EFFECT OF THREE DIFFERENT EXTENDERS IN SLOW FREEZING PROTOCOL ON POST-THAW QUALITY OF DOG SEMEN*

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Abstract

Forty dog semen ejaculates were diluted in three extenders at the rate of 1:3, and subjected to slow freezing in a programmable freezer. Tris and Triladyl extenders were superior and significantly different from Laiciphos-488 (p< 0.01) on post-thaw values for motility, live sperm and membrane integrity in slow freezing protocol.

Keywords: *Dog semen, Semen extenders, Slow freezing rate, Sperm motility, Acrosome integrity, Sperm membrane integrity*

Artificial insemination in dogs has gained momentum among dog breeders at the national and international level. This turn of events necessitated a detailed investigation on dog semen to find out its suitability for cryopreservation. Although, many investigations have been carried out in bovines on the efficacy of various semen extenders, such studies are scarce in dogs. Hence, the aim of the study was to identify a suitable extender to maintain the fertilizing capacity of the post-thawed canine spermatozoa and to evolve proper semen evaluation methods for assessing the post-thaw sperm viability in slow freezing protocol.

Materials and Methods

Semen was collected twice a week from 10 non-descript mongrel dogs. 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 clear, graduated semen collection cups. The pre-sperm and post-sperm fractions were discarded. The sperm-rich fraction was transferred to a water bath at 37^{0}C for further processing.

Tris fructose-citric acid extender, Triladyl extender (Minitub GmbH, Tiefenbach, Germany) and Laiciphos-488 extender (IMV International Corp. L" Aigle Cedex, France) were used to dilute the semen samples before freezing. Tris extender was prepared by adding Tris (hydroxymethyl-aminomethane), Fructose, Citric acid, Distilled water, Penicillin, Streptomycin, Egg yolk and Glycerol at the rate of 2.9g, 1.25g, 1.32g, 100ml, 1,00,000 IU, 100 mg, 20 per cent and 9 per cent W/V respectively, as per Yubi *et al.* (1987). Triladyl extender was prepared with 1 part of Triladyl liquid, 1 part of egg yolk and 3 parts distilled water. Laiciphos - 488 had diluent A

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and B. Diluent A consisted of 2.5 g Laiciphos-488 powder and 20 ml 40^{0}C distilled water. Diluent B comprised of 2.5 ml egg yolk and 5 ml distilled water at 50°C**.** Diluent A and B were mixed carefully to get a final volume of 27.5 ml and glycerol was added at the rate of 9 per cent W/V. The pH of all the three extenders was adjusted to 7.0 and were kept in separate water bath maintained at 37^{0}C before semen collection.

The sperm-rich fractions of 40 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. The diluted semen samples were transferred to a flask containing ice and were immediately transferred into cold handling chamber at 5^{0}C. French mini straws (0.25ml) of different colours (one colour for each extender) 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 Poly vinyl alcohol powder to make the laboratory seal. Then they were immersed in a water bath at 5°C to enable proper sealing and equilibration. Uniform equilibration period of four hours was given to all the extenders (Nair *et al*., 1999). 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 5^{0}C. At the end of the equilibration period pre-freeze semen evaluation was carried out for assessing motility, live and dead sperms, abnormal sperms, acrosome and membrane integrity.

Semen samples extended with the three diluents were frozen using a programmable freezer (M/S Planner product Ltd., UK.) by adopting a slow (40 ejaculates) freezing protocol. It was done at the reduction rate of 2^{0}C min^{-1} from 5^{0}C to - 15^{0}C, 5^{0}C min^{-1} from -15^{0}C to - 40^{0}C and 10^{0}C min^{-1} from – 40^{0}C to 100^{0}C (Nair, 1996). After freezing, the frozen straws were collected by a gloved hand into labelled goblets filled with liquid nitrogen and transferred to liquid nitrogen container at -196^{0}C for storage. Thawing of frozen semen was carried out in a water bath at 37^{0}C for one minute (Nair *et al.,* 1999). Post-thaw semen evaluation after 24 h of frozen storage was carried out as per the procedures followed for pre-freeze diluted semen samples. The statistical analysis was done to test the significant difference among the extenders in all the parameters (Snedecor and Cochran, 1989).

