Characterization of mongrel dog seminal plasma proteins and their correlation with semen characteristics

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Abstract The objective of this study was to characterize mongrel dog seminal plasma proteins with SDS-PAGE and to determine the correlation between different proteins and semen characteristics. Ejaculate volume, sperm motility, total sperm count, live sperm percent, percentage abnormalities and membrane integrity (hypo osmotic swelling test) were assessed in 5 ejaculates of seven dogs. For each dog, seminal plasma was also pooled from five ejaculates and proteins were separated by SDS-PAGE using polyacrylamide concentration of 13% and 15% in separating gels. There was considerable variation in the semen characteristics among the ejaculates of different dogs. Total protein content of seminal plasma varied from 12.06 to 21.3 mg/ml in different dogs. The number of protein bands ranged from 10-12 in different dogs. The proteins with mol wt of 42, 33, 29, 24 and 14.0 kDa were major proteins in seminal plasma of mongrel dog and ranged from 61.3% to 74.3% in different dogs. There were differences in live sperm percent (CD 5% =21.7) and HOS positive spermatozoa (CD 5% =21.01) among different dogs. There was strong correlation between volume of semen and total sperm concentration (r= +0.87), whereas the correlation between motility vs HOST (r=+0.58) and motility vs live percent (r=+0.60) was moderate. A positive correlation was observed between concentrations of 82, 70, 24, 14 kDa proteins vs percent motility, live sperm percent and percent HOS positive spermatozoa. Our study confirmed the previous conclusion that HOS-test could also be included in routine evaluation of semen. The positive correlation of 14, 24, 70 and 82 kDa proteins with semen characteristics also inferred the role of these proteins in the fertility of mongrel dog semen, which needs to be worked out further for their role in the process of fertilization.

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Introduction

Seminal plasma is a complex mixture and can affect sperm morphology, motility, acrosome reaction and fertility (Mann and Mann 1981). In recent years, several seminal plasma proteins have been identified, isolated and characterized. Literature suggests that the protein composition of seminal plasma is different among species and some seminal plasma proteins are associated with fertility in various species (Yue et al., 2009). It has been established that bovine seminal plasma proteins bind to the sperm surface and modulate sperm function (Manjunath and Therien, 2002). In stallion HSP-1 of 72 kDa is proved to be positively correlated with fertility (Calvette et al., 1995 and Brandon et al., 1999). Best quality of semen of Alpine American goat semen is attributed to the presence of proteins with 13 kDa and 45 kDa (Souza et al., 2009). It has also been reported that goat seminal plasma contains a group of proteins that are structurally related to BSP proteins detected in bull, boar and stallion (Villemure et al., 2003). An increasing number of seminal plasma

proteins, such as insulin-like growth factor-I, alpha2-macroglobulin and the enkephalin-degrading enzymes, have been shown to be associated with sperm motility (Glander et al., 1996; Raszusti et al., 2004 and Wennemuth et al., 1997). Prostate secretion / seminal plasma proteins have been identified and characterized in different breeds of dogs (Dubiel 1974 and Bruschi et al. 1979; Stubbs and Resnick 1978; de Souza et al., 2002 and de Souza et al., 2007). deSouza et al., 2007 also found significant correlation of two proteins (67 kDa & 58.6 kDa) with semen characteristics.

Semen analysis is a valuable diagnostic tool to assess the fertility status of the male. However, the prediction of potential fertility of a male on the basis of a single assay is not reliable. Each sperm cell consists of multiple sub cellular compartments with different functions, all of which must be intact for successful fertilization (Amann and Graham, 1993). In recent years, more attention has been given to evaluating sperm membrane integrity as it is of fundamental importance in the fertilization process. Jeyendran *et al*. (1984) developed a hypo-osmotic swelling test (HOS) to evaluate sperm membrane function of human spermatozoa. Since its development, HOS test has been used for evaluation of sperm membrane integrity in bovines (Rota *et al*., 2000), equine (Neild *et al*., 2000), canine (Rodriguez-Gil *et al*., 1994), porcine (Perez-Llano *et al*., 2001) and rainbow trout (Cabrita *et al.,* 1999).

