Haryana Vet. (April, 2021) **60(SI)**, 60-63

Research Article

MORPHOLOGICAL AND FUNCTIONAL PARAMETERS AND THEIR CORRELATION IN CRYOPRESERVED CANINE SEMEN

RAM RANJAN PATTI[1], N. ARUNMOZHI*, P. SRIDEVI, A. GOPINATHAN[1], B.S. PRADEEP NAG, K. VIJAYARANI[2] and K. KRISHNAKUMAR

Department of Veterinary Gynaecology and Obstetrics, [1]Department of Animal Genetics and Breeding, [2]Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai-600 007, India

Received: 06.08.2020; Accepted: 18.08.2020

ABSTRACT

The correlation between different seminal parameters is important, since based on the evaluation of one parameter, other parameters could be predicted fairly. Semen was collected through digital manipulation from six dogs of different breeds aged between 2 to 6 years and subjected to conventional method of cryopreservation. Fresh and frozen semen were assessed for motility, concentration, viability, morphological abnormality, acrosomal, plasma membrane and DNA integrity. The mean percentage of all the functional and morphological characteristics of semen viz. progressive motility, viability, abnormality, acrosomal and plasma membrane integrity was significantly higher ($P<0.05$) in fresh semen compared to the frozen thawed semen with no significant difference in DNA integrity between the fresh and frozen thawed spermatozoa. The interrelation among the functional and morphological parameters of canine frozen thawed semen in the present study showed highly significant ($P<0.01$) correlation between functional membrane integrity and motility and positive correlation with viability and abnormality.

Keywords: Canine, Correlation, Fresh semen, Frozen semen, Seminal parameters

In canines, cryopreservation of semen can be used to retain and preserve the fertility in various occasions such as infertility, for genetic improvement of important breeds to increase breeding efficiency etc. Conventional parameters have restricted scope in evaluation of semen as they help only to assess the structural integrity of the cell (Nelid *et al.*, 1999). Knowing the value of one score allows perfect prediction of the score on the second variable if two parameters correlate perfectly. The present study highlights about the morphological and functional parameters of fresh and frozen semen in canine and their inter relation.

MATERIAL AND METHODS

Semen was collected from six dogs by digital manipulation technique (Linde-Forsberg, 1991) with or without the presence of estrus bitch. Semen was initially evaluated for physical characteristics viz. volume, colour, consistency, mass activity and concentration and subsequently for morphological and functional parameters. Semen was frozen by conventional method of freezing after diluting it with TRIS-egg yolk extender. Fresh and frozen semen were further subjected to evaluate progressive motility, viability, abnormality, acrosomal integrity, functional membrane and DNA integrity. Post thaw semen evaluation was done 24 hours after cryopreservation.

Progressive motility and viability and abnormality were evaluated as per the standard procedures described by Rota *et al.* (1995) and Johnston *et al.* (2001), respectively. Acrosomal integrity of sperm cells was evaluated using Giemsa stain.

$$\text{Percentage of sperm showing intact acrosome} = \frac{\text{No. of sperms with intact acrosome}}{\text{Total no. of sperms counted}} \times 100$$

Functional Membrane integrity was assessed by Hypo-osmotic sperm swelling (HOS) test as described by Jayendran *et al.* (1984).

$$\text{Percentage of sperm showing HOS reaction} = \frac{\text{No. of curied tails sperms counted}}{\text{Total no. of sperms counted}} \times 100$$

Integrity of sperm DNA was assessed by using Acridine orange staining as described by Chohan *et al.* (2004).

$$\text{Percentage of sperm showing intact DNA} = \frac{\text{No. of sperms with intact DNA}}{\text{Total no. of sperms counted}} \times 100$$

Statistical analysis was performed using SPSS 22.0 version, Mean ± SE was derived in both the groups and student's t test was performed to know the difference between two groups for various parameters. Correlation was performed between the parameters to assess the relationship between the assessed parameters.

