Investigation of Various Cryopreservation Methods for Improvement of Canine Sperm Survival

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ABSTRACT

Cryoprotectants are key factors affecting sperm survival during sperm cryopreservation. Apart from glycerol, the commonly used cryoprotectant, other permeating cryoprotectants (i.e. dimethyl sulfoxide, ethylene glycol and propylene glycol) have been tested in sperm freezing of various animal species. However, the efficiency of these permeating cryoprotectants (i.e. glycerol, dimethyl sulfoxide, ethylene glycol and propylene glycol) on canine sperm freezing has not been reported. In the present study, the cryoprotective effects of four cryoprotectants on Kunming dog sperm cryopreservation have been investigated based on two freezing extenders Tris-citric acid-fructose (TCF) and Lactose-Glucose DM (LG-DM). The performance of different concentration of cryoprotectants in Kunming canine sperm freezing was also been investigated. Sperm motility, motility recovery rate, plasma membrane and acrosomal integrity were examined to evaluate cryopreserved sperm function. The results showed that: Glycerol and ethylene glycol exhibited best cryopreserving effects while propylene glycol and DMSO showed little cryoprotection for Kunming canine spermatozoa, though propylene glycol displayed better effectiveness than that of dimethyl sulfoxide. Although 5% glycerol and 5% ethylene glycol yielded more than 40% of frozen-thawed sperm motility, which meets the need for i*n vitro* fertilization (IVF) or artificial insemination (AI), 5% glycerol was the best for Kunming dog sperm freezing and had a significant difference compared with other treatments ($P<0.05$). On the contrary, propylene glycol or dimethyl sulfoxide was found to be unsuitable for the sperm freezing in Kunming dogs. Additionally, the function of permeating cryoprotectant appeared to be independent of extender types.

Keywords: Kunming Dogs; Sperm; Freezing; Permeating Cryoprotectant.

INTRODUCTION

Effective cryopreservation of spermatozoa provides a useful way to preserve genetic resources, especially for some endangered and/or precious animals. Animal breeding can also be carried out using frozen semen via artificial insemination (AI). However, sperm cryopreservation usually results in low sperm viability and fertility, which then affects the efficiency of AI (1,2,3,4,5,6,7). Being a model animal used in semen freezing, dogs are attracting more and more interest (8). Cryopreservation of canine sperm might offer exchange of genetic material as well as lead to improvement in the breeding of working dogs (9). Unlike many other working dogs used in China, the Kunming dog is the sole breed developed independently by China and is widely used by Chinese military and police in searching, rescuing and guarding. Thus, the cryopreservation of Kunming dog spermatozoa is of great

importance in these two aspects. Recently, we have successfully frozen this animal semen with glycerol (unpublished). In order to improve the quality of cryopreserved sperm, various cryoprotectants, among which glycerol is a common one, have been inevitably employed in sperm freezing. It is reported dog sperm can be cryopreserved in media containing 2%-8% glycerol without damage (10, 11), but the toxicity of glycerol on spermatozoa has been reported (12, 13, 14), and sperm injury caused by glycerol was detected (15). As a result, attempts to diversify with effective cryoprotectants in sperm cryopreservation have been made for some mammalian species. As previously reported, ethylene glycol could significantly improve post-thaw sperm motility in dog (16, 17), stallion (18), rhesus macaque (19) and cynomolgus monkey (20). Dimethyl sulfoxide (DMSO) was used for sperm freezing in rabbits (21,22), cynomolgus monkeys (23), dogs (24) and Tasmanian devil (25). In a few studies, propylene glycol was used for the freezing of cynomolgus monkey (20) and rabbit (26) spermatozoa. As in different species, freezing extenders and procedures were involved in those studies, the cryoprotectant concentrations and the relative results varied greatly. In addition, the effects of four permeating cryoprotectants (i.e. glycerol, DMSO, ethylene glycol and propylene glycol) on dog sperm freezing have not been compared yet.

In the present study, the comparison of four permeating cryoprotectants for Kunming dog sperm cryopreservation has been investigated based on two extenders TCF and LG-DM. At the same time, the freezing effects of various concentrations for each cryoprotectant on canine spermatozoa have been examined.