Ever increasing number of dogs is a public menace. Several measures are being tried to control population of dogs. Identification of seminal plasma proteins have also considerable merit and can be potentially used to predict fertility and perhaps to increase fertility or as a contraceptive. Therefore, the present study was performed to investigate the seminal plasma protein profile and correlation, if any, between seminal plasma proteins and semen characteristics in mongrel dog.

Material and Methods

Animals

Seven healthy adult mongrel dogs weighing about 16-20 Kg and of 2-3 years age were selected for semen collection. Starting 2-4 weeks prior to semen collection, the dogs were housed in concrete floored kennels with access to outside runs and fed commercial dog feed (nutripet). The water was available at libitum. Deworming and vaccination for rabies and six viral diseases of all the dogs were done.

Semen collection and evaluation

The semen was collected by manual manipulation in clean graduated tube attached to a glass funnel. Five ejaculates were collected from each dog at a minimum interval of 4 days. Semen analysis was done immediately after semen collection. All the chemicals were purchased from Sigma Chemicals and Sisco Research Laboratories.

Volume was measured in a graduated tube. A drop of semen, diluted in PBS, pH 7.4 was placed on clean pre warmed glass slide, covered with a cover slip. Video recording of motile semen was done at 400 X using Olympus microscope (CH-21) and digital Camera. About 100 motile and non motile sperms in different recorded fields were counted and percentage of motile spermatozoa was calculated. Sperm concentration was evaluated with the help of haemocytometer.

A drop of semen was mixed with a drop of eosin: nigrosin, kept at 37^{o}C for 2 min, smears were prepared on clean glass slides. Air dried slides were examined at 1000 X for live (unstained) and dead (pink) spermatozoa. About 100 live and dead spermatozoa were counted in different fields and percentage of live spermatozoa was calculated.

50µl of semen was mixed with 250µl of 60mM HOS solution and incubated at 37^{o}C for one hour. A control was run in PBS, pH 7.4. A drop of semen covered with a cover slip was examined under microscope at 400 X. About 100 coiled and uncoiled spermatozoa were observed in control as well as HOS solution in different fields and percentage of HOS positive spermatozoa were calculated by using the formula:-

$$\frac{\textit{No. of coiled spermatozoa in HOS sol - No. of coiled spermatozoa in control X100}}{\textit{Total Spermatozoa}}$$

Preparation of Seminal Plasma Samples

Seminal plasma was separated by centrifuging at 3000rpm for 5 min (Remi,

CPR-24) and stored at -20^{o}C. The samples were thawed and total protein in seminal plasma of five ejaculates of each dog was determined (Lowry et al. 1951). Samples were diluted in ultra-purified water at a concentration of 75 µg/10 µl (for 15% gel) and 100 µg/10 µl (for 13% gel) of total protein and mixed with sample buffer (Tris HCl 0.125M, pH 6.8, 10% sucrose, 2% SDS, 10% 2-mercaptoethanol and 0.1% bromophenol blue) vortexed and held in boiling water bath for 2-3 minutes.

SDS-Polyacrylamide denaturing gel electrophoresis (SDS-PAGE)

One dimensional PAGE of seminal plasma was performed under denaturing conditions by the method of Laemmli et al. (1971) in a vertical system using two gel concentrations i.e. 13% and 15%. A 5% stacking gel was used in all runs. A Protein molecular weight marker (broad band, 3.0-205 kDa, Bangalore genei was also run along the samples. The electrophoresis system was connected to a power supply (Consort EB 232) with a constant current of 30 mA and maximum voltage of 200V for 2h for 13 % gel and 2.5 h for 15% gel.

Gels were fixed in fixing solution i. e. Methanol: Acetic acid: Formaldehyde: distilled water (50ml: 12ml: 50µl: 38 ml), stained with 0.1% silver nitrate. Then gels were pretreated with 0.1% sodium thiosulphate and developed with 2% sodium carbonate solution. Reaction was stopped with the fixing solution. Gel images were captured on Syngene gel doc Gene snap image acquisition software. The image analyzer corrected the densitometry of each band, discounting the optical density of background.

