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Cryoprotective Activity of Glycerol and Ethylene Glycol: Dynamics with Canine Sperm Integrity

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ABSTRACT

Background: Different cryoprotectants had been used for safe gaurding the post thaw Canine sperm cell motility. Glycerol and ethylene glycol may be used as cryoprotectants. However, scanty reports are available on use of different concentrations of glycerol, ethylene glycol and the combination of glycerol and ethylene glycol as cryoprotectants for canine spermatatozoa.

Methods: In present study, the semen samples were collected from 4 different adult spitz male dogs. The diluted semen was divided into four aliquots, Group I: 8% glycerol (control); Group II: 4% glycerol; Group III: 5% ethylene glycol; Group IV: 4% glycerol + 4% ethylene glycol.

Result: The result showed that post-thaw sperm motility in canine semen with 5% ethylene glycol was recorded as 36.83±1.26%. For Group I (8% glycerol) and Group II (4% glycerol), the same was 34.25±0.95% and 26.50±0.81%, respectively whereas Group IV (4% glycerol + 4% ethylene glycol) it was 29.25%. However, there was no significant correlationship between abnormal sperm count and live spermatozoa; abnormal sperm count and dead spermatozoa; dead spermatozoa and DNA integrity and in between HOST (hypo-osmotic swelling test) and acrosomal integrity of spermatozoa.

Key words: DNA integrity, HOST, Ethylene glycol, Sperm integrity, Sperm viability.

INTRODUCTION

Veterinary practitioners may perform semen analysis as part of a complete breeding soundness examination to evaluate the suitability and quality of semen for AI, preserved by chilling or freezing (Kustritz, 2007).The genetic diversification in canines is possible in any corner of the world by using cryopreserved semen for artificial insemination (AI). The rate of successful AI is more than 70% (Mason and Rous, 2014; Hollinshead and Hanlon, 2017). Sometimes, improper storage and thawing of cryopreserved semen reduce the sperm viability. The process of cryopreservation will reduce sperm motility, plasma-acrosomal reaction and cause premature sperm cell death (Lucio *et al.,* 2016). Many studies were conducted to improve the sperm viability during freezing. Several chemical and synthetic molecules were used as cryoprotectants. Glycerol, dimethylsulfoxide, Nmethylformamide, N-N-dimethylformamide, ethylene glycol are the most widely used cryoprotectants during the preservation of dog sperm (Kusum *et al.,* 2020). Most of the previous studies reported favourable results upon hypoosmotic swelling test (HOST) at post thaw (Futino *et al.,* 2010). Ethylene glycol (5%) or glycerol (5%) had positive correlation with sperm viability at thawing by maintaining the sperm motility and membrane integrity (Rota *et al.*, 2006). In another study it was found that ethylene glycol had a lower impact than glycerol on canine sperm motility (Cavalcanti *et al.*, 2002).

Meagre studies were conducted on the canine sperm viability, motility, DNA integrity after supplementaion of different concentrations of glycerol, ethylene glycol and combined glycerol and ethylene glycol to the diluent for cryopreservation of canine semen. Hence, the present study was designed to determine the effect of 8% glycerol (Control), 4% glycerol, 5% ethylene glycol and 4% glycerol + 4% ethylene glycol as cryoprotectants upon the integrity of canine sperm membrane, acrosome and DNA. Further, in the present study it was hypothesised that the combination of glycerol and ethylene glycol as cryoprotectant improved the post thaw quality of canine spermatozoa.

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MATERIALS AND METHODS

Chemicals and reagents

Glycerol and Ethylene glycol were purchased from Sigma Aldrich, USA.

Experimental animals and semen sample collection

Four healthy Spitz adult male dogs were selected for the study. All the dogs were maintained on uniform balance diet and managemental practices in the Kennels stationed at Animal Nutrition Division of ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, India. The present study was done during the period from June, 2016 to August, 2018. A total forty-eight ejaculates (second fraction) were collected from 4 adult Spitz male dogs, *i.e.*, twelve ejaculates from each dog. Semen was collected by digital manipulation, as described by Linde-Forsberg *et al.* (1999).

Experimental analysis

Initial evaluation of the ejaculates was carried out and the samples with (criteria for selection to be cited) were utilized for further processing. Semen was diluted with Tris fructose egg yolk glycerol diluent, as suggested by Davis *et al.,* (1963). The diluted semen was divided into four (aliquots) groups as Group I: 8% glycerol (control); Group II: 4% glycerol; Group III: 5% ethylene glycol; Group IV: 4% glycerol + 4% ethylene glycol. Based on the preliminary studies for the average concentration of spermatozoa in the sperm rich fraction, a dilution rate of 1:2 was used in the present study. The dilution rate would ensure a minimum sperm concentration of 100 million/ml of diluted semen (Christiansen, 1984).