MATERIALS AND METHODS

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless indicated otherwise.

Semen collection and processing

Six Kunming dogs between 2 to 4 years old, which had been proved to be fertile via natural mating, were used for semen collection in the study. Only the second sperm-rich fraction of each ejaculate was collected (27). The sperm motility, concentration, plasma membrane and acrosomal integrity were examined immediately. The semen with motility of ≥70% and concentration of ≥200×10^6 spermatozoa/mL were used for freezing.

Diluents and Extenders

The medium for semen dilution and sperm washing was Tris buffer, prepared by dissolving 2.4 g Tris (hydroxymethyl, aminomethane), 1.4 g citric acid, 0.8 g glucose, 0.06 g penicillin and 0.1 g streptomycin sulfate in 100ml of distilled water (28).

Two freezing extenders, Tris-citric acid-fructose (TCF) and Lactose-Glucose DM （LG-DM） were used in the study. TCF was composed of 3.025g Tris (hydroxymethyl, aminomethane), 1.70g citric acid, 1.25g fructose, 0.1g penicillin, 0.1g streptomycin sulfate, 20 ml egg yolk in 80 ml distilled water (29). LG-DM contained 2.75g Glucose, 5.5g Lactose, 6.32 mg penicillin, 5mg streptomycin sulfate, 10ml egg yolk in 90 ml distilled water (30). Fresh chicken eggs laid within 8 hours were used for egg yolk preparation and the procedure was taken from the description of Li *et al*. (30).

Semen freezing and thawing

After collection, the semen was diluted 4-fold with Tris buffer, then centrifuged at 700 ×g for 5 min (room temperature) and the sperm pellets were re-suspended in TCF or LG-DM at a concentration of 2000 million spermatozoa/ml. Canine semen freezing was performed as previously described (30). Briefly, spermatozoa was diluted with TCF or LG-DM (1:9) without cryoprotectant and kept at 4°C for 2h. An equal volume of pre-cooled (4°C) corresponding extenders containing glycerol, ethylene glycol, DMSO or 1,2- propylene glycol (1%, 3%, 5%, 7% or 9% (v/v)) was added to the sperm suspension for each respective extender. The addition was carried out stepwise (five times) at intervals of 5 to 6 min within 30 min. The spermatozoa were equilibrated at 4°C for 30 min. Just before the end of equilibration, the spermatozoa were drawn into 0.25-ml plastic straws (IMV, L'Aigle, France) with the final concentration of 100×10^6 spermatozoa/mL, sealed with a straw heater (Heat Sealer, PFS -300; Zhejiang jiangnan industrial co., Ltd., Zhejiang, China). Following that, the straws were frozen by being placed horizontally on a rack 5 cm above the surface of liquid nitrogen. Ten minutes later they were submerged directly into liquid nitrogen (LN_2) for storage. After being stored in LN_2 for more than 7 days, the straws containing frozen spermatozoa were put into 37°C water bath for 1 minute to thaw.

Table 1. Post-thaw motility, motility recovery rate, membrane integrity and acrosomal integrity of spermatozoa cryopreserved with different cryoprotectants at different concentrations in TCF extender.