RESULTS AND DISCUSSION

The physical parameters such as volume, colour and consistency, gross motility and concentration of fresh semen are presented in table 1. The mean volume of sperm rich fraction of semen in the present study was 2.54±0.22 ml. The colour of sperm rich fraction in the present study varied from white to milky white. The consistency of the second fraction of semen varied from thin to medium and the mean value of initial or gross motility and spermatozoa concentration were 4±1 and 428.27±49.22 million per ml. The mean volume of sperm rich fraction of semen in the

*Corresponding author: drarunmozhivet@gmail.com

Table 1

Physical properties of sperm rich fraction of fresh canine semen

Parameters/Variable	Value	Range
Volume (ml)	2.54 ± 0.22	1.5-3.5
Colour	Greyish white to white	
Consistency	Thin to Medium	
Concentration ($\times 10^6$/ml)	428.57 ± 49.22	280-660
Mass motility (0-5)	4 ± 1	3-5

present study was in accordance with Domoslawska *et al.* (2013). However, Silva *et al.* (2003) have recorded a lower mean volume. The higher mean volume recorded in the present study might be due to the differences in the breed, age, size of the dog and frequency of semen collection. The colour of sperm rich fraction in the present study was similar to the reports of Silva *et al.* (2003). The concentration reported in the present study was in agreement with Sathiamoorthy (2007). On the contrary, lower concentration was reported by Prinosilova *et al.* (2006). Comparatively higher mean value of sperm concentration observed in the present study might be due to the use of mostly larger breeds of dogs for the semen collection, since sperm production is directly related to the testicular size, larger breeds might have larger testicular diameter and higher sperm production (Rijsselaere *et al.*, 2007).

The morphological and functional characteristics of fresh and frozen thawed canine semen are presented in table 2. The mean percentage of post thaw motility and viability obtained in the present study were 42.92 ± 2.77 and 55.00 ± 3.62, respectively. The abnormality percentage of the frozen thawed semen was 9.67 ± 0.90 in the present study. The mean percentage of sperm having DNA integrity in fresh and frozen thawed canine semen was 99.08 ± 0.24 and 98.42 ± 0.24, respectively. The mean percentage of all the functional and morphological characteristics of semen viz. progressive motility, viability, abnormality, acrosomal and plasma membrane integrity was significantly higher (P<0.05) in fresh semen compared to the frozen thawed semen, except DNA integrity which was found non-significant.

Evaluation of the morphological and functional characteristics of fresh and frozen thawed canine semen showed that the post thaw motility obtained in the present study was similar to the reports of Prinosilova *et al.* (2006), Sathiamoorthy (2007). However, Kurien *et al.* (2012) have recorded lower values. Wide variation in the post thaw motility between the present study and other reports might be due to the variations in the freezing protocols used and different breeds used in the study (Feldman and Nelson, 1996). Differences in post thaw motility might also be due to factors like initial motility, sperm concentration and individual variation (Pena and Linde-Forsberg, 2000). The abnormality percentage of the frozen thawed semen was much lower than that reported by Sathiamoorthy (2007). The lower percentage of abnormalities recorded in the present study might be due to the healthy dogs selected after initial screening based on semen evaluation and also because of the seasonal influence. Local inflammatory conditions associated with heat stress or hyperthermia, infection in the reproductive tract, decreased LH and testosterone secretion and change in the owner or environment might be the causes of sperm morphology in the dog.

