. and Iaffaldano N. (2013). Cryopreservation of rabbit semen: comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor freezing and vitrification. *Theriogenology.* **79(3),** 508-516.

Rota A., Milani C., Cabianca G. and Martini M. (2006). Comparison between glycerol and ethylene glycol for dog semen cryopreservation. *Theriogenology.* **65(9),** 1848-1858.

Shin J.M., Gwak J.W., Kamarajan P., Fenno J.C., Rickard A.H. and Kapila Y.L. (2016). Biomedical applications of nisin. *J. Appl. Microbiol*. **120(6),** 1449-1465.

Silva E.C., Cajueiro J.F., Silva S.V., Vidal A.H., Soares P.C. and Guerra M.M. (2012). *In vitro* evaluation of ram sperm frozen with glycerol, ethylene glycol or acetamide. *Anim. Reprod. Sci.* **132(3),** 155-158.

SPSS Inc. (2011). Statistical Package for Social Sciences Study. SPSS for Windows, Version 25. Chicago SPSS Inc., USA.

Swelum A.A., Mansour H.A., Elsayed A.A. and Amer H.A. (2011). Comparing ethylene glycol with glycerol for cryopreservation of buffalo bull semen in egg-yolk containing extenders. *Theriogenology.* **76(5),** 833-842.

Tvrdá E., Bučko O., Rojková K., Ďuračka M., Kunová S., Kováč J., Benko F. and Kačániová M. (2021). The efficiency of selected extenders against bacterial contamination of boar semen in a swine breeding facility in western slovakia. *Animals.* **11(11),** 3320-3331.

Vernet P., Fulton N., Wallace C. and Aitken R.J. (2001). Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. *Biol. Reprod.* **65(4),** 1102-1113.

Vicente J.S. and Viudes C. (1996). A sucrose-DMSO extender for freezing rabbit semen. *Reprod. Nutr Dev.* **36(5),** 485-492.

Wu Z., Zheng X., Luo Y., Huo F., Dong H., Zhang G., Yu W., Tian F., He L. and Chen J. (2015). Cryopreservation of stallion spermatozoa using different cryoprotectants and combina soybean lecithin as an alternative to avian egg yolk in the cryopreservation of fish sperm. *Cryobiology.* **67(1),** 91-94.

Zhang L., Wang Y., Sohail T., Kang Y., Niu H., Sun X., Ji D. and Li Y. (2021). Effects of taurine on sperm quality during room temperature storage in Hu sheep. *Animals.* **11(9),** 2725-2732.

Zhao J.Q., tions of cryoprotectants. *Anim. Reprod. Sci.* **163,** 75-81.

Wündrich K., Paasch U., Leicht M. and Glander H.J. (2006). Activation of caspases in human spermatozoa during cryopreservation-an immunoblot study. *Cell Tissue Bank.* **7(2),** 81-90.

Yildiz C., Bozkurt Y. and Yavas I. (2013). An evaluation of Xiao G.L., Zhu W.L., Fang D., Li N., Han C.M. and Gao Q.H. (2021). Ram semen preserved at 0 °C with soybean lecithin Tris-based extender substituted for egg yolk. *Anim. Biosci.* **34(2),** 192-197.