EFFECTS OF DIFFERENT COOLING RATES USING PROGRAMMABLE FREEZER ON POST THAW SURVIVAL OF DOG SPERMATOZOA*

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ABSTRACT

Semen collected by digital manipulation from eighteen healthy and sexually matured dogs of three different breeds viz., Labrador Retriever (n=8), German Shepherd (n= 6), Boxer (n = 4) aged between 2 to 6 years was diluted with Tris - fructose - citric acid extender containing 8% glycerol and 20% egg yolk at the rate of 1:2 and packed in French mini straws. They were frozen at the three different cooling rates of programmable freezing viz. Slow (R1), Medium (R2) and Fast (R3) at -1p C/min for 30min, -5p C/min for 10min and -10p C/min for 7min respectively. The mean percentages of post-thaw motility, live spermatozoa, normal spermatozoa, acrosomal integrity and plasma membrane integrity were significantly higher ($P \leq 0.05$) following slow cooling rate (R1) than other rates of programmable freezing. Hence, slow cooling rate (R1) of programmable freezing is suggested to be optimum for freezing dog semen.

Key words: Spermatozoa, Acrosomal integrity, Programmable freezing.

INTRODUCTION

In dogs, the cryopreservation of semen is becoming increasingly popular as it allows for the transportation of genetic material both within and between countries, reduces animal stress, disease risk and animal shipping cost. However, pregnancy and whelping rates are generally lower with frozen semen than with chilled extended semen (Farstad, 1984).This lower fertility was mainly due to cryoinjury that occurred during freezing which caused decline in post-thaw motility, alteration in the acrosomal and plasma membrane integrity. As most of the damage to the spermatozoa occurred during the critical temperature at which water was converted to solid state, optimizing the cooling rate in this critical zone (5p C to -2p C) would minimize the cryoinjury. With the advent of programmable freezing, it has now been possible to have control over the cooling rate leading to higher post-thaw survivability of spermatozoa. Hence, the present study was taken up to evolve suitable freezing protocol for dog semen by comparing different cooling rates of programmable freezing and to study the effect of cryopreservation on the freezability of dog semen by assessing the post-thaw viability.

MATERIALS AND METHODS

Semen from 18 healthy and sexually matured dogs of three different breeds viz., Labrador Retriever (n=8), German Shepherd (n= 6), Boxer (n = 4) aged between 2 to 6 years owned by private dog breeders in and around the city was collected by digital manipulation. The sperm

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rich second fraction was analyzed to determine its Volume, Motility, Concentration (using Haemocytometer), Live and Abnormal spermatozoa (Eosin – Nigrosin staining), Acrosomal integrity (Spermac staining) and Plasma membrane integrity (HOS test).

The semen samples were extended with Tris-fructose -citric acid extender containing 8% glycerol and 20% egg yolk and diluted in the ratio of 1:2 or 1:3 (based on the sperm concentration of the ejaculate).The extended semen samples were immediately transferred into cold handling cabinet at 5p C and equilibrated for 2 hr. The French mini straws (0.25 ml) of different colours for different cooling rates were used to load the diluted semen samples. After filling, the open end of the straws were sealed by dipping in polyvinyl alcohol powder and immersed in a water bath at 5p C to enable proper sealing and equilibration.The equilibrated straws arranged on freezing rack were subjected to programmable freezing (Programmable freezer, Kryo 10 series III, Planer, U.K) using three different cooling rates, viz. Slow (R1), Medium (R2) and Fast (R3) (Nair,1996 and Kurien,2000) as follows,

1. Slow cooling rate (R1)

Start temp	:	5°C	Hold 2 Min		
Rate	:	-1°C/minute	Temp	:	5°C to-20°C
Rate	:	-20°C/minute	Temp	:	-20°C to-50°C
Rate	:	-30°C/minute	Temp	:	-50°C to-100°C

2. Medium cooling rate (R2)

Start temp	:	5°C	Hold 2 min		
Rate	:	-5°C / minute	Temp	:	5°C to –20°C
Rate	:	-20°C/minute	Temp	:	-20°C to –50°C
Rate	:	-30°C/minute	Temp	:	-50°C to –100°C

3. Fast cooling rate (R3)

Start temp	:	5°C	Hold 2 min		
Rate	:	-10°C / minute	Temp	:	5°C to –20°C
Rate	:	-20°C/minute	Temp	:	-20°C to –50°C
Rate	:	-30°C/minute	Temp	:	-50°C to –100°C