The acrosomal integrity of frozen thawed semen in the present study was in agreement with Kurien *et al.* (2012) and higher than Sathiamoorthy (2007). Higher percentage of acrosomal integrity in frozen thawed semen might be due to higher percentage of spermatozoa with intact acrosomes in fresh semen. The mean percentage of spermatozoa showing plasma membrane integrity in frozen semen was in agreement with the values reported by

Table 2

Morphological and functional parameters of fresh and frozen (Mean ± SE)

Groups	Motility (%)	Viability (%)	Morphological Abnormality			Acrosome Integrity (%)	Plasma membrane integrity (%)	DNA Integrity (%)
			Major (%)	Minor (%)	Total			
Fresh Semen	80.83 ± 2.00**	89.00 ± 0.74**	2.67 ± 0.33**	3.08 ± 0.30*	5.75 ± 0.48**	91.92 ± 0.85**	73.83 ± 1.07**	99.08 ± 0.24^{NS}
Frozen Control	42.92 ± 2.77	55.00 ± 3.62	5.00 ± 0.62	4.67 ± 0.42	9.67 ± 0.90	79.92 ± 0.60	53.67 ± 1.84	98.42 ± 0.24
t- Value	-11.08**	-9.10**	3.32**	3.06**	-9.47**	-11.54**	-9.47**	-1.98^{NS}

Values bearing *(P<0.01) and ** (P<0.05) in different columns differed significantly with frozen semen

Table 3

Correlation matrix showing coefficients of correlation among seminal parameters of frozen canine semen

	Motility	Viability	Abnormality	AI	PMI	DNAI
Motility	1	.628	.362	-.600	.969**	.242
Viability		1	-.144	-.694	.611	-.080
Abnormality			1	.129	.509	.401
AI				1	-.641	.283
PMI					1	-.126
DNAI						1

Sathiamoorthy (2007) but lower than that reported by Kurien *et al.* (2012). Difference in the mean percentage of functional membrane integrity as reported by different authors might be due to difference in breed susceptibility, age of animals, nutritional status and season along with method and frequency of semen collection (Davis, 1999).

The mean percentage of all the functional and morphological characteristics of semen viz. progressive motility, viability, abnormality, acrosomal and plasma membrane integrity was significantly higher ($P<0.05$) in fresh semen when compared to the frozen thawed semen. The significant difference reported in progressive motility, viability and abnormality between fresh and frozen thawed semen could be due to the insults that the sperms undergo during cryopreservation. Cryopreservation of semen involves cooling, freezing and thawing which causes remarkable decrease in quality of semen due to osmotic stress, cold shock, intracellular ice crystal formation and excessive production of ROS (Chatterjee and Gagnon, 2001).

Freezing and thawing process leading to vast structural and functional alterations like hydration dependent phase changes and lateral phase separation of membrane components, intramembranous particle aggregation, loss of membrane permeability fusion which may be a result of membrane cracking and resealing (Watson *et al.*, 1992), could be a contributing factor for the significant difference in the functional membrane integrity. Cryopreservation procedure also disrupts the cytoplasmic membrane of the spermatozoa resulting in loss of intracellular solute. This accounted for the ability of spermatozoa to curl because the damaged membrane would be unable to support osmotic swelling (Kumi-Diaka, 1993). There was no significant difference in the percentage of intact DNA between fresh and frozen-thawed canine semen. Similar results were reported by Martin *et al.* (2004) in cattle. No significant difference in DNA integrity between the fresh and frozen thawed spermatozoa in the present study might be because the nuclear packing in the spermatozoa is more stable and DNA organization is highly condensed and unique (Yanagimachi, 1994).

The interrelation among the functional and morphological parameters of canine frozen thawed semen in the present study showed highly significant ($P<0.01$) correlation between functional membrane integrity and motility and positive correlation with viability and abnormality (Table 3). Non-significant positive correlation was observed in motility with DNA integrity and abnormality. The viability was also positively correlated to motility and functional membrane integrity. Non-significant positive correlation of abnormality with motility, acrosomal integrity, functional membrane integrity and DNA integrity was observed. DNA integrity was positively correlated with motility, abnormality and acrosomal integrity at non-significant level. Negative correlation was observed between acrosomal integrity and functional membrane integrity and between viability and abnormality at non-significant level.