Cryoprotective Solution		Motility (%)	Motility recovery rate (%)	Membrane Integrity(%)	Acrosomal Integrity(%)
Fresh sperm		80.02±4.35		91.22±1.96	94.48±0.94
Gly(v/v%)	1	39.63±1.09^{a}	49.66±3.15^{a}	44.74±0.99^{a}	83.34±2.07^{a}
	3	43.41±1.41^{b}	54.40±3.66^{b}	46.99±2.02^{ab}	85.55±1.57^{b}
	5	48.75±3.69^{c}	61.03±5.05^{c}	52.81±4.00^{c}	89.09±1.57^{c}
	7	42.22±1.58^{b}	52.88±3.22^{ab}	47.82±3.26^{b}	87.40±2.29^{c}
	9	30.45±1.84^{d}	38.22±3.82^{d}	35.35±2.63^{d}	83.21±1.73^{a}
DMSO(v/v%)	1	11.94±1.67^{ab}	14.92±1.80^{ab}	18.70±2.16^{ab}	81.35±2.29^{a}
	3	13.47±1.17^{ac}	16.87±1.61^{ac}	19.00±1.74^{ac}	80.39±1.80^{ab}
	5	14.46±2.11^{c}	18.04±2.21^{c}	21.96±2.40^{d}	82.19±2.60^{a}
	7	11.60±1.39^{b}	14.51±1.56^{b}	19.91±2.35^{ad}	80.73±3.76^{ab}
	9	9.22±1.58^{d}	11.53±1.87^{d}	17.16±1.89^{bc}	78.43±1.71^{b}
EG(v/v%)	1	40.50±1.75^{ab}	50.69±2.73^{ab}	46.02±0.8^{2ab}	84.07±0.55^{a}
	3	43.19±1.75^{bc}	54.11±3.47^{a}	48.67±2.45^{a}	87.74±2.10^{b}
	5	43.99±3.01^{c}	55.20±5.89^{a}	48.67±3.12^{a}	86.81±2.76^{b}
	7	38.62±3.85^{a}	48.37±5.35^{b}	43.16±4.31^{b}	84.22±1.49^{a}
	9	33.72±3.77^{d}	42.20±4.64^{c}	38.69±2.61^{c}	82.70±1.74^{a}
PROH(v/v%)	1	35.39±1.49^{a}	44.40±3.84^{a}	39.58±1.15^{a}	83.81±1.61^{ab}
	3	30.35±3.09^{b}	38.13±5.31^{b}	35.07±4.11^{b}	84.38±3.50^{a}
	5	24.84±1.43^{c}	31.16±2.89^{c}	29.96±2.43^{c}	81.24±2.29^{bc}
	7	20.65±1.00^{d}	25.91±2.36^{d}	25.72±1.02^{d}	80.84±2.09^{c}
	9	17.66±1.47^{e}	22.16±2.58^{d}	23.85±1.88^{d}	80.47±3.24^{c}

Gly indicates glycerol; DMSO, dimethylsulfoxide; EG, ethylene glycol; and PROH, 1,2- propylene glycol. Groups with different superscript letters in the same column for same cryoprotectant are significantly different (P<0.05,n=10). Only statistical differences to p<0.05 were examined.

Sperm quality assessment

Sperm motility

Using a pre-warmed hemocytometer, the percentage of sperm motility was evaluated by counting at least 200 spermatozoa per group.

Sperm recovery rated based on motility

Recovery rate was calculated by comparing the motility of pre-freezing (Mpr) and post-thawed (Mps) spermatozoa. Where Mpr and Mps are the sperm motility percentages before and after freezing, then the recovery rate would calculated as Mps/Mpr×100%.

Sperm membrane integrity

Sperm membrane integrity was measured by means of a dual DNA staining technique as Cai *et al.* as described (31). Briefly, a 6-ml volume of Hoechst 33342 (H342) (1 mg/ml) and an 8-ml volume of propidium iodide (PI) (1 mg/ml) were added to each sperm sample (1 ml). Each sperm suspension was mixed three times by gentle pipetting, and was then incubated in a 37°C water bath for 15 min. A minimum of 200 spermatozoa were counted for each sample.

Sperm acrosomal integrity

Acrosome status was determined by means of fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA) and the acrosome staining process described by Esteves *et al.* (32). Under a fluorescence microscope, spermatozoa with intact acrosome showed uniform apple-green fluorescence in the acrosomal region of the sperm head, while acrosome-reacted spermatozoa showed little or no green fluorescence in the equatorial segment of the acrosome. During the experiment, a minimum of 200 spermatozoa were counted for each sample.

Statistical Analysis

All data are expressed as the mean±SD. The percentage of sperm motility, sperm membrane and acrosomal integrity were analyzed by analysis of variance (ANOVA), Fisher's protected least significant difference (LSD) test and the One-Samples Test. Values of P<0.05 were considered statistically significant.