Statistical Analysis

Standard error, coefficient of variation, critical difference and pearson's correlation were calculated for all parameters.

Results

Total protein content of seminal plasma varied from 12.06 ± 4.78 to 21.3 ± 7.17 mg/ml in different dogs (Table 1). Average protein content in the seminal plasma was observed to be 15.28 ± 6.91. The differences in seminal plasma protein were statistically significant. CV indicated variation in the protein content of seminal plasma.

Table1. Total protein (Mean±SD) in seminal plasma and spermatozoa and their correlation with sperm characteristics.

Dog No	Seminal Plasma (mg/ml) (SPP)
1	12.92± 7.93
2	17.1± 2.89
3	12.06± 4.78
4	13.30± 1.98
5	15.74± 5.56
6	14.82± 8.22
7	21.30± 7.17
Mean of 7dogs	15.28±6.91
CD (5%)	7.95

SDS-PAGE of seminal plasma was performed on 10%, 12%, 13%, 15% and 17% gels, but proteins were best separated only on 13% and 15% gels. The number of protein bands ranged from 10-12 in different dogs (Table 2). <6.0 kDa and >33 kDa proteins were better separated on 13% gels. Whereas the proteins in the range of 6.0-29 kDa were separated more clearly on 15% gels. The variation in the mol wt and amount of different proteins was observed among the dogs. Due to variation in the mol wt and amount of proteins, the seminal plasma of all the dogs was pooled and also analyzed. SDS-PAGE of pooled seminal plasma of seven dogs indicated the presence of 107, 82, 70, 33, 29, 24, 17, 14, 10, 6, 4, 3 kDa proteins in the seminal plasma of mongrel dog. The proteins with mol wt of 42, 33, 29, 24 and 14.0 kDa were major proteins in seminal plasma of seven dogs and ranged from 61.3% to 74.3%. Images of 13% and 15% gels are shown in Fig. 1&2.

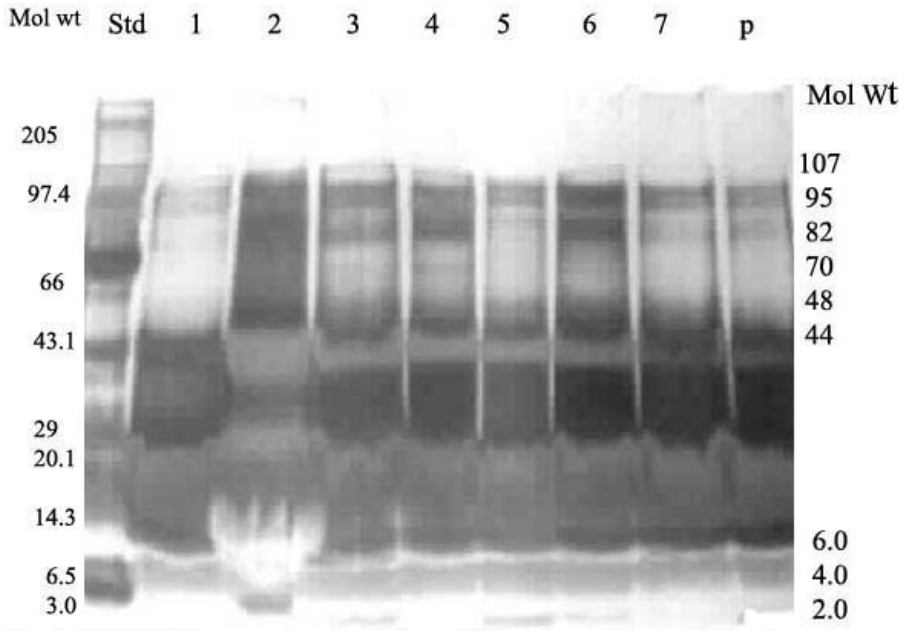

Fig.1. SDS-PAGE pattern of seminal plasma (SP) proteins of Mongrel dog on 13% gel. 1-7, dog no.; P, pooed SP of 7 dogs, Std, standard.