Results and Discussion

Among thethree extenders used, Triladyl and Laiciphos-488 were commercial extenders which have the advantage of easy preparation. All the pre-freeze and post- freeze values of semen characteristics are presented in the table. The pre-freeze values for the percentage of motility, live sperm, abnormal sperm, intact acrosome and hypo-osmotic swelling response studied with Tris, Triladyl and Laiciphos-488 did not differ significantly. This could be due to uniform concentration of sperms by split ejaculate technique, uniform dilution, uniform egg yolk level, uniform equilibration period and use of same cryoprotectant in all the three extenders. The post-thaw values obtained for the above five parameters studied in each extender differed significantly ($p \leq 0.01$) from its corresponding pre-freeze values. The possibility of sub-lethal modification of the sperm membranes that could occur during cryopreservation, would make the sperms reactive to environment after thawing process (Nair *et al.*,1999).

Post-thaw motility:

The post-thaw motility after 24 h of frozen storage in Tris was 35.25 $\pm$ 0.99 per cent, was in agreement with Olar *et al.* (1989) who recorded a post-thaw motility of 35 percent in Tris extender, whereas Dobrinski *et al.* (1993) recorded a low post-thaw motility of 30.30 $\pm$ 1.40 in Tris extender for slow freezing. The differences in post-thaw motility between the present study and other findings could be attributed to the difference in freezing protocol and breed variations. The post-thaw motility after 24 h in Triladyl was 32.50 $\pm$ 1.26 per cent which was in agreement with Dobrinski *et al.* (1993) who recorded a post-thaw motility of 32.2 $\pm$ 1.5 in Triladyl extender for slow freezing. The post-thaw motility after 24 h in Laiciphos- 488 was 24.00 $\pm$ 0.92 was

Table: Effect of slow freezing of dog semen in different semen extenders (Mean ± SE)

Extenders	Pre-freeze evaluation					Post – thaw evaluation (24 hours)				
	Motility (%)	Live sperm (%)	Abnormal sperm (%)	Intact Acrosome (%)	Hypo-osmotic swelling response (%)	Motility (%)	Live sperm (%)	Abnormal sperm (%)	Intact Acrosome (%)	Hypo-osmotic swelling response (%)
Tris (n=40)	75.50 ± 0.99	79.33± 1.17	9.13 ± 0.11	92.30 ± 0.23	87.40 ± 0.90	35.25^{a} ± 0.99	39.50^{a} ± 1.00	18.73 ± 0.17	64.78 ± 0.33	49.88^{a} ± 0.71
Triladyl (n=40)	75.75 ± 0.99	79.54 ± 1.23	9.18 ± 0.11	92.20 ± 0.17	87.38 ± 0.98	32.50^{a} ± 1.26	37.43^{a} ± 1.08	18.95 ± 0.15	64.43 ± 0.26	48.05^{a} ± 0.90
Laiciphos-488 (n=40)	75.75± 0.99	79.37± 1.10	9.55 ± 0.11	92.18± 0.24	87.65± 0.91	24.00^{b} ± 0.92	31.01^{b} ± 0.68	19.60 ± 0.16	62,86 ± 0.41	43.03^{b} ± 0.57

Mean values between columns having different superscript differ significantly (P< 0.01)

in agreement with Yubi *et al.* (1987) who recorded a post-thaw motility of 24.17 ± 17.0 per cent in Lactose diluent. The differences in post-thaw motility between the present study and other findings could be on account of were attributed to difference in freezing protocol.

Post-thaw live spermatozoa:

One of the main reasons for the considerable variation in conception rate using frozen semen is due to the difference in the percentage of post-thaw live spermatozoa in dog semen. In this study the post-thaw live spermatozoa were 39.50 ± 1.00, 37.43 ± 1.08 and 31.01 ± 0.68 per cent in Tris, Triladyl and Laiciphos-488 extenders, respectively. However, Nair (1996) recorded a high post-thaw live sperm percentage of 66.38 ± 1.28 in Tris extender after programmable freezing. Perusal of available literature revealed no information on percentage of post-thaw live spermatozoa in Triladyl and Laiciphos-488 extenders.

