Effect of three different semen extenders and fast freezing rate on post–thaw viability of dog semen*

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Abstract: Semen collection was done twice in a week from 10 non-descript dogs by digital manipulation technique. The pre-sperm, sperm-rich and post-sperm fractions were collected separately in semen collection cups and the sperm-rich fraction was transferred to a water bath kept at 370°C for further processing. Other two fractions were discarded. Tris fructose-citric acid, Triladyl and Laiciphos-488 extenders were used to dilute the semen samples. The pH of all the three extenders was adjusted to 7.0 and they were kept in separate water bath maintained at 370°C before the semen collection. The sperm-rich fractions of 30 semen samples were diluted in three extenders at the rate of 1:3 by split sample technique and kept in a flask containing ice and then immediately transferred into cold handling chamber at 50°C. After filling the semen samples in the French straws, the open ends of the straws were dipped in poly vinyl alcohol powder to make the laboratory seal. Uniform equilibration period of four hours were given to all the semen samples diluted in different extenders. Semen samples extended with the three diluents were subjected to fast freezing using a programmable freezer. After freezing, the frozen straws were collected by a gloved hand into the labelled goblets filled with liquid nitrogen and transferred to liquid nitrogen container at -1960°C for storage. The post-thaw motility, live spermatozoa, abnormal spermatozoa, acrosome integrity, hypo-osmotic swelling response in Tris, Triladyl and Laiciphos-488 extenders after 24 hours of storage were 38.21 ± 1.11, 35.12 ± 1.15 and 30.33 ± 0.58, 41.57 ± 1.26, 40.53 ± 1.18 and 33.57 ± 0.67, 19.47 ± 0.17, 19.47 ± 0.19 and 20.00 ± 0.15, 64.77 ± 0.37, 64.30 ± 0.32 and 64.13 ± 0.34 per cent and 51.63 ± 0.77, 50.07 ± 0.92 and 45.07 ± 0.54 per cent, respectively. All the pre-freeze values for the five parameters studied in Tris, Triladyl and Laiciphos-488 did not differ significantly. It was concluded that Tris extender was superior to Triladyl and Laiciphos-488 in fast freezing.

Key words: Semen extenders, Fast freezing rate, Post-thaw viability, Dog

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INTRODUCTION

Over the last 10 to 15 years canine breeding has rapidly developed into a commercial business and there has been a tremendous increase in the breeding of pedigreed dogs. Today dog breeding has attained a disciplined way by registering pups with the kennel club, selection of pedigreed dogs, maintenance of highly pedigreed stud dogs and even importing dogs from abroad at higher cost. Although about 400 breeds are recognized by the breed registering authorities of many countries, some rare breeds of dog are at the verge of extinction. Under these circumstances assisted reproductive techniques such as cryopreservation of dog semen and AI may be helpful to preserve and propagate the valuable gametes of rare breeds of dogs (CITES 1998 and IUCN 1996). Further long storage of dog sperm by cryopreservation method will pave the way for increased representation of the stud dog in areas where he will never travel and even for a long time after his existence. So this study was undertaken to assess the effect of extenders in short and long term storage of dog sperm cryopreservation by fast freezing protocol. Semen samples extended with the three diluents were subjected to fast freezing using a programmable freezer. Pre-freeze and post-thaw semen evaluations after 24 hours storage for motility, live and dead sperms, abnormal sperms, acrosome and membrane integrity were done.

MATERIALS AND METHODS

Semen collection was done twice in a week from 10 non-descript dogs by keeping them to stand on a non-slippery floor, in a quiet place of the kennel. The digital manipulation technique described by Allen (1991) with minor modifications was adopted to collect semen from the dogs without a teaser bitch. The pre-sperm, sperm-rich and post-sperm fractions were collected separately in a clear, graduated semen collection cups. The pre-sperm and post-sperm fractions were discarded. The sperm-rich fraction was transferred to a water bath kept at 370C for further processing. 

Semen diluents: Tris fructose-citric acid extender, Triladyl extender (Minitub GmbH, Tiefenbach, Germany) and Laiciphos-488 extender (I.M.V International Corp. L’ Aigle Cedex, France) were used in this study, to dilute the semen samples for freezing. Tris extender was prepared as per Yubi et al. (1987). The pH of all the three extenders was adjusted to 7.0 and they were kept in separate water bath maintained at 370C before the semen collection.

Dilution of semen: The sperm-rich fractions of 30 semen samples were diluted in three extenders at the rate of 1:3 by split sample technique (Kadirvel, 1998). The dilution rate ensured average sperm concentration of 100 million/ml of diluted semen (Yubi et al.,1987). The diluted semen samples were transferred to a flask containing ice.

Semen packaging: The diluted semen samples kept in a flask containing ice, were immediately transferred into cold handling chamber at 50C. French mini straws (0.25 ml) of different colours (one colour for each extenders) were used to pack the diluted semen samples. After filling the semen samples in the straws, the open ends of the straws were dipped in
polyvinyl alcohol powder to make the laboratory seal. Then they were immersed in a water bath at 5°C to enable proper sealing and equilibration.

**Equilibration period:** Uniform equilibration period of four hours were given to all the extenders (Nair et al., 1999).

**Drying of straws:** At the end of equilibration period, straws were collected from the cold water bath and dried by the pre-cooled towel. The dried straws were arranged in freezing rack at 50°C.

**Pre-freeze semen evaluation:** At the end of the equilibration period pre-freeze semen evaluation was carried out to assess motility, live and dead sperms, abnormal sperms, acrosome and membrane integrity and as per the standard procedures (Kadirvel, 1998).