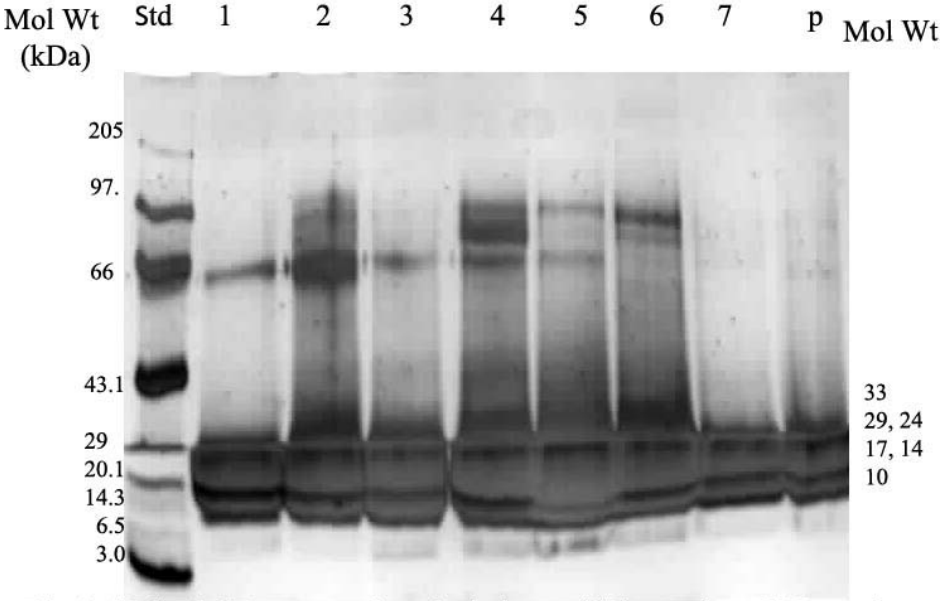

Fig. 2. SDS-PAGE pattern of seminal plasma (SP) proteins of Mongrel dog on 15% gel. 1-7, dog no.; P, pooled SP of 7 dogs; Std, standard.

Table 2. Protein profile of seminal plasma of different dogs, detected by SDS-PAGE on 13% and 15% gels.

Band No	Dog No							
	1	2	3	4	5	6	7	Pooled
1	107	107	107	107	107	107	107	107
2	95	--	--	--	95	95	--	--
3	82	82	82	--	--	82	82	82
4	--	--	--	70	--	70	70	70
5	--	--	48	48	48	--	--	--
6	42	--	--	---	--	42	--	--
7	33	33	33	33	--	33	33	33
8	29	29	29	29	29	29	29	29
9	24	24	24	24	24	24	24	24
10	17	17	17	17	17	17	17	17
11	14	14	14	14	14	14	14	14
12	10	10	10	10	10	10	--	10
13	-	6	6	6	--	--	--	6
14	4	--	4	4	4	4	4	4
15	2	2	2	2	2	2	2	2

Values are molecular weights of proteins in kDa

Semen characteristics are shown in Table 3. Volume of semen, sperm motility (%), sperm count/ml, total sperm count, live sperm percent and HOS (%) ranged from 0.83 ± 0.05 to 2.15 ± 0.04; 46 ± 2.09 to 80.07± 0.63; 121.5 x 10^6 ±7.19 to 255.3 x 10^6 ±9.65; 181.9 x10^6 ± 6.16 to 485.9 x 10^6 ±22.55; 47.21 ± 1.69 to 82.72 ± 0.97% and 29.0 ± 1.68 to 73.27 ± 0.52 respectively (Table 3). There was considerable variation in the semen characteristics among the ejaculates of different dogs. The differences in live sperm percent (CD 5% =21.7) and HOS positive spermatozoa (CD 5% = 21.01) were significant among different dogs. There was strong correlation between volume of semen and total sperm concentration (r= +0.87), whereas the correlation between motility vs HOST (r=+0.58) and motility vs live percent (r=+0.60) was moderate.

