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Effect of Cryopreservation on Biological Markers of Sperm Function and their Correlation in Different Dog Breeds

By Pathania A, Ranjna S Cheema & Sharma A
Guru Angad Dev Veterinary and Animal Sciences University

Abstract- In the present study, we compared motility, viability, plasma membrane integrity (PMI), acrosome integrity (AI), inner mitochondrial membrane potential (IMMP), ATP and LDHC in freshly extended (FE,) and frozen-thaw (FT) semen among the dogs and breeds. Freshly extended semen in Tris-citric acid-fructose-egg yolk plasma-glycerol extenderwas also subjected to in vitro capacitation and acrosome reaction. Pearson correlation coefficient among the sperm attributes of FE and FT semen has been evaluated.Sperm attributes in FE and FT semen vary significantly ($P<0.05$) among the dogs. There was a significant ($P\leq0.05$) decline in motility, viability PMI, AI and high IMMP (HIMMP)during freezing-thawing process, in contrast, in ATP, LDHC and medium IMMP(MIMMP)decline was non-significant ($P\geq0.05$). There was a loss of 21.7-36.7%, 20.9-36.6%, 20.3-53.6%, 18.9-43.9%, 16.1-36% in motility, viability, PMI, AI and HIMMP during freezing-thawing process. A loss in ATP and LDHC content was also observed during freezing-thawing process. Incubation of washed spermatozoa of 13 dogs in canine TALP medium for 6 hrs resulted in a decline in motility, viability, MIMMP and an increase in HIMMP and percentage of the altered acrosome.

Keywords: biological markers, correlation, cryopreservation, semen, dogs.

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Effect of Cryopreservation on Biological Markers of Sperm Function and their Correlation in Different Dog Breeds

Cryopreservation of Canine Semen

Pathania A [α], Ranjna S Cheema [σ] & Sharma A [ρ]

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Keywords: biological markers, correlation, cryopreservation, semen, dogs.

Author α ρ: Department of Bioinformatics, College of Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004.

Corresponding Author σ: Principal Scientist Department of Bioinformatics, College of Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141004, Punjab, India.
e-mail: ranjna.cheema@gmail.com

I. Introduction

In mammals, the success of fertilization mainly depends on gamete fertilization potential and subsequently upon sperm and oocyte quality. Sperm's contribution to fertilization is generally estimated through the evaluation of sperm attributes. A loss of fertility potential is associated with manipulation and preservation techniques. In dogs, the success of artificial insemination depends on optimal insemination period and the use of high-quality semen, as low-quality semen can significantly decrease the effectiveness of insemination (Niżański et al. 2004). Semen quality is generally assessed by evaluating motility, sperm concentration and morphology. But, motility, concentration and morphology are insufficient to predict fertility and to detect sub- fertility in males. Sometimes sperm function tests viz, hypo-osmotic swelling test (HOST), viability, acrosome integrity (AI) and Hemi-zona assays are also evaluated. Some of these parameters are correlated with fertility though it does not predict male fertility (Petrunkina et al. 2007; Sutovsky and Lovercamp 2010; Dyck et al. 2011). Concentration, morphology, motility and the acrosome status analyzed under light microscopy are considered to be important in evaluating the fertilizing ability of spermatozoa. However, functional assays, such as the zona pellucidabinding assay, the Hemi-zona essay or the hypoosmotic swelling test, are better correlated with the artificial insemination outcome than the results of conventional semen evaluation (Petrunkina et al. 2007; Sutovsky and Lovercamp 2010).

Inability of the *in vitro* assessment methods to accurately predict spermatozoa fertility may be credited to the complexity and multifactorial nature of male fertility. Attempts had been made to escape these limits, which led to the introduction of some advanced tests. These tests included the use of fluorescent markers to assess the acrosomal status, the use of vital staining for mitochondrial activity, the use of particular fluorochromes to detect altered sperm chromatin or DNA integrity along, several molecular regulators of thermal and oxidative stress. Understanding the main factors involved in sperm fertility and how fertility

changes or are influenced by sperm manipulation e.g., cryopreservation and sperm-sorting, would allow to 1) improve the extenders, 2) accurately estimate sperm fertility and 3) predict sperm survival after processing. It is well - known and accepted that cryopreservation damages the sperm, with many of cells losing their fertility potential after freezing/thawing. Further, individual variations also exist on sperm resistance to cell damage during these procedures, justifying that some males are "better freezers" than others, even if no differences are found in fresh semen quality assessment[46,32]. Therefore, in the present study, functional sperm attributes in freshly extended (FE) and frozen-thaw (FT) semen of different dogs among the breeds were compared. At the same time, correlation among the sperm attributes of FE and FT semen have been evaluated.