RESULTS

Comparative study of four permeating cryoprotectants for Kunming dog sperm in TCF extender

As shown in Table 1, when glycerol concentration increased from 1% to 5%, the post-thaw sperm motility, motility recovery rate, membrane integrity and acrosomal integrity increased. However, those sperm parameters declined when glycerol concentration increased from 5% to 9%. Five percent glycerol yielded the optimal freezing efficacy. When spermatozoa were frozen in DMSO, all sperm parameters with the exception of acrosomal integrity were

extremely low (9.22%-21.96%), regardless of the gycerol concentration.

The freezing efficacy of ethylene glycol was generally similar to glycerol, but the maximum post-thaw sperm motility given as 5% ethylene glycol was 43.99%, less than that of 5% glycerol which was 48.75%. The freezing efficacy of propylene glycol (with the exception of acrosomal integrity) was better than that of DMSO but was inferior to that of glycerol or ethylene glycol.

In general, the post-thaw sperm motility, motility recovery rate, membrane integrity and acrosomal integrity exhibited by glycerol or ethylene glycol were higher than that of propylene glycol or DMSO. Under the same concentration, 5% for instance, all sperm parameters (except for acrosomal integrity) of spermatozoa frozen by four cryoprotectants were significantly different from each other ($P<0.05$). The cryopreservation efficiency for the four cryoprotectants was glycerol> ethylene glycol> propylene glycol> DMSO (Figure 1, $P<0.05$).

Comparative study of four permeating cryoprotectants for Kunming dog sperm in LG-DM extender

Post-thaw motility, motility recovery rate, membrane integrity and acrosomal integrity of spermatozoa cryopreserved with different cryoprotectants and concentrations are shown in Table 2.

As shown in Table 2, when glycerol concentration increased from 1% to 5%, the post-thaw sperm motility, motility recovery rate, membrane integrity and acrosomal integrity increased. However, those sperm parameters declined when glycerol concentration increased from 5% to 9%. 5% glycerol yielded the optimal freezing efficacy. When spermatozoa were frozen in DMSO, all sperm parameters with the exception of acrosomal integrity were extremely low (8.14%-20.53%), regardless of its concentration.

The freezing efficacy of ethylene glycol was generally similar to glycerol, but the maximum post-thaw sperm motility given by 5% ethylene glycol was 45.31%, less than the result of 5% glycerol which was 50.40%; The freezing efficacy of propylene glycol (with the exception of acrosomal integrity) was better than that of DMSO but was poorer than that of glycerol or EG.

In general, the post-thaw sperm motility, motility recovery rate, membrane integrity and acrosomal integrity exhibited by glycerol or ethylene glycol were higher than that of propylene glycol or DMSO. Under the same concentration, 5% for instance, all parameters (except for acrosomal integrity) of spermatozoa frozen by four cryoprotectants were significantly different from each other ($P<0.05$). The cryopreservation efficiency for the four cryoprotectants was glycerol> ethylene glycol> propylene glycol> DMSO (Figure 2, $P<0.05$).

Kunming dog sperm stained with fluorescein isothiocyanate conjugated peanut agglutinin (FITC-PNA)

As shown in Fig. 3, spermatozoa with intact acrosome (NR) showed uniform apple-green fluorescence in the acrosomal region of the sperm head, while acrosome-reacted spermatozoa (AR) showed little or no green fluorescence in the equatorial segment of the acrosome.

DISCUSSION

In the present study, we investigated the efficiency of four permeating cryoprotectants on canine sperm cryopreservation. Though glycerol is the most widely used cryoprotectant for sperm freezing, its negative effect was also reported (33, 34, 35). Other chemicals as substitutes were considered promising for glycerol in sperm cryopreservation. For example, Rota *et al.* (16, 17) reported post-thaw sperm motility of 5% ethylene glycol was higher than that of 5% glycerol in their dog experiment. The cryopreservation result of glycerol in our work was similar to that of Rota *et al.,* but the ethylene glycol result was far less than theirs. We suggest that different results stem from different freezing procedures. The major differences in two studies lies in the pattern of sperm cooling and the exposure time of sperm in ethylene glycol. In our study, dog semen was cooled (at 4°C) in extender without ethylene glycol for 2 hours, then ethylene glycol was added and the semen was equilibrated in 5% ethylene glycol for half an hour (at 4°C) before freezing. In the experiment of Rota *et al.,* however, semen was firstly cooled (also at 4°C) in extender containing 3% ethylene glycol for a shorter period of time (only one hour). Then a higher concentration of ethylene glycol (7%) was added and the semen was frozen afterwards. During sperm freezing, when a permeating cryoprotectant is added to sperm suspension, sperm cells begin to expel internal water therefore causing the cell to shrink. Then, the permeating cryoprotectant could enter the cells and help