There was no significant correlation between total protein concentration and semen characteristics. There was weak correlation (r=+0.37/0.38) between 82 kDa protein and percent motile/ HOS Positive spermatozoa.

The correlation between 70 kDa protein and percent motility, live and HOS positive spermatozoa was very weak (r=+0.06), moderate (r=+0.52) and low (r=+0.26). A weak (r=+0.36) and moderate (r=+0.62/0.46) correlation was observed between 24 kDa protein and percent motile and live/ HOS positive spermatozoa. The correlation between 14 kDa protein and percent motile, live and HOS positive spermatozoa was also weak (r=+0.33, +0.25 and +0.18).

Discussion

In this study, we have focused on the assessment of quality of semen devoting attention to the semen characteristics and seminal plasma proteins. There is variation in the semen characteristics among

ejaculates of same dog as well as different dogs as reported by de Souza et al. (2007).

There was variation in seminal plasma protein concentration among individual dogs as observed by de Souza and Lopes (2002) in mixed breed dogs. The high mol wt proteins were in small amounts whereas

Table3. Semen characteristics of mongrel dog (Mean ± S.E.)

Dog No.	Volume of Semen	Motility	Sperm Conc. × 10^6/ml	Total Sperm Conc. × 10^6	Live Spermatozoa (%)	HOS +ve Spermatozoa (%)
1	2.15 ± 0.04	75.35 ± 0.69	226 ± 8.49	485.9 ± 22.55	71.06 ± 0.55	72.36 ± 1.08
2	1.43 ± 0.05	59.43 ± 1.85	182.4 ± 13.38	260.8 ± 27.29	82.72 ± 0.97	65.40 ± 0.07
3	0.83 ± 0.05	54.0 ± 2.79	318.1 ± 20.24	264.0 ± 17.12	65.14 ± 1.67	29.80 ± 1.55
4	1.66 ± 0.06	77.25 ± 0.51	213.7 ± 7.26	354.7 ± 21.51	81.23 ± 0.38	59.40 ± 1.30
5	1.30 ± 0.09	46.0 ± 2.09	141.0 ± 8.86	183.3 ± 14.89	47.21 ± 1.69	29.0 ± 1.68
6	1.37 ± 0.04	80.07 ± 0.63	132.8 ± 5.72	181.9 ± 6.16	72.72 ± 1.15	73.27 ± 0.52
7	1.53 ± 0.20	58.72 ± 2.63	121.5 ± 7.19	185.9 ± 13.49	76.05 ± 1.03	25.78 ± 1.83
	NS	NS	NS	NS	CD(5%)21.7	CD(5%)21.01

those with low mol wt were at much higher concentration in seminal plasma of seven dogs, which has also been observed by the de Souza and Lopes (2002) in mixed breed dogs.

In seminal plasma, three protein fractions were identified using electrophoresis in fertile dogs (Dubiel 1974). The dogs with reduced fertility also had three fractions of proteins. Bruschi et al. (1979) also reported three fractions of proteins separated from canine seminal plasma but did not determine association between these proteins and fertility or semen characteristics. 42, 33, 29, 24 & 14.0 kDa proteins account for 61.3-74.3% of total protein in the seminal plasma of dog. Subunits H & L of similar protein with mol. wt. of 15 and 12-14 kDa have been explained as an enzyme arginine esterase (Frenette et al., 1985) which accounts for 79% of proteins secreted by canine prostate and 30% of canine seminal plasma proteins (Calvette et al., 1995). The mol wt of this protein was estimated at 29.5 kDa by sephadex G-100 gel filtration and 25 kDa by SDS-PAGE. Canine arginine esterase is considerably homologues to a family of heparin-binding proteins of equine seminal plasma (SSps-7, Souza et al., 2006). Further 15.6 kDa protein was investigated as a heparin binding protein, because of its affinity for heparin (Moore and Hibbitt 1976). Therefore, 14 and 24, 29 kDa proteins, detected in the seminal plasma of mongrel dog during present study may be similar to that identified by Souza et al. (2006).