II. Materials and Methods

a) Maintenance of dogs and semen collection

All the procedures were approved by the CPCSEA, New Delhi, vide F. No 25-19-2018-CPCSEA, dated 22/11/2018. Semen was collected from Labrador Retriever (6), Pug (4), Pomeranian (1), Golden Retriever (1), German shepherd (2) and Shih Tzudogs (2).Six Labrador Retriever and Pug dogs were maintained in individual pens in the university dog house. Labrador (500 g) and Pug (200 g) dogs were fed daily cooked feed supplemented with vitamins and trace minerals twice daily and water provided ad libitum. Dogs were given regular exercise of walking / running for one hour daily in the morning and evening. Deworming of dogs was done and they were vaccinated for rabies, CDV, CAV2, CPV, CPI and CAV1. Pomeranian, Golden Retriever, German shepherd and Shih Tzudogs were pet dogs within a three km of the university. Semen was collected by digital stimulation method at an interval of 3-5 days. Only sperm - rich fraction was used for evaluation and semen freezing.

b) Experimental design

Three ejaculates per dog (n=13 dogs) were evaluated and cryopreserved individually in Tris citric acid-fructose-egg yolk plasma-glycerol (TCFEYP) extender[5,19]). Freshly extended (FE) and frozen-thaw (FT) semen was evaluated for motility, viability, plasma membrane integrity (PMI), AI, inner mitochondrial membrane potential (IMMP), ATP and lactate dehydrogenase (LDHC) concentration, freshly extended semen was also evaluated for rate of induced *in vitro* capacitation/ acrosome reaction.

c) Chemicals and Reagents

All the chemicals were procured from Sigma Aldrich, Thermo scientific, Himedia and BR Biochem. All the reagents were prepared in water filtered through RO-Synergy- Millipore water purification system. Kits for LDHC and ATP assay were procured from Sigma Aldrich and My BioSource, respectively.

d) Analysis of sperm attributes in freshly ejaculated and frozen-thaw semen

i. *Motility and Viability*

Motility was noted by the wet mount method. A drop of semen on a slide, covered with a coverslip was observed microscopically using CCTV. A total of 200 motile and non-motile sperms were observed on the monitor and the percent of motile spermatozoa was calculated. For viability, a drop of semen was added to a drop of 0.5 % aqueous eosin in normal saline, mixed for 60 sec followed by adding a drop of 10 % nigrosin on a slide. Mixed for another 60 sec, and a smear was prepared, dried and observed under a binocular microscope (Olympus) at 1000x. About 200 sperms stained white (live) and pink (dead) were counted in different fields and percentage of live spermatozoa was calculated (Fig 1A).

e) Plasma membrane integrity (PMI)

HOST was performed to analyze the integrity of sperm membrane. The hypo-osmotic solution (300mOsm/L) was prepared by dissolving 735 mg sodium citrate and 1.351 g fructose in 100 ml DDW. Semen (10 µl) was mixed with 100 µl of 60 mOsmol (20 ml of 300 mOsmol and 80 ml DW) HOS solution and incubated at 37°C for 30 min. One drop of incubated semen was placed on a slide, covered with coverslip and examined under bright - field microscope at 400 x for coiled tailed spermatozoa. A total of 200 spermatozoa with coiled and uncoiled tails were observed under different fields and percentage of coiled tailed spermatozoa was calculated. Similarly, 10µl of semen was incubated in phosphate buffer saline (PBS) under similar conditions and percentage of coiled tailed spermatozoa was calculated (Fig 1B). The number of coiled tailed spermatozoa in PBS was deducted from the number in hypo-osmotic solution and the resultant figure was taken as the HOS-reactive spermatozoa.

f) Acrosome integrity(AI, Feng et al., 2007)

