Original Research

Effects of Conventional Method of Freezing on Post Thaw Characteristics of Dog Spermatozoa

A. Sabarinathan*, T. Sathiamoorthy, K. Kulasekar, M. Parthiban, S. Rangasamy and D. Gopikrishnan

Department of Veterinary Gynaecology and Obstetrics, Madras Veterinary College, Chennai-7, Tamil Nadu, INDIA

***Corresponding author:** dr.sabarinathan36@gmail.com

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Abstract

Semen samples were collected from twenty healthy and sexually mature dogs of different breeds by digital manipulation. Out of twenty dogs fifteen had sired a litter before and remaining five dogs had not been tested. The physical and morphological characteristics of fresh semen samples viz. volume, colour, consistency, pH, percentage of gross motility, spermatozoal concentration, percentage of live, abnormality, acrosomal integrity and plasma membrane integrity of sperms were evaluated. The semen samples with acceptable quality were cryopreserved with Tris egg yolk extender by conventional freezing method. The physical and morphological characteristics such as post thaw motility, live and dead, abnormality, acrosomal integrity and plasma membrane integrity of frozen semen were evaluated 24 hours after freezing. The effects of conventional method of freezing on spermatozoa were assessed.

Key words: Conventional Freezing, Dog, Spermatozoa, Tris Egg Yolk Extender

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Introduction

Successful cryopreservation of germ cell is important to preserve the genetic pool of several species. Canine semen is known for freezing damages, which has a limited concern for long term germ cell preservation (Sabarinathan *et al.*, 2016). The ultimate goal of freezing of semen samples are for long time storage for future use. There are different methods by which semen samples are cryopreserved. Cryopreservation of dog semen allows easy transportation of genetic materials between the countries and more importantly to get rid of sexually transmitting diseases. Though modern methods of freezing were

evaluated and utilized for dog semen freezing the study was aimed at conventional method to assess their freezing ability and their effects on characteristics of semen.

Materials and Methods

Semen samples were collected from dogs aged between 2- 6 years by digital manipulation technique described by Linde-Forsberg (1991) in the presence or absence of estrus bitch. The dog penis is vigorously massaged through the prepuce at the level of bulbous glandis (caudal most aspect of the prepuce) until the partial erection develops then prepuce is retracted over the bulbous glandis and firm constant pressure applied over the bulbous glandis by pressing the penis between index finger and thumb. Occurrence of pelvic thrusting and commencement of ejaculates consist of first fraction (sperm poor), second fraction (sperm rich), third fraction (prostatic fluid) were collected separately in clear, graduated, sterile semen collection cups. The pre-sperm and post-sperm fractions were discarded while, sperm rich second fraction was subjected to evaluation. Immediately after collection, the sperm rich fraction was evaluated to assess its ability to be preserved based on volume, colour, initial motility, sperm concentration, viability, abnormality, acrosome integrity and plasma membrane integrity. The volume of sperm rich fraction was directly assessed from the graduated semen collection cup. The colour was assessed directly from the collection cup immediately after collection and was classified as white to milky white. The consistency was assessed directly by holding the collection cup against a good light and was graded as thin to medium.

A drop of neat semen was kept on a clean, dry, pre-warmed glass slide, covered with a clean glass coverslip and examined under microscope to assess the percentage of progressive motile spermatozoa (Thomas *et al.*, 1993). Semen samples containing a minimum of 70 per cent of spermatozoa with normal, vigorous and forward linear motion were utilized for further processing. The concentration of spermatozoa in the sperm rich fraction was assessed by Neubauer haemocytometer. Sperm viability was assessed by Nigrosin-Eosin staining technique. The percentage of spermatozoa with abnormal morphology was evaluated using Nigrosin – Eosin stained smears and examined under microscope (100 X).Acrosome integrity was assessed after smeared slides were fixed in 5 per cent formaldehyde and stained with 3 per cent Giemsa stain. The hypo-osmotic swelling test was performed to assess membrane integrity (Jeyendran *et al*., 1984) by mixing 1 ml of hypo-osmotic solution (fructose – 13.5 g and sodium citrate – 7.35g dissolved in 1000 ml of distilled water with osmolality of 150 mosm/l) in a test tube with 0.1 ml of the ejaculate and incubating the mixture for 30 minutes at 37^{o}C. After incubation, smears were prepared on glass slides dried. The slides were dried and a minimum of two hundred spermatozoa was counted for typical "tail curling" (Fig. 1) under microscope (100 X).