There was no significant correlation between total protein concentration and semen characteristics of mongrel dog as reported by de'Souza et al. (2007). In this study a

positive correlation was found between concentration of four protein bands i.e.14 kDa; 24 kDa, 70 kDa and 82 kDa and percent motile; viabile and HOS positive spermatozoa of different dogs. Similarly de Souza et al., (2007) correlated optical density of 67 & 58.6 kDa proteins with semen characteristics in dog. A linear correlation between motility of both fresh and frozen thawed spermatozoa and the relative concentration of 19.6 & 15.3 kDa proteins was observed in stallion (Amann et al. 1987). The correlation between 82, 70, 24 and 14 kDa proteins of dog spermatozoa with sperm motility during the present studies justifies the findings of Bass et al. (1983), which indicated the presence of a motility-stimulating factor in the low molecular weight fraction of seminal plasma. Killian et al., (1993) correlated the differences in non-return rates with the amounts of four proteins in seminal plasma.

An increasing number of seminal plasma proteins, such as insulin-like growth factor-I, alpha 2-macroglobulin and the enkephalin-degrading enzymes, have also been shown to be associated with sperm motility (Glander et al., 1996; Raszusti et al., 2004 and Wennemuth et al., 1997). The best quality of Alpine American goat semen was attributed to the presence of 13 and 45 kDa proteins (Souza et al., 2009). Yue et al., (2009) also indicated that ram seminal plasma contain specific proteins which are associated with fertility and semen characteristics and they also suggested the possibility of utilizing these proteins in developing a reliable and simple method to determine the ram fertility or semen quality. Therefore, any of 14, 24, 70 and 82 kDa proteins of mongrel dog showing correlation with semen characteristics in the present study may be a valuable marker of fertility.

During the present studies a moderate correlation was observed between HOS positive spermatozoa and progressive motility/ viability. Motility and other seminal characteristics are correlated to *in vitro and in vivo* fertility in dogs (Jeyendran et al., 1992, Revell & Mrode, 1994). Using the 60 mosmol fructose solution, Kuni Diaka (1993) observed a high correlation (r = 0.94) between the percentage of curled spermatozoa and sperm motility, because sperm motility/ viability is partly dependent on membrane transport (membrane integrity. Kuni Diaka (1993) also concluded that the HOS-test may be a useful addition to standard semen analysis for the identification of male dogs that may be subfertile despite a normal spermiogram. The results of Lodhi et al (2008) also showed highly significant correlation between HOS-test score and progressive motility (%), sperm viability (%) and morphologically normal spermatozoa (%) both in Nili- Ravi buffalo and Sahiwal cow bull semen. These studies inferred that HOS-test could be a valuable and practical tool to know the functional capacity of fresh Nili-Ravi buffalo and Sahiwal cow bull spermatozoa.

These observations are also in agreement with previous work on human (Jeyendran *et al*., 1984), equine (Mantovani *et al.*, 2002) and goat (Fonseca *et al*., 2005) semen samples. Jeyendran et al. (1992) found in dogs that changes provoked by the hypo-osmotic environment could be used for predicting sperm quality. Findings of Venzik et al. (2003) showed the usability of HOST for interpreting the membrane resistance of sperm and also indicated the statistical significance of comparing the results with the categories of live and dead sperm. The correlation between HOS-test and motility/viability during the present studies also confirmed the statistical significance of comparing these sperm parameters. Recently Gamel et al (2010) observed no significant correlation between semen characteristics and conception rate in spite of a significant correlation between HOS–test and sperm progressive motility, viability and abnormalities in buffalo.