AI of spermatozoa was assessed using Coomassie brilliant blue stain(CBB, R-250). Fixative was prepared by dissolving 5gm of Paraformaldehyde in 80ml PBS, pH 7.4 with continuous stirring at 60°C. Added 1N NaOH dropwise till the solution becomes transparent and pH reaches to 7.4. Added 500 µl Triton X-100 and final volume was made up to 100ml with distilled water. Fixative was stored in aliquots at -20°C for further use. Coomassie brilliant blue stain was prepared by dissolving 0.25 g of CBB powder in 10% acetic acid and methanol. A smear of washed semen was prepared on a clean a glass slide and air – dried and fixed the smear in fixative for 15 mins. Washed the slide with PBS, pH 7.0, incubated the smear in CBB for 5 min and again washed with DW. Observed the air-

dried the slide under bright- field microscope (Olympus) at 1000X. About 200 spermatozoa with intact (blue) and damaged (unstained) spermatozoa were counted in different fields and the percentage of spermatozoa with intact acrosomes was calculated. Results are expressed as the percentage of spermatozoa with intact acrosomes.

g) Inner Mitochondrial membrane Potential

It was measured using a fluorescent carbocyanine dye, JC-1 (JC-1 stain kit, Sigma –Aldrich), Semen (10µl.) was mixed with 1µl of JC-1 dye and incubated at37°C for 20 min. After incubation a drop of 10µl semen was placed on glass slide and covered with coverslip. Slide was observed under a fluorescent then the semen is observed under fluorescent microscope (Olympus CX-24) at 400X. About 200 spermatozoa with high IMMP (HIMMP, red / orange), medium (MIMMP) and low (LIMMP) were counted in different fields and the percentage of spermatozoa with HIMMP and MIMMP was calculated (Fig 1C).

h) In vitro Capacitation and acrosome reaction

Basic TALP was prepared by dissolving NaCl (488 mg), KCl (35.6 mg), $CaCl_2$ (17.49 mg), Na_2CO_3 (316 mg), KH_2PO_4 (16.3 mg), Sodium pyruvate (2.75 mg), Sodium lactate (241.5 mg) in 80 ml DW. Adjusted the pH to 7.2 and the final volume was made up to 100 ml with DW. Energy TALP was prepared by dissolving bovine serum albumin (40 mg), glucose (5 mg) and heparin (10 µl of 1mg/ml stock solution) in 10 ml basic TALP just before use. Mixed gently 0.5 ml semen (>60% motility) with 0.5 ml TALP and centrifuged at 1000 rpm for 3 min. Discarded the supernatant and dissolved the loose pellet in energy TALP to get a final sperm concentration 150 x 10^6 / ml. Sperm suspension was incubated at 37°C for 6 hrs and observations were taken for motility, viability, acrosome reaction and IMMP at 2, 4 and 6 hrs as per the protocols explained above.

i) Preparation of sperm extract for ATP and LDHC concentration

Spermatozoa (500 x10^6) were washed twice with PBS, pH 7.4 at 3000 rpm for 5 min. Sperm pellet was suspended in 1.0 ml of 2 % SDS in 62.5 mM Tris-HCl buffer containing 10 µl protease inhibitors (cocktail, BWR). Sperm suspension was sonicated at 20 watts for 3 x 20 secs, centrifuged at 3000 rpm for 15 min. Pellet was discarded and the supernatant was passed through 3 kDa protein concentrators by centrifugation at 10000 rpm for 30 min. Both filtrate (containing ATP) and concentrate (containing proteins) were stored at -20°C.

j) ATP assay (ATP Colorimetric/Fluorometric Assay Kit, Biovision)

For the standard curve, diluted 10 µl of the ATP Standard with 90 µl of DW to generate 1 mMATP standard, mixed well. Added 0, 2, 4, 6, 8, 10 µl into a series of wells and adjusted volume to 50 µl with ATP Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of ATP. Added 50µl each of the standard and sample to the wells. Prepared a reaction mixture by thoroughly mixing 44 µl ATP assay buffer, 2 µl each of ATP converter, probe and developer for per well. Mix well. Add 50 µl reaction mixture to each well containing the ATP Standard and test samples. Samples were incubated at room temperature for 30 min., protected from light. Measured absorbance at OD 570 nm.ATP concentration in samples was calculated from the standard curve plotted OD vs ATP concentration of standards (Fig2 A)

k) Enzyme - linked immuno-absorbent assay for LDHC (Canine ELISA kit, G Bioscience)