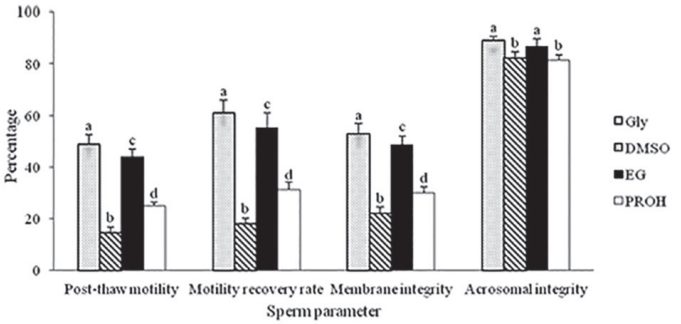

Figure 1. Post-thaw motility, motility recovery rate, membrane integrity and acrosomal integrity of spermatozoa cryopreserved with different cryoprotectants at the same concentration (5%) in TCF extender.

Gly indicates glycerol; DMSO, dimethylsulfoxide; EG, ethylene glycol; and PROH, 1,2- propylene glycol. Different letters above the columns for each parameter are significantly different (P<0.05).

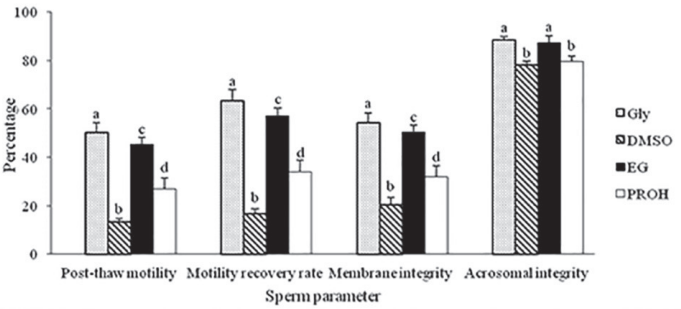

Figure 2. Post-thaw motility, motility recovery rate, membrane integrity and acrosomal integrity of spermatozoa cryopreserved with different cryoprotectants at the same concentration (5%) in LG-DM extender.

Gly indicates glycerol; DMSO, dimethyl sulfoxide; EG, ethylene glycol; and PROH, 1,2- propylene glycol. Different letters above the columns for each parameter are significantly different (P<0.05).