**Freezing rate:** Semen samples extended with the three diluents were subjected to fast freezing using a programmable freezer (M/S Planner product Ltd., UK.). Fast freezing was done at the reduction rate of 30°C min⁻¹ from 50°C to -150°C, 100°C min⁻¹ from -150°C to -400°C and 500°C min⁻¹ from 400°C to -1000°C (Rota, 1998). After freezing, the frozen straws were collected by a gloved hand into the labelled goblets filled with liquid nitrogen and transferred to liquid nitrogen container at -1960°C for storage.

**Post-thaw evaluation after 24 hours:** Post-thaw semen evaluation for motility, live and dead sperms, abnormal sperms, acrosome and membrane integrity as per the standard procedures (Kadirvel, 1998).

**Statistical analysis:** The statistical analysis was done to test the significant difference among the extenders in all the parameters in short time and long time storage. The tests of significance and analysis of variance were carried out as per Snedecor and Cochran (1989).

**RESULTS AND DISCUSSION**

The cryoprotective efficacy of three extenders viz., Tris, Triladyl and Laiciphos-488 during fast freezing were studied in 30 dog ejaculates. All the pre-freeze values for the five parameters studied in Tris, Triladyl and Laiciphos-488 did not differ significantly. This might be due to the uniform concentration of sperms by split ejaculate technique, uniform dilution rate, egg yolk level, equilibration period and use of same cryoprotectant in all the three extenders. All the post-thaw values obtained for the five parameters studied in each extender differed significantly (p< 0.01) from their corresponding pre-freeze values. Nair et al. (1999) stated that sub-lethal modification of the sperm membranes which could have occurred to the sperm during cryopreservation which made them reactive to environment after thawing process.

**Post-thaw motility:** In this study, the post-thaw motility after 24 hours in Tris, Triladyl and Laiciphos-488 was 38.21 ± 1.11, 35.12 ±1.15 and 30.33 ± 0.58 per cent, respectively. Dobrinski et al., (1993) recorded a low post-thaw motility in Tris extender at fast freezing. Rota (1998) recorded a high post-thaw motility of 58.9 per cent in Tris extender. The variations in post-thaw motility in various studies were attributed to difference in equilibration period, the rate of fast freezing and thawing rate.

**Post-thaw live spermatozoa:** In fast freezing, the post-thaw live spermatozoa
were 41.57 ± 1.26, 40.53 ± 1.18 and 33.57 ± 0.67 per cent in Tris, Triladyl and Laiciphos-488 extenders, respectively. Nair (1996) recorded a post-thaw live spermatozoa of 30.03 ± 0.36 per cent in Tris extender after fast freezing. Silva and Verstegen (1995) observed a high post-thaw live spermatozoa percentage of 80 in both Tris and Laiciphos extenders. The wide variation with the present study might be due to variation in the equilibration period and thawing rate followed between the two studies.

**Post-thaw abnormal spermatozoa:** In fast freezing the post-thaw abnormal spermatozoa were 19.47 ± 0.17, 19.47 ± 0.19 and 20.00 ± 0.15 per cent in Tris, Triladyl and Laiciphos-488 extenders, respectively. The predominant type of abnormal spermatozoa noticed includes detached heads and tails, presence of proximal and distal protoplasmic droplets, bent mid piece, coiled tails and bent tails. This observation was in agreement with Yubi et al. (1987) who recorded 20.5 ± 2.12 per cent post-thaw abnormality in Tris extender for Labrador dogs. However, Nair, (1996) recorded a higher post-thaw abnormality of 43.89 ± 0.55 per cent in Tris extender in fast freezing which was higher than the present study. This might be due to controlled rate of fast freezing in a programmable freezer followed in the present study.

**Post-thaw acrosome integrity:** In fast freezing the post-thaw acrosome integrity recorded in this study was 64.77 ± 0.37, 64.30 ± 0.32 and 64.13 ± 0.34 per cent in Tris, Triladyl and Laiciphos-488 extenders respectively. Similar result of 69.00 ± 16.00 per cent was obtained by Pefia et al., (1998) for post-thaw acrosome integrity for semen diluted in Tris based extender.

**Post-thaw hypo-osmotic swelling response (HOST):** Assessment of membrane integrity along with motility can be a valuable information in judging the quality of frozen semen sample. In this study the post-thaw hypo-osmotic swelling response of 51.63 ± 0.77, 50.07 ± 0.92 and 45.07 ± 0.54 in per cent were recorded in Tris, Triladyl and Laiciphos-488 extenders, respectively. Nair (1996) recorded a lower percentage of HOST response (43.52 ± 1.61 per cent) in Tris extender. This could be attributed to difference in programmed fast freezing and thawing rate adopted in the present study. Rota (1998) recorded a plasma membrane integrity of 61.10 ± 5.80 per cent in Tris extender which was higher than the present study. This may be due to the method employed in the assessment of membrane integrity.

In fast freezing study, it was observed that the post-thaw motility, live spermatozoa, hypo-osmotic swelling response in Tris and Triladyl extenders was significantly different (p < 0.01) from its corresponding values in Laiciphos-488 extender. In a similar study higher post-thaw motility for Tris extender than Lactose extender was recorded by Yubi et al. (1987) and Olar et al. (1989). This might be due to the fact that Tris and Triladyl extenders were Tris based and was resistant to cold shock (Olar et al., 1989). More over the differences in the type of sugar present in the extender available for energy and their metabolism by spermatozoa influences the quality of spermatozoa. However, there was no
significant difference among the three extenders in post-thaw values for abnormality and acrosome integrity. Controlled rate of fast freezing in a programmable freezer, uniform buffering capacity of all the extenders, uniform packaging and uniform thawing rate followed might have maintained the morphology of spermatozoa during cryopreservation. The study indicated that Tris extender was superior to Triladyl and Laiciphos-488 in fast freezing. Further, Triladyl extender was better than Laiciphos-488 in fast freezing.

References