Extended semen was filled in french medium straws and was equilibrated at 5°C for 4 hrs (Kordan *et al.*, 2010). After equilibration, straws were kept in liquid nitrogen vapour around 4-6 cm above the liquid nitrogen level for 10-15 minutes. After that, the straws were plunged into liquid nitrogen in cryo for storage. The abnormalities include micro, macro, pear-shaped or detached in the head region, swollen, abaxially attached, double or zig-zagmidpiece in middle piece region and coupled tail over in the tail region. Besides the proximal and distal droplets, the membrane integrity hypo-osmotic swelling test was done after following England and Plummer's (1993) method. Acrosomal integrity was assessed by the method described by Watson (1975).

DNA integrity of spermatozoa (Double-strand breaks) was estimated by a single-cell alkaline microgel electrophoresis method (Comet assay). The slide was stained with propidium iodide (20 µg/ml) while others with ethidium bromide (50 µl at 20 µg/ml) for 3 minutes and later washed with cold water to remove the excess stain. Coverslips were applied and observed immediately under fluorescent microscopy (Rijsselaere *et al.*, 2005). 50 cells were analyzed using a visual scoring system and the cells were graded (Grade 1-4) according to the extent of damage (Tail extent, nucleus head diameter) in each group. DNA integrity of spermatozoa is evaluated as described by Singh *et al.* (2003).

Statistical analysis

Analysis of variance among the experimental treatments were compared with means by Duncans multiple range test (DMRT). Further, correlation coefficient between various sperm parameters was also determined, as per the procedures described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The post-thaw sperm motility percentage in canine semen added with 5% ethylene glycol was 36.83±1.26. While the same for 8% and 4% glycerol concentrations was 34.25±0.95% and 26.50±0.81%, respectively. However, with combination of 4% glycerol and 4% ethylene glycol the post thaw sperm motility percentage was 29.25±0.94. Similar findings were also reported by Kurien (2012).

Spermatozoa had a higher osmotic tolerance to quick addition and removal of ethylene glycol than to the glycerol (Rota *et al.,* 2010). The possibility of ethylene glycol to cause fewer osmotic lesions had been proposed for stallion spermatozoa. Macias *et al.* (2012) observed that dog spermatozoa's response to osmotic shock was controlled by the activity of the potassium channel and was diminished in the presence of an intact cytoskeleton. Ethylene glycol might alter the functioning of potassium channel, their activation mechanism of fluxes of ions and organic osmolytes. Spermatozoa showed different volumetric variations under similar hypo-osmatic conditions, suggestive of individual differences in cytoskeleton characteristics and potassium channel activity (Yeste *et al.,* 2019). Plasma membrane lipid peroxidation (LPO) is a physiological event in which radicals from oxygen metabolism and reactive oxygen species (ROS) reacted with membrane lipids in an oxidative process that primeed the spermatozoa for fertilization (Ortega-Ferrusola *et al.,* 2017). However, higher levels of ROS destroyed the structure of lipid matrix, leading to a loss of membrane integrity. In frozen/thawed dog semen, LPO increased with increase in intracellular hydrogen peroxide levels and DNA fragmentation compared to fresh semen (Rocco *et al.,* 2018). Present study observed positive correlation between dead sperm count and DNA grade 2 damage (0.61; $P \leq 0.05$) and in between HOST and progressive motility (0.61; $P \leq 0.05$). In Group I aliquots no correlation was recorded between dead sperm and grade 1 DNA integrity, abnormal spermatozoa and DNA intactness, abnormal spermatozoa and live sperm count. However, in the present study observed a positive correlation between dead sperm count and DNA grade 2 damage (0.61; $P \leq 0.05$) and in between HOST and progressive motility (0.61; $P \leq 0.05$) in Group I aliquots (Table 1). In 4 % glycerol (Group II) 4.5.3 4 per cent glycerol (group II) aliquots a negative correlation was observed between live and dead sperm count (-0.74; $P \leq 0.01$); abnormal sperm count and live sperm (-0.71; $P \leq 0.01$); DNA intactness (grade-1) and HOST (-0.60; $P \leq 0.05$); dead sperm count and acrosomal integrity (-0.63; $P \leq 0.05$); spermatozoal abnormality and HOST (-0.82;

P≤0.01) and in between abnormality and acrosomal integrity (-0.74; *P≤0.01*) (Table 2). However, a positive correlation occurred between acrosomal integrity and grade 2 (DNA intactness) (0.58; *P≤0.05*); acrosomal integrity and HOST (0.77; *P≤0.01*); HOST and live sperm count (0.89; *P≤0.01*) and in between acrosomal integrity and live sperm count (0.68; *P≤0.05*) in Group II aliquots.