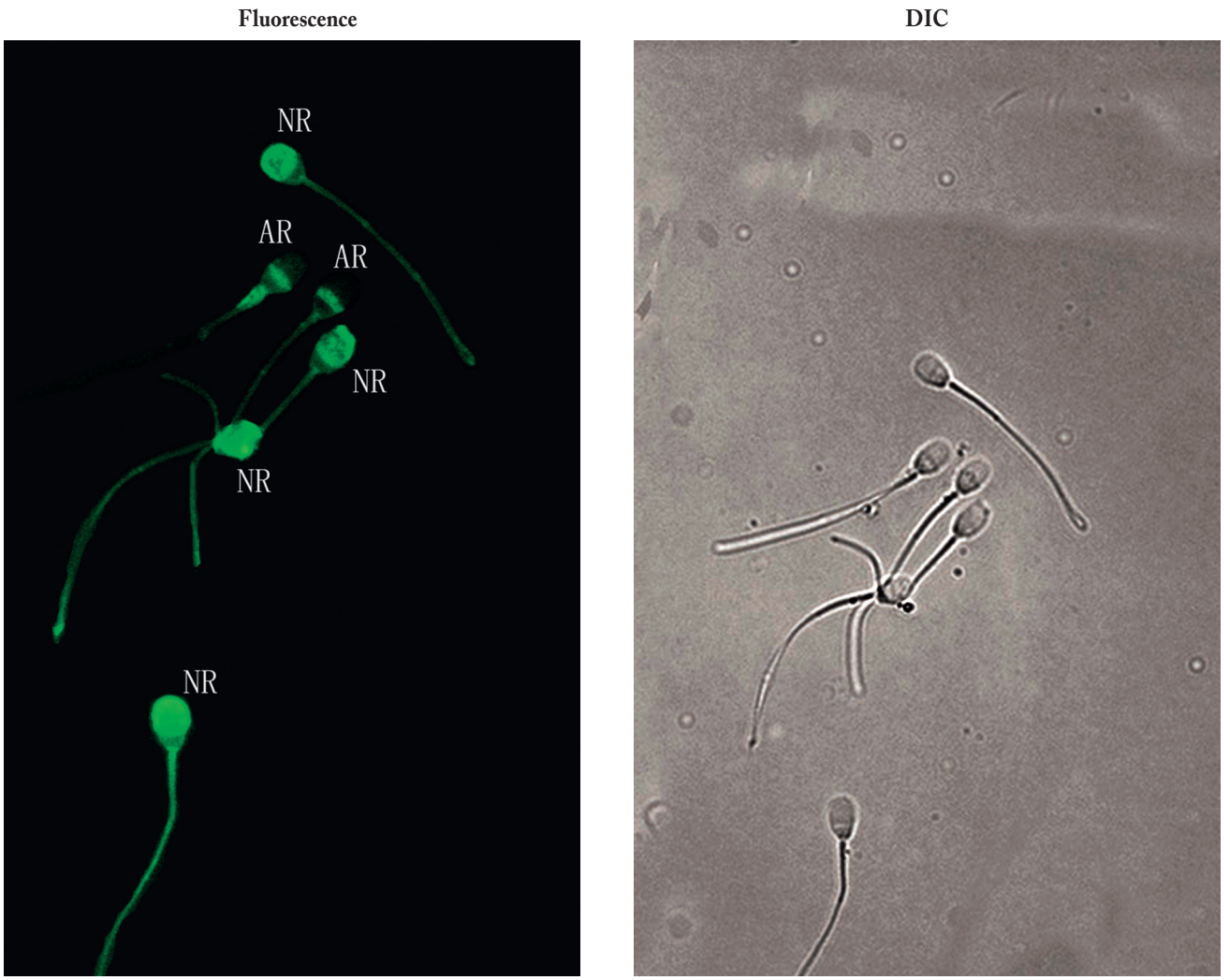

Figure 3. Canine sperm stained with FITC-PNA. Sperm were fixed and stained with FITC-PNA as described in the Materials and Methods. Sperm with intact acrosome (NR) and acrosome reacted sperm (AR) were observed. Representative pictures are shown.

Table 2. Post-thaw motility, motility recovery rate, membrane integrity and acrosomal integrity of spermatozoa cryopreserved with different cryoprotectants at different concentrations in LG-DM extender.