The correlation between different seminal parameters is important, since based on the evaluation of one parameter, a fair prediction of other parameters can be achieved. The interrelation among the functional and morphological parameters of canine frozen thawed semen in the present study showed highly significant ($P<0.01$) correlation between functional membrane integrity and motility and positive correlation with viability and abnormality (Table 3). Similar findings of highly significant correlation of functional membrane integrity with motility and viability was reported by Lodhi *et al.* (2008) in buffalo, Sharma *et al.* (2012) in bull, Bohlooli *et al.* (2012) and Rajashri *et al.* (2017) in ram. The positive correlation recorded between motility, viability and functional membrane integrity was expected, since integrity of plasma membrane is related to all these parameters (Brito *et al.*, 2003). Motility is the function of intracellular adenosine triphosphate (ATP) content. So functional membrane integrity if affected will lead to rapid leakage of intracellular ATP through the damaged plasma membrane either due to death or an isosmotic condition (Bohlooli *et al.*, 2012). Significantly higher positive correlation between motility and functional membrane integrity in the present study is thus justified.

Non-significant positive correlation was observed in motility with DNA integrity and abnormality. Also viability was positively correlated to motility and functional membrane integrity. Non-significant positive correlation of abnormality with motility, acrosomal integrity, functional membrane integrity and DNA

integrity was observed. DNA integrity was positively correlated with motility, abnormality and acrosomal integrity at non-significant level. Negative correlation was observed between acrosomal integrity and functional membrane integrity which is contrary to the results reported by Sharma *et al.* (2012) in bull and Rajashri *et al.* (2017) in ram. The negative correlation observed in the present study might be due the effect of freezing and thawing procedures since the present study was carried out in frozen semen. A negative correlation was observed between viability and abnormality indicating death of the defective or abnormal sperms, even though the correlation was non-significant statistically.

CONCLUSION

The results of the present study showed a positive correlation of functional membrane integrity with motility and viability in cryopreserved canine semen. DNA integrity had also shown a positive correlation with motility and acrosomal integrity, but at non-significant level. Further studies with larger sample size is warranted to explain the correlation pattern of the seminal parameters in canine.

ACKNOWLEDGEMENT

The authors acknowledge the necessary supports and facilities provided by the Dean, Madras Veterinary College and the Director of Clinics, Tamil Nadu Veterinary and Animal Sciences University, Chennai to carry out the present work.

REFERENCES

Bohlooli, S., Cedden, F., Bozoglu, S., Razzaghzadeh, S. and Pishjang, J. (2012). Correlation between conventional sperm assay parameters in cryopreserved ram semen. *Annals Biologic. Res.* **3**: 884 - 889.

Brito, L.F., Barth, A.D., Bilodeau-Goessel, S., Panich, P.L. and Kastelic, J.P. (2003). Comparison of methods to evaluate plasmalemma of bovine sperm and their relationship with in-vitro fertilization rate. *Theriogenology.* **60**: 1539-1551.

Chatterjee, S. and Gagnon, C. (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. *Mol. Reprod. Dev.* **59**: 451–458.

Chohan, K.R., J.T. Griffin and D.T. Carrell (2004). Evaluation of chromatin integrity in human sperm using acridine orange staining with different fixatives and after cryopreservation. *Andrology.* **36**: 321–326.

Davis, M. (1999). Equine artificial insemination. Cabi Publishing.

Domoslawska, A., Zdunczyk, S., Nizanski, W. and Janowski, T. (2013). Assessment of semen quality in infertile dogs using computer-assisted sperm analysis by the Hamilton-Thorne Semen Analyser. *Bull. Vet. Inst. Pulawy.* **57(3)**: 429-432.

Feldman, E.C. and Nelson, R.W.(1996). Clinical and diagnostic evaluation of the male reproductive tract. In: Canine and feline endocrinology and reproduction. W.B. Saunders, Philadelphia, pp. 673-690.

Jayendran, R.S., der Ven, H.H.V., Perez-pelaez, M., Crabo, B.G. and Zaneveld, L.J. (1984). Development of an assay to assess the functional integrity of human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.* **70**: 219–228.