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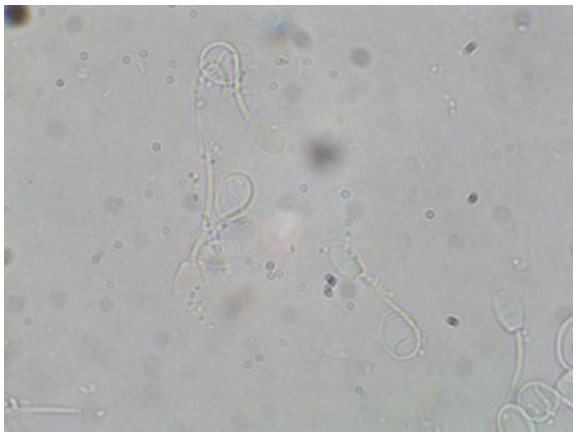

Fig.1: Hypo-osmotic swelling response – Typical tail curling

Cryopreservation of Semen

Analyzed semen samples were subjected to conventional method of freezing. Tris-egg yolk extender was used to dilute the semen samples for freezing. Extender composition utilized as described by Silva *et al.* (2003). The sperm rich fraction of semen was diluted with the extender in the ratio of 1:2. The dilution rate was adjusted based upon the sperm concentration of an ejaculate, to ensure that average sperm concentration of 200 million sperms / ml of diluted semen. The diluted semen samples were kept in a cold handling chamber at 5^{o}C. French mini straws (0.25 ml) of different colours were used and semen samples were filled in the straws by manual suction. Briefly the open ends of the straws were sealed by dipping in poly vinyl alcohol powder and placed at 5^{o}C to enable proper sealing and equilibration. Semen straws were equilibrated at 5^{o}C for a period of 2 h in cold handling chamber. After the equilibration period the straws were collected from the cold handling chamber.

Conventional freezing at slow cooling rate was done using a styrofoam box containing liquid nitrogen to a level of 6 cm and the straws were arranged on a freezing rack at 6 cm above the liquid nitrogen. Freezing was done by holding the straws horizontally and exposing in the vapour for 30 min until frozen (Silva *et al*., 2003).After freezing, the frozen straws were immediately transferred into goblets filled with liquid nitrogen and stored at –196^{o}C in liquid nitrogen storage container. After 24 h of storage the semen samples were thawed at 37^{o}C in a water bath for 30 seconds and subjected to post thaw evaluation. Post thaw semen evaluation for motility, viability, morphology, acrosomal integrity and plasma membrane integrity was done as per the standard procedures followed for fresh semen.

Result and Discussion

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The physical, morphological and functional characteristics of fresh and frozen thawed semen viz. volume, colour, consistency, pH, percentage of gross motility, spermatozoal concentration, percentage of live sperms, abnormal sperms, percentage of acrosomal integrity and plasma membrane integrity from twenty male dogs of different breeds were evaluated and the results are presented in Table 1 and 2.

Table 1: Characteristics of fresh dog semen evaluation

Characteristics of Semen (n=20)	Mean ± SE	Range	
		Minimum	Maximum
Volume (ml)	1.71 ± 0.18	0.50	4.0
Colour and consistency	White to milky white and thin to medium		
Motility (%)	78 ± 1.37	70	90
Sperm concentration(millions/ml)	332.5 ± 18.90	160	520
Live spermatozoa (%)	77.23 ± 2.53	57.47	96.78
Abnormal spermatozoa (%)	15.42 ± 1.56	6.31	30.87
Acrosomal integrity (%)	73.62 ± 1.54	58.50	83.20
Plasma membrane integrity (%)	89.59 ± 1.62	72.46	98.03

The first and third fraction of semen was clear and watery while the second fraction (sperm rich fraction) utilized for cryopreservation varied from white to milky white in colour. The initial motility of neat semen observed in this study varied from 70 to 90 per cent with a mean value of 78 per cent which was similar to the report of Prinosilova *et al*. (2006) but higher values (97.9%) were reported by Silva *et al*. (2003). Slight variation between different authors might be due to individual scoring ability on motility percentage.