Post-thaw abnormal spermatozoa:

It has been suggested that abnormalities may reduce post-thaw semen quality and fertility. In this study the post-thaw abnormal spermatozoa were 18.73 ± 0.17, 18.95 ± 0.15 and 19.60 ± 0.16 per cent in Tris, Triladyl and Laiciphos-488 extenders, respectively. The predominant type of abnormal spermatozoa noticed included 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 Retriever dogs. However, Nair (1996) recorded a high post-thaw abnormal sperm percentage of 29.02 ±1.95 in Tris extender after programmable freezing.

Post-thaw acrosome integrity:

It has been claimed that acrosomal status of frozen thawed spermatozoa is an important indicator of its fertilizing potential. In this study post-thaw intact acrosome percentage of 64.78 ± 0.33, 64.43 ± 0.26 and 62.86 ± 0.41 in Tris, Triladyl and Laiciphos-488 extenders respectively was recorded. Ferguson *et al.* (1989) recorded a high post-thaw acrosome intact percentage of 71 in Tris extender which was in contrary to the present observation. The difference in this study was attributed to the difference in freezing methods and thawing rates. Perusal of literature revealed no information on post-thaw intact acrosome percentage in Triladyl and Laiciphos-488 extenders.

Post-thaw hypo-osmotic swelling response (HOST):

The integrity of sperm plasma membrane after freezing and thawing is an another indicator of the functional capacity of the spermatozoa. The hypo-osmotic swelling responses recorded in this study were 49.88 ± 0.71, 48.05 ± 0.90 and 43.03 ± 0.57 in Tris, Triladyl and Laiciphos-488 extenders, respectively. Nair (1996) recorded a higher hypo-osmotic swelling response of 67.6 ± 1.02 percentage.

It was observed in the present study that post-thaw motility, live spermatozoa, hypo-osmotic swelling response in Tris and Triladyl extenders of slow freezing protocol were significantly different ($p<0.01$) from its corresponding values in Laiciphos-488 extender. However, there was no significant

difference between Tris and Triladyl extenders. Similar result of a higher post-thaw motility for Tris extender than Lactose extender was recorded by Yubi *et al.* (1987) and Thomas *et al.* (1993). This might be due to the fact that Tris and Triladyl extenders were Tris based and were resistant to cold shock (Olar *et al.,* 1989). More over sugar available for energy was in the form of simple sugar (fructose). In the Laiciphos-488 extender less vigorous forward progression of spermatozoa and poor visibility of motility was observed due to die thick consistency and higher viscosity of the extender (Yubi *et al.,* 1987). The sugar available for energy was lactose (disaccharide). The lactose contained glucose and galactose which on glycolysis produced pyruvate. This compound in the absence of oxygen was converted into lactic acid (Olar *et al.,* 1989). The lactic acid produced in Laiciphos-488 was double the quantity when compared to Tris and Triladyl extenders. The increased amount of lactic acid might have reduced the pH of the medium which in turn drastically reduced the motility, live sperms and hypo-osmotic swelling response in Laiciphos-488 extender. However, there was no significant difference between the three extenders in post-thaw values for abnormality and acrosome integrity. This might be attributed to controlled rate of slow freezing in a programmable freezer, uniform buffering capacity of all the extenders, uniform packaging and uniform thawing rates which might have maintained the morphology of spermatozoa during cryopreservation. The above findings clearly indicated that Tris was superior to Triladyl and Laciphos-488 in slow freezing. The Triladyl extender was much better than Laciphos-488 in the preservation of dog semen at slow freezing rate and in maintaining the fertilizing capacity of cryopreserved sperms. It was also found that assessment of sperm abnormality, live sperm and dead sperm count, acrosomal integrity, membrane integrity along with motility gave better assessment of post-thaw sperm viability.

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