Cryoprotective Solution		Motility(%)	Motility recovery rate (%)	Membrane Integrity (%)	Acrosomal Integrity (%)
Fresh sperm		79.55±2.26		89.99±3.05	95.22±0.79
Gly(v/v%)	1	40.59±2.07^{ab}	51.03±2.30^{ab}	45.65±1.14^{ab}	85.31±2.21^{ab}
	3	44.67±2.61^{c}	56.18±3.43^{c}	49.77±1.84^{c}	87.02±1.69^{bc}
	5	50.40±3.90^{d}	63.35±4.57^{d}	54.25±3.84^{d}	88.72±0.93^{c}
	7	43.36±1.82^{bc}	54.53±2.50^{bc}	47.23±1.16^{bc}	87.61±1.86^{c}
	9	35.28±2.75^{e}	44.36±3.39^{e}	39.95±3.19^{e}	83.57±2.31^{a}
DMSO(v/v%)	1	12.21±0.90^{ab}	15.38±1.46^{ab}	19.29±1.70^{ab}	78.86±2.65^{a}
	3	12.78±1.37^{ab}	16.10±2.01^{bc}	20.13±2.88^{b}	78.88±2.29^{a}
	5	13.42±1.31^{a}	16.89±1.80^{b}	20.53±2.88^{b}	78.35±1.38^{a}
	7	11.54±1.03^{b}	14.51±1.16^{ac}	18.23±1.81^{ab}	78.41±3.12^{a}
	9	8.14±1.29^{c}	10.22±1.49^{d}	16.98±2.30^{a}	77.41±3.23^{a}
EG(v/v%)	1	38.98±1.61^{a}	49.00±1.45^{a}	45.23±1.98^{a}	82.81±2.87^{a}
	3	42.60±2.51^{b}	53.54±2.77^{b}	47.90±1.38^{b}	85.78±2.81^{bc}
	5	45.31±2.79^{c}	56.96±3.15^{c}	50.22±2.84^{c}	87.38±2.57^{b}
	7	40.23±2.05^{a}	50.60±2.92^{a}	45.31±2.35^{a}	84.67±2.09^{ac}
	9	35.41±1.43^{d}	44.54±2.01^{d}	39.77±1.74^{d}	82.66±1.55^{a}
PROH(v/v%)	1	34.66±1.94^{a}	43.56±1.76^{a}	39.74±1.78^{a}	82.93±1.92^{ab}
	3	30.21±3.18^{b}	37.93±3.39^{b}	34.83±2.84^{b}	81.47±2.05^{bc}
	5	26.99±4.37^{b}	33.87±5.05^{b}	31.96±4.57^{b}	79.67±2.16^{cd}
	7	21.51±4.95^{c}	26.95±5.78^{c}	27.33±4.95^{c}	78.83±2.13^{d}
	9	18.24±4.37^{c}	22.86±5.17^{c}	24.35±3.31^{c}	78.54±3.32^{d}

Gly indicates glycerol; DMSO, dimethylsulfoxide; EG, ethylene glycol; and PROH, 1,2-propylene glycol. Groups with different superscript letters in the same column within each cryoprotectant are significantly different ($P<0.05$, n=10).

avoid lysis. In this way, a permeating cryoprotectant prevents sperm cells from damage. Although glycerol and ethylene glycol belong to the same category of chemicals, ethylene glycol enters sperm cells more quickly than glycerol as the former has a lower molecular weight (36, 37). Consequently it takes less time for ethylene glycol to cause a sperm cell to resume its shape compared with glycerol. Apart from lower molecular weight, ethylene glycol has higher permeability coefficient than glycerol, making it more effective for sperm freezing (36, 37). To test this, by adding glycerol or ethylene glycol with the protocol of Rota *et al* (16, 17). We obtained similar results (the post-thaw sperm motilities were 45% and 57%, respectively) to that of Rota *et al.* In addition, the studies (ours and that of Rota *et al.*) also indicating that the same concentrations of glycerol or ethylene glycol, both of which are ideal cryoprotectants for canine sperm freezing, may exhibit significant freezing efficiency via different protocols. It seems a feasible alternative to substitute ethylene glycol for glycerol in sperm cryopreservation, at least for canine species, as some researchers speculated that ethylene glycol is less toxic than glycerol to sperm cells (16, 38, 39). Toxicity test of ethylene glycol for canine sperm cells need to be carried out in the future.

Our study showed that 5% glycerol or 5% ethylene glycol yields the best freezing results in Kunming dog sperm compared with other concentrations. The fact that ethylene glycol could cryopreserve dog semen well is consistent with the findings in stallion or ram semen cryopreservation (40, 41, 42). On the contrary, the result from Awad's study is quite different. Awad found that glycerol is much better than ethylene glycol in bull sperm freezing (43). Based on these findings, we postulate that the role of ethylene glycol in sperm freezing may also be species-specific.