Our study confirmed the previous conclusion, given by (Jeyendran *et al*., 1984), equine (Mantovani *et al.*, 2002) and goat (Fonseca *et al*., 2005), dog (Jeyendran et al., 1992) and Venzik et al. (2003) that HOS-test could also be included in routine evaluation of semen. The positive correlation of 14, 24, 70 and 82 kDa proteins with semen characteristics also inferred the role of these proteins in the fertility of mongrel dog semen, which needs to be worked out further for their role in the process of fertilization.

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References

Amann R P and Graham J K. Spermatozoal function. In: Equine Reproduction, Mcinnon, A. O., Voss, J. L. (eds.), Lea and Febiger, London, UK, 1993; 715-745.

Amann R P, Cristanelli M J, Squires M L. Proteins in stallion seminal plasma. Equine Reproduction 1V. J Reprodn Fert (Suppl) 1987; 35: 113-120.

Bass J W, Molan P C, Shannon P. Factors in seminal plasma of bulls that affect the viability and motility of spermatozoa. J Reprod Fert 1983; 68: 275-280.

Brandon, C I, Heusner G L, Caudle A B and Fayrer-Hosken R A. Two dimensional polyacrylamide gel electrophoresis of equine seminalmplasma proteins and their correlation with fertility. Theriogenology 1999; 52: 863-873.

Bruschi J H, Mendes M C, Viana E S, Abreu J J, Megale F. Teores de acido citric, fructose, protein total e seu fracionamento eletroforetico no semen do cao pastor alemao normal. *Arqu Esc da veter da UFMG* 1979; 31: 13-17.

Cabrita E R, Alvarez E, Anel and Herraez M P. The hypo-osmotic swelling test performed with coulter counter: a method to assay functional integrity of sperm membrane in rainbow trout. Anim Reprod Sci 1999; 55: 279-287.

Calvete J, Sanz L, Reinert M, Dostalova Z, Topfer-Peterson E. Heparin-binding proteins on bull, boar, stallion and human spermatozoa. Mem Mus Nat Hist Nat *1995;* 166: 515-524.

de Souza F F, Baretto C S, Lopes M D. Characterstics of seminal plasma proteins and their correlation with canine seminal analysis. Theriogenology 2007; 68: 100-106.

de Souza F F, Lopes M D. Massa molar das proteinas do plasma seminal canino: dados preliminares. Revis Brasi de Reprod Ani 2002*;* 26: 75-77.

Dubiel A. Electrophoretic studies of dog semen plasma in both fertile and sterile dogs. Pol Arch Wetery 1974; 17: 699-706.

Fonseca J F, Torres C A A, Maffili V V, Borges A M, Santos A D F, Rodrigues M T and Oliveira R F M. The hypoosmotic swelling test in fresh goat spermatozoa. Anim. Repod 2005; 2: 139-144.

Frenette G, Dube J Y, Marcotte J R. Arginine esterase from isolated dog prostate secretory granules is fully active enzymatically. Can J Physiol and Pharmacol 1985; 63: 1603-1607.

Gamal A, El-Sisy Reda I, El-Sheshtawy, Alaa A Mohamed and Walid S. El-Nattat. Correlations between semen parameters and conception rate in buffaloes. Global Veterinaria 2010; 5: 15-21.

Glander H J, Kratzsch J, Weisbrich C, Birkenmeier G. Insulin-like growth factor-I and alpha 2-macroglobulin in seminal plasma correlate with semen quality. Human Reprod 1996; 11: 2454–2460.

Jeyendran R S, van der Ven, H H, Perez-Pelaez M, Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characterstics. J Reprod Fert *1984;* 47: 219-228.

Jeyendran R S, Ven Ded Ven HH, and Zaneveld L J D. The Hypoosmotic swelling test; an update. Arch Androl 1992; 29: 105-116.

Killian G J, Chapman D A, Rogowski, L A. Fertility associated proteins in Holstein bull seminal plasma. Biol Reprod 1993; 49: 1202-1207.

Kumi Diaka, J. Subjecting canine semen to the hypo-osmotic test. Theriogenology 1993; 39: 1279-1289.