In 5% ethylene glycol aliquots (Group III) 4.5.4 5 percent ethylene glycol (group III) a negative correlation existed between HOST (-1.00; *P≤0.01*) and dead sperm count (-0.66; *P≤0.05*); DNA grade 1 integrity and grade 3 (-0.72; *P≤0.01*) as well as in between live and dead counts. However, there was no significant correlation between abnormal sperm count and live spermatozoa, abnormal sperm count and dead spermatozoa, dead spermatozoa and DNA integrity and in between HOST and acrosomal integrity (Sicherle*et al.,* 2020). Similar types of findings were reported by Gharajelar *et al.* (2016) and Lecewicz *et al.* (2018). Although there was a significant (*P≤0.01*) difference in acrosomal integrity, livability, abnormal sperm count, grade 1, 2 and 4 DNA integrity among different cryoprotectants in the study of Sanchez *et al*. (2011). There was a higher (*P≤0.01*) dead sperm count and grade-3 DNA intactness, lower HOST and post-thaw motility in group I (8% glycerol) as compared to other groups. In the present study, there was a positive correlation between post-thaw motility, HOST and live spermatozoa (Lucio *et al.,* 2016) also found significant positive correlation between HOST and live sperm count (r = 0.66; *P≤0.05*); HOST and progressive motility (r = 0.76; *P≤0.01*); progressive motility and grade 1 DNA integrity (r = 0.62; *P≤0.05*) and in between abnormality of sperm and grade 3 DNA intactness (r = 0.60; *P≤0.05*) (Table 3). 4.5.5 4 per cent ethylene glycol + 4 per cent glycerol (group V) Spermatozoal abnormality was positively correlated with the number of dead spermatozoa (r = 0.67; *P≤0.05*) in frozen semen where 4% ethylene glycol and 4% glycerol (group V) cryoprotectant were used. However, live sperm count were negatively correlated with dead sperm count (r = -0.97; *P≤0.01*) and the abnormality (r = -0.60; *P≤0.05*). DNA integrity was assessed by comet assay and different grade of DNA intactness was recorded (Table 4). There was negative correlation (r = -0.62; *P≤0.01*) recorded between

Table 1: Correlation coefficient between conventional, function and structural characteristics of spermatozoa in group I (control) of four Spitz dogs.

	CH1	CH2	CH3	CH4	CH5	CH6	CH7	CH8	CH9	CH10
CH1	1									
CH2	-0.49	1								
CH3	0.50	**-0.78****	1							
CH4	-0.05	0.30	-0.43	1						
CH5	**0.61***	**0.59***	-0.49	0.21	1					
CH6	0.11	**0.66***	-0.38	0.49	0.02	1				
CH7	-0.03	-0.01	-0.03	0.04	-0.08	0.06	1			
CH8	0.50	-0.54	**0.61***	-0.51	-0.51	-0.17	-0.42	1		
CH9	-0.17	0.06	0.00	0.45	0.26	0.18	-0.57	0.06	1	
CH10	-0.12	0.37	-0.48	0.29	0.08	0.11	-0.28	0.44	-0.02	1

CH1: Post-thaw motility; CH2: Live spermatozoa; CH3: Dead spermatozoa; CH4: Abnormal spermatozoa; CH5: HOST; CH6: Acrosomal integriy; CH7: DNA 1st grade; CH8: DNA 2nd grade; CH9: DNA 3rd grade; CH10: DNA 4th grade; *Significant at 5% level, ** Significant at 1% level.

Table 2: Correlation coefficient between conventional, function and structural characteristics of spermatozoa in group II of four Spitz dogs.

	CH1	CH2	CH3	CH4	CH5	CH6	CH7	CH8	CH9	CH10
CH1	1									
CH2	0.09	1								
CH3	0.21	**-0.74****	1							
CH4	0.35	**-0.71****	0.32	1						
CH5	0.39	**0.89****	-0.48	**-0.82****	1					
CH6	0.35	**0.68***	**-0.63***	**-0.74****	**0.77****	1				
CH7	-0.11	-0.55	0.37	0.56	**-0.60***	0.35	1			
CH8	0.10	0.20	-0.30	0.53	-0.34	**0.58***	-0.41	1		
CH9	-0.25	0.15	0.05	-0.17	0.34	0.12	-0.45	0.27	1	
CH10	-0.08	0.23	0.38	-0.13	0.15	0.15	-0.48	0.07	-0.10	1

CH1: Post-thaw motility; CH2: Live spermatozoa; CH3: Dead spermatozoa; CH4: Abnormal spermatozoa CH5: HOST; CH6: Acrosomal integriy; CH7: DNA 1st grade; CH8: DNA 2nd grade; CH9: DNA 3rd grade; CH10: DNA 4th grade; *Significant at 5% level, ** Significant at 1% level.