Olar reported that the motility of spermatozoa cryopreserved in DMSO was reduced dramatically (24). In our experiment, the post-thaw sperm motility (less than 15%) for DMSO was far less than other three cryoprotectants, implying that DMSO is not suitable for Kunming dog sperm cryopreservation. Si *et al.* showed that the effect of glycerol was better than DMSO in rhesus monkey sperm freezing (44). However, this cryoprotectant had been successfully employed for sperm freezing in bulls (45), rabbits, (21, 22, 26) and cynomolgus monkeys (23). It seems reasonable to infer that the effects of DMSO as a sperm cryoprotectant may be related to animal species and/or different freezing procedures. The mechanism of the failure of DMSO to cryopreserve Kunming dog semen may be due to its toxic effects rather than osmotic effects. Since according to the study of Songsasen *et al.*, the motility of canine sperm exposed to hypertonic solution of DMSO decreased considerably after dilution into isotonic solution, owing to having a lower molecular weight than that of glycerol, it seems that

DMSO would be unlikely to produce an osmotic shock for spermatozoa (46). Although post-thaw sperm motility given by DMSO was very low in the present study, the acrosomal integrity of frozen-thawed spermatozoa was scarcely affected, so motility might be a more sensitive indicator of sperm function (20).

Another permeating cryoprotectant we investigated in this study is propylene glycol which showed a better effect in sperm freezing than that of DMSO, in contrast, the freezing effect of propylene glycol was significantly poorer than that of glycerol or ethylene glycol. We point out that the cryoprotective effect of propylene glycol in our study is equivalent to the report of Nur *et al.* in which ram sperm cryopreservation was conducted (47). Compared with the study of Li *et al.,* which examined cryoprotective effects of these four permeating cryoprotectants on cynomolgus monkey (*Macaca fascicularis*) sperm cryopreservation, the efficiencies of glycerol and ethylene glycol in our study were similar to that of Li *et al.* (20). As for DMSO and propylene glycol, our results were different from their study: In our study, the cryoprotective effect of propylene glycol was better than that of DMSO compared to Li *et al.* We postulate species difference may account for the inconsistency.

The present study demonstrated that 4 cryoprotectants used here either performed similar freezing effects for canine spermztozoa rather in TCF or LG-DM, suggesting the action of a permeating cryoprotectant is mainly associated with its internal chemical feature instead of extender types.

Our laboratory has been focusing on sperm cryopreservation in wild and domestic animals. We have compared freezing effects of permeating cryoprotectants on spermatozoa of primate animals (20) as well as dogs (the present study) and found that the effects of glycerol or ethylene glycol were significantly better than that of DMSO or propylene glycol regardless of the animal species. Furthermore, the frozen-thawed sperm motility with glycerol or ethylene glycol could reach 40% or more in any species, which means those cryopreserved spermatozoa can be used in the practice of IVF and/or AI. Considering the similar freezing effects of glycerol, DMSO, ethylene glycol and propylene glycol on sperm cryopreservation in primate animals and dogs, we speculate that primates and Kunming dog spermatozoa might share similar response to these permeating cryoprotectants.

In the present study, six fertile Kunming dogs whose ages are 2-4 years were used as semen donors. In many other canine sperm cryopreservation studies, the number of animals is from 2 to 12 and quite a few laboratories use 5-6 dogs for each study. Additionally, the age of dogs in those previous studies is between 2-7 years old. Thus, the number or the age of dogs in our study is similar to other studies. In addition, both FITC-PSA and FITC-PNA can be used in evaluating acrosomal integrity for mammalian spermatozoa. FITC-PNA staining has been performed in assessing sperm acrosome status for various species including Kunming dogs in our laboratory and the staining results are quite clear, reliable and easy to determine. Therefore, FITC-PNA was used to dye sperm acrosomes in this study. Spermatozoa should be permeabilized by methanol before staining, as PNA cannot penetrate an intact membrane.

ACKNOWLEDGEMENTS

This study was supported by National Natural Science Foundation of China (31460308) and the Opening Fund of Yunnan Key Laboratory of Primate Biomedical Research (Yun Zhongke 2016-No.008).The authors wish to thank Dr, Enid Arcelay from University of Puerto Rico, Mayagüez for her serious revision of the manuscript.

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