Johnston, S.D., Kustritz, M.V.R. and Oslon, P.V.N. (2001). Semen collection, evaluation and preservation. In: canine and Feline Theriogenology. Philadelphia, W.B. Saunders, pp. 287-386

Kumi-Diaka J. (1993). Subjecting canine semen to the hypo-osmotic test. *Theriogenology.* **39**: 1279–1289.

Kurien, M. O., Katheresan, D., Selvaraju, M. and Pattabiraman, S.R. (2012). Effect of three different extenders in slow freezing protocol on post-thaw quality of dog semen. *J. Vet. Anim. Sci.* **43**: 11-14.

Linde- Forseberg, C. (1991). Achieving pregnancy using frozen or chilled extended semen. *Vet. Clin. North Am. Small Anim. Pract.* **21**: 467-485.

Lodhi, L.A., Zubair, M., Qureshi, Z.I., Ahmad, I. and Jamil, H. (2008). Correlation between hypoosmotic swelling test and various conventional semen evaluation parameters in fresh Nili-Ravi buffalo and Sahiwal cow bull semen. *Pak. Vet J.* **28**: 186-188.

Martin, G., Sabido, O., Durand, P. and Levy, R. (2004). Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol. Reprod.* **71(1)**: 28-37.

Neild, D., Chaves, G., Flores, M., Mora, N. Beconi, M. and Aguero, A. (1999). The hypo-osmotic swelling test in equine spermatozoa. *Theriogenology.* **51(4)**: 721-727.

Pena, A. and Forsberg, C.L. (2000). Effect of spermatozoal concentration and post-thaw dilution rate on survival of after thawing of dog spermatozoa. *Theriogenology.* **54**: 703-718.

Prinosilova, P. Vinkler, A. and Veznik, V. (2006). Morphological image of fresh and cryopreserved dog semen evaluated by the strict analysis of sperm morphology method, using sperm quality analyzer (SQA IIc) evaluation. *Acta Vet. Brno.* **75**: 393-401.

Rajashri, M., Reddy, K.R., Kumari, G.A., Kumari, A.N. and Kesharwani, S. (2017). Correlation between hypo osmotic swelling test and other seminal characteristics of Deccani ram semen. *J. Exp. Biol. Agric. Sci.* **5(2)**: 195-200.

Rijsselaere, T., Maes, D., Hoflack, G., De Kruif, A. and Van Soom, A. (2007). Effect of body weight, age and breeding history on canine sperm quality parameters measured by the Hamilton-Thorne analyser. *Reprod. Domest. Anim.* **42(2)**: 143-148.

Rota, A., Storm, B. and Linde-forsberg, C. (1995). Effects of seminal plasma and three extenders on canine semen stored at 4 °C. *Theriogenology.* **44**: 885-900.

Sathimoorthy T. (2007). Fertility in bitches fallowing intravaginal and intrauterine insemination with fresh and frozen semen. Ph.D. thesis submitted to Tamil Nadu Veterinary Animal Sciences University, Chennai, India.

Sharma, M., Singh, M., Kapoor, S. and Jasial, S. (2012). Inter-relationship between some routine semen evaluation parameters in Jersey × local hill cattle crossbred bulls. *Open Vet. J.* **2**: 26-31.

Silva, A.R., Cardoso, R.C., Uchoa, D.C. and Silva, D.M. (2003). Quality of canine semen submitted to single or fractionated glycerol addition during the freezing process. *Theriogenology.* **59**: 821-829.

Watson, P.F., Kunze, E., Cramer, P. and Hammerstedt, R.H. (1992). A comparison of critical osmolality and hydraulic conductivity and its activation energy in fowl and bull spermatozoa. *J. Androl.* **13(2)**: 131-138.

Yanagimachi, R. (1994). Fertility of mammalian spermatozoa: its development and relativity. *Zygote.* **2(4)**: 371-372.