Table 3: Correlation coefficient between conventional, function and structural characteristics of spermatozoa in group III frozen-thawed semen.

	CH1	CH2	CH3	CH4	CH5	CH6	CH7	CH8	CH9	CH10
CH1	1									
CH2	0.17	1								
CH3	0.17	**-1.00****	1							
CH4	0.09	-0.52	0.52	1						
CH5	**0.76****	**0.66***	**-0.66***	-0.20	1					
CH6	0.23	0.52	-0.52	-0.56	0.50	1				
CH7	**0.62***	0.30	-0.30	-0.52	-0.27	0.32	1			
CH8	0.52	-0.20	0.20	-0.02	0.26	-0.01	-0.27	1		
CH9	0.45	-0.11	0.11	**0.60***	0.29	-0.40	**-0.72****	-0.03	1	
CH10	0.25	0.15	-0.15	-0.22	0.27	0.22	-0.44	-0.15	0.03	1

CH1: Post-thaw motility; CH2: Live spermatozoa; CH3: Dead spermatozoa; CH4: Abnormal spermatozoa; CH5: HOST; CH6: Acrosomal integriy; CH7: DNA 1st grade; CH8: DNA 2nd grade; CH9: DNA 3rd grade; CH10: DNA 4th grade; *Significant at 5% level, **Significant at 1% level.

Table 4: Correlation coefficient between conventional, function and structural characteristics of spermatozoa in group IV frozen-thawed semen.

	CH1	CH2	CH3	CH4	CH5	CH6	CH7	CH8	CH9	CH10
CH1	1									
CH2	0.48	1								
CH3	-0.57	**-0.97****	1							
CH4	-0.48	**-0.60***	**0.67***	1						
CH5	0.48	0.53	**-0.60***	-0.42	1					
CH6	**0.63***	0.39	-0.44	**-0.66***	0.29	1				
CH7	-0.16	-0.39	0.33	0.19	-0.15	0.31	1			
CH8	-0.09	-0.11	0.08	-0.35	0.16	-0.07	-0.41	1		
CH9	**-0.62***	**-0.67***	**0.62***	-0.35	0.10	0.31	**-0.62***	0.04	1	
CH10	-0.13	-0.09	0.08	0.41	0.14	**-0.66***	-0.44	-0.17	-0.13	1

CH1: Post-thaw motility; CH2: Live spermatozoa; CH3: Dead spermatozoa; CH4: Abnormal spermatozoa; CH5: HOST; CH6: Acrosomal integrity; CH7: DNA 1st grade; CH8: DNA 2nd grade; CH9: DNA 3rd grade; CH10: DNA 4th grade; *Significant at 5% level, **Significant at 1% level.

DNA 1st grade and DNA 3rd grade; and in between grade 3 DNA intactness and live spermatozoa count (r = -0.67; *P≤0.05*). In the present study, we also negative correlation observed between progressive motility and grade 3 DNA integrity (r = -0.62; *P≤0.05*); acrosomal integrity and grade 4 DNA (r = -0.66; *P≤0.05*) HOST and dead sperm count (r = -0.60; *P≤0.05*) and in between acrosomal integrity and abnormal sperm (r = -0.66; *P≤0.05*) (Steckler *et al*., 2015). However, there was positive correlation recorded between dead sperm count and grade 3 DNA integrity (r = 0.62; *P≤0.05*); abnormal and dead sperm count (r = 0.67; *P≤0.05*) and in between acrosomal integrity and progressive motility (r = 0.63; *P≤0.05*). These results are following the findings of Kusum *et al.* (2012) and Stuart *et al*. (2019).

CONCLUSION

The study reveals the application of glycerol and ethylene glycol in different concentrations to know the cryo-protective activity on canine sperm activity at post-thawing. Canine semen was diluted with 8% and 4% glycerol, 5% ethylene glycol and 4% glycerol + 4% ethylene glycol. From the present study, it was found that ethylene glycol is a better cryoprotectant than glycerol in terms of the highest post-thaw motility (36.83±0.73%). Positive correlation was also observed between dead sperm count and DNA grade 2 intactness (group I), dead sperm count and grade 3 DNA intactness (group IV), acrosomal integrity and post-thaw motility (group IV).Therefore, 5% of ethylene glycol may serve as the best cryoprotective agent among all other designed concentration.

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