Straws were exposed to one freezing protocol at a time. After freezing, the straws were immediately transferred into goblets and stored at -196° C in LN2 storage container. After 24 hrs of storage, the semen straws were thawed in a water bath at 37° C for one min and post thaw semen evaluation for motility, live spermatozoa, abnormal spermatozoa, acrosomal integrity and plasma membrane integrity were done as per the procedure followed for fresh semen samples. The data were statistically analyzed using Completely Randomized Design as per Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The physical, morphological and functional characteristics of fresh and frozen thawed semen are presented in table. The post-thaw values of motility, live spermatozoa, morphological normal spermatozoa, acrosomal integrity and plasma membrane integrity were found to be significantly higher($P \leq 0.05$) with slow (R1) cooling rate than with medium (R2) and fast (R3)cooling rates of programmable freezing .Significantly ($p \leq 0.05$) higher post-thaw motility found with slow cooling rates than with medium and fast cooling rate of programmable freezing. was in agreement with Smith and Graham (1984) and Rota *et al.* (2005). Hay *et al.* (1997) also reported that intracellular ice crystal formation during rapid freezing had a lethal effect on the motility of sperms. Canconnon and Battista (1989) suggested that 30 to 50 % sperm motility in frozen semen was considered acceptable and motility more than 50% was ideal for AI with canine frozen semen.

The mean post-thaw live spermatozoa per cent obtained with slow cooling rate was higher than the value reported by Kurien (2000) for slow cooling rate in programmable freezing. This might be due to the variation in the equilibration period and thawing rates.The mean percentage of post-thaw abnormal spermatozoa in slow cooling rate was lower than the medium and fast cooling rates of programmable freezing. Mortan and Bruce (1989) reported a higher incidence of sperm abnormalities with a reduction in post thaw motility. But the limit at which fertility shows a significant reduction is unknown (Oettle, 1993).

The mean percentage of post-thaw acrosomal integrity for slow cooling rate in the present study was higher than with medium and fast cooling rates of programmable freezing, but lower than the value reported by Nair (1996), Kurien (2000) and Ponglowhapan *et al.* (2004). The lower values obtained could be due to the highly precise Spermac staining technique used to evaluate the acrosomal integrity of frozen thawed spermatozoa. Oettle (1986) and Hey *et al.* (1997) reported that the Spermac stain provided a better resolution and accurate assessment of the membrane changes without being influenced by the type of extender used. Hence its use has proved to be advantageous in demonstrating changes in the acrosome which may occur during the process of dilution, cooling, freezing and thawing of dog semen.

The integrity of the sperm plasma membrane is also an important parameter in the assessment of sperm viability, since it is essential for general cell function (Strom et al., 1997). In the present study, the mean sperm plasma membrane integrity (HOS reactive sperms)was found to be higher in slow cooling rate than with medium and fast cooling rates of programmable freezing. This was similar to the results reported by Nair (1996) and slightly higher than the values recorded by Kurien (2000). Such response of canine spermatozoa to the HOS test was similar to that of bovine spermatozoa (Drevius, 1972).

The results therefore suggest that the slow cooling rate of programmable freezing was found to be optimum for freezing of dog semen and post thaw viability of spermatozoa was better assesed by live spermatozoa, acrosomal integrity using Spermac staining technique and plasma membrane integrity by HOS test.

TABLE: EFFECT OF CRYOPRESERVATION ON DIFFERENT CHARACTERISTICS OF DOG SEMEN (MEAN ± SE)

Parameters	Fresh / Pre Freeze	Post-thaw following Programmable freezing		
		Slow(R1)	Medium(R2)	Fast(R3)
Motility (%)	75.00 ± 1.17	51.66 ± 1.22^{a}	35.00 ± 1.66^{b}	30.26 ± 1.29^{b}
Live spermatozoa (%)	81.94 ± 1.47	60.55 ± 1.79^{a}	40.38 ± 1.68^{b}	32.77 ± 1.57^{b}
Abnormal spermatozoa (%)	12.16 ± 0.83	36.83 ± 1.11^{a}	42.16 ± 0.9^{b}	48.33 ± 0.94^{c}
Acrosomal integrity (%)	76.83 ± 2.63	46.38 ± 1.46^{a}	31.50 ± 1.98^{b}	28.38 ± 1.20^{b}
Plasma membrane integrity (%)	86.16 ± 1.57	53.00 ± 1.01^{a}	37.77 ± 1.73^{b}	34.27 ± 1.56^{b}

Mean bearing different superscript within the same row differ significantly ($P \leq 0.05$)

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