Laemmli U K. Cleavage of structural proteins during the assembly of bacteriphage $T_{4.}$ *Nature* 1970; 227: 680-685.

Lodhi L A, Zubair M, Qureshi Z I, Ahmad I and Jamil H. Correlation between hypo-osmotic swelling test and various conventional semen evaluation parmeters in fresh Nili Ravi buffalo and Sahiwal cow bull semen. Pakistan Vet. J 2008; 28: 186-188.

Lowry O H, Rosenbrough W G, Farr A L, Randall R J. Protein measurement with the follin phenol reagent. J Biol Chemistry; 1951; 193: 265-275.

Manjunath P and Therien I. Role of seminal plasma phospholipid- binding proteins in

sperm membrane lipid modification that occurs during capacitation. J Reprod Immunol 2002, 53: 109-111.

Mann T, Lutwak-Mann C. Male Reproductive Function and Semen General Features of the Seminal Plasma, 2nd ed Springler- Verlag. New York 1981; 28-34.

Mantovani R A, Rota, M E, Falomo, L Bailoni and Vincenti L. Comparison between glycerol and ethylene glycol for the cryopreservation of equine spermatozoa: semen quality assessment with standard analyses and with the hypoosmotic swelling test. Reprod Nutr Develop 2002; 42: 217-226.

Moore H D, Hibbitt KG. The binding of labeled basic proteins by boar spermatozoa. J Reprod Fert 1976; 46: 71-76.

Neild D G, Chaves M, Flores M H, Miragaya A, Gonzalez and Aguero A. The hypo-osmotic swelling test and its relationship to fertility in the stallion. Andrologia 2000; 32: 351-55.

Perez-Llano B, Lorenzo J L, Yenes P, Trejo A and Garcia-Casado P. A short hypo-osmotic swelling test for the prediction of boar sperm fertility. Theriogenology 2001; 56: 387-398.

Razusta J, Valdivia A, Fernandez D, Agirregoitia E, Ochoa C, *et al*. Enkephalin-degrading enzymes in normal and subfertile human semen. J Androl 2004; 25: 733–739.

Revell S G, Mrode R A. An osmotic resistance test for bovine semen. Ani Reprod Sci 1994; 36: 200-203.

Rodriguez-Gil J E, A, Monserrat and Rigau T. Effects of hypo-osmotic incubation on acrosome and tail structure of canine spermatozoa. Theriogenology 1994, 42: 815-829.

Rota A N, Penzo L, Vincenti and Mantovani R. Hypo-osmotic swelling as a screening assay for testing in vitro fertility of bovine spermatozoa. Theriogenology 2000; 53: 1415-1420.

Souza A F, de; Leitão M, da C G, Batista A M, Porto A L F, Lima Filho J L de; Guerra, M M P Proteins of goat seminal plasma related with precipitation index and semen quality. Ciência Rural 2009; 39: 1155-1161.

Souza F F, Martins M I M, Fernandes C E S, Ribolla P E M, Lopes M D. Heparin-binding proteins of canine seminal plasma. Theriogenology 2006; 66: 1606-1609.

Stubbs A J, Resnick M I. Protein electrophoresis patterns of canine prostatic fluid-effect of hormonal manipulation. Invest in Urol 1978; 16: 175-178.

Veznik Z, Suecova A, Zajicova A and Prinosilova P. Functional evaluation of dog ejaculates with priority given to the aspect of acrosome integrity. Vet Med-Czechg 2003; 8: 221-228.

Villemure M, Lazure C and Manjunath P. Isolation and characterization of gelatin binding proteins from goat seminal plasma. Reprod Biol Endocrinol 2003; 1: 39-50.

Wennemuth G, Schiemann PJ, Krause W, Gressner AM, Aumüller G. Influence of fibronectin on the motility of human spermatozoa. *Int J Androl* 1997; 20: 10–16.

Yue W, Shi L, Bai Z, Ren, Zhao Y. Sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis of ram seminal plasma proteins and their correlation with semen characteristics. Ani Reprod Sci 2009; 116: 386-391.