The mean spermatozoal concentration in the present study was 332.5 million/ml with the range of 160 to 520 million/ml of semen. Lower values (276.3) were reported by Prinosilova *et al*. (2006) whereas higher spermatozoal concentration of 780.3 and 775.0 million/ml was reported by Rijsselaere *et al*. (2002) and Silva *et al*. (2003), respectively. Feldman and Nelson (1996) reported that the variation in sperm concentration was influenced by the breed, age, sexual activity or frequency of collection and season of the year. The mean percentage of live spermatozoa in the present study was 77.23 which were similar to the report of Kurien *et al*. (2012) with a range of 57.47 to 96.78. But the live spermatozoa obtained in the present study are lower than the values reported by Rijsselaere *et al*. (2002) and Prinosilova *et al*. (2006). The major sperm abnormalities observed in this study were bent tail, coiled tail, coiled mid piece and proximal cytoplasmic droplets. Simple coiled tails were among the most common sperm defects. Often, an increased prevalence of such defects in the ejaculates was associated with a one or more of a variety of non-genetic etiologies (Chenoweth, 2005).

Abnormal sperm morphology in the dog may be due to heat stress associated with local inflammatory conditions (Oettle and Soley, 1986) or hyperthermia, infection of the reproductive tract, decreased LH

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and testosterone secretion (Kawakami *et al*., 1998) or iatrogenic (Freshman, 1989). Pena (2004) suggested that healthy dogs transferred to a new environment or to a new owner may show a transient increase in sperm abnormalities probably due to increased endogenous corticoid secretion. Characteristics of frozen thawed semen are depicted in Table 2.

Table 2: Characteristics of frozen thawed dog semen evaluation

Characteristics of Semen (n=18)	Mean ± S.E	Range	
		Minimum	Maximum
Post thaw motility (%)	40.55 ± 3.28	10	60
Live spermatozoa (%)	47.87 ± 2.76	19.1	63.33
Abnormal spermatozoa (%)	20.58 ± 1.74	8.44	31.25
Acrosomal integrity (%)	51.54 ± 2.13	34.07	63.54
Plasma membrane integrity (%)	50.24 ± 2.80	14.29	61.51

The mean percentage of post thaw motility obtained from this study was similar to the values reported by Prinosilova *et al*. (2006) (41.5%) and Sathiamoorthy (2007) (42.14%). But, Bencharif *et al*. (2010) (30%) and Kurien *et al*. (2012) reported lower values (35.25%) in Tris extender. However, Umamageswari *et al*. (2012) recorded higher post thaw motility in Tris extender following slow freezing protocol. The mean percentage of live spermatozoa in the semen cryopreserved with Tris egg yolk extender was higher than the report of Kurein *et al*. (2012). However, the higher values (60.55%) were reported by Umamageswari *et al*. (2012). The mean percentage of abnormal spermatozoa obtained in this study was in accordance with value of Kurien *et al*. (2012) and lower than the report of Sathiamoorthy (2007). The mean percentage of plasma membrane integrity or tail curling observed in this study was similar to the result of Umamageswari *et al*. (2012) (53%) with slow freezing whereas lower values (49.88%) were reported by Kurien *et al*. (2012).

The variation in the characteristics of the frozen semen might be due to sensitivities of the different dog's semen to the freezing procedure (Rota *et al*., 2005). Further, Thurston *et al*. (2001) explained in their review that the semen of certain bulls froze poorly, which indicated the existence of variation between males within a species. The authors also illustrated inter-individual differences that affect post-thaw fertility using heterospermic insemination studies with cryopreserved bull spermatozoa. The results of this study showed that dog semen could be successfully frozen by conventional slow freezing method.

Conclusion

This study revealed that cryopreservation of dog semen with Tris egg yolk extender by conventional freezing method was found to be optimum for freezing of dog semen. Therefore we suggest that conventional method of freezing with Tris egg yolk extender can be successfully utilized for freezing of dog semen.

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