Prepared all reagents, standard solutions and samples as per manufacturer's instructions. Added 50 µl standard to standard wells. Added 40 µl sample and 10 µl anti-LDHC to sample wells. A control without sample was also run. Then added 50 µl streptavidin-HRP to all wells except control. Covered the plate with a sealer and incubated for 60 min at 37°C. Removed the sealer and washed the plate with 300 µl wash buffer. Blotted the plate on absorbent paper and added 50 µl each of substrate A and B to each well. Covered the plate and incubated for 10 min at 37°C in the dark. Added 50 µl stop solution and absorbance was read at 450 nm in an ELISA reader (TECAN). LDHC concentration in samples was calculated from the standard curve plotted OD vs LDHC concentration of standards (Fig 2 B).

l) Cryopreservation of semen

Tris-citric acid-fructose buffer (TCF, Tris, 3.08 g; citric acid, 1.78 g; fructose, 1.25 g/100 ml, pH 7.2, gentamycin, 5mg) was prepared, filtered through 0.2-micron membrane filter and supplemented with 15% egg yolk plasma (EYP) and 7 % glycerol (G). TCFEYPG Extender was always prepared fresh and again filtered through 0.45-micron membrane filter. Semen of each dog exhibiting >70% motility was mixed with an extender in the ratio of 1:1 and equilibrated at 37°C for 10 min. The semen suspension was centrifuged at 1000 rpm for 3min and the loose pellet was suspended in the extender to get a final concentration of 200 x10^6 spermatozoa/ml. Extended semen was equilibrated at 37°C for 15 min. During this period, it was analysed for motility, viability, membrane/AI, IMMP, ATP, LDHC and *in vitro* capacitation/acrosome reaction. Then, filling and sealing of straws was done manually at room temperature (25 °C). Straws were kept at 4°C in cold handling cabinet for equilibration. Straws racked in the floating rack, equilibrated for 4 hrs were kept in LN2 vapours in a manual freezer for 10 min before plunging into liquid nitrogen. Semen was thawed after 24 hrs at 37 °C for 30 sec and analyzed for motility, viability, membrane/AI, IMMP, ATP and LDHC.

III. Results and Discussion

a) *Analysis of motility, viability, plasma membrane and acrosome integrity in freshly extended semen*

Values for motility, viability, PMI and AI varied from 66.7±4.4 – 83.3±1.7%, 72.9±1.6 – 89.5±1.5%, 60.6±1.8 – 87.6±1.6%, 66.5±3.2 – 90.0±1.4%, respectively among the dogs of four breeds (Table 1). All sperm attributes vary significantly ($P<0.05$) among the dogs irrespective of breed. Although number of dogs was not similar in all breeds, but still, significant ($P<0.05$) difference was observed among four breeds. Motility in normal canine semen is between 70-90% (Johnston et al. 2001; Ignee Ouada and verstegen, 2001). It has also been proposed that fertile dog should have at least 70% total sperm motility (Larson, 1980). It is also stated that good - quality canine semen should have at least 80% morphologically normal and viable spermatozoa (Johnston et al. 2001). Based on motility and viability, semen of 10 dogs out of 13 was of good - quality (Table 1).

HOST is considered as a suitable assay to test male fertility (Stanger et al. 2010; Baiee et al. 2017). PMI is essential for sperm fertilizing capacity (Rigsselaere et al., 2005). Sperm without a functionally intact membrane is defined as deteriorated and is not capable of fertilizing an ovum (Graham and Moce, 2005). Semen evaluation conducted on pooled fresh semen of 20 dogs of different breeds revealed average values of motility, viability and mitochondrial potential as 76.3%, 69% and 67.4% respectively (Abedin et al.2020). Contrary to their observation viability was higher in all the breeds, whereas PMI was higher in Labrador, Pug and lower in Pomeranian and German Shepherd breeds in the present study. However, values of motility in four breeds correspond to the observation of Abedin et al. 2020). The mean rates of progressive motility (87.2±5.0%) and viability (82.66±2.8%) observed in pooled semen samples of two German Shepherd, two Golden Retrievers and two Labrador dogs were similar to our observations on four separate breeds (Cheuqueman et al., 2012).

Earlier acrosome reaction make sperm infertile and hence, evaluation of AI in fresh as well as frozen-thaw semen before assisted reproduction procedure (Silva and Gadella, 2006) is essential. AI observed in Labrador (82.6%), Pug (75.8%) and German shepherd breeds (84.9%) separately was slightly lower than observed in pooled samples of different breeds (Veznik et al. 2003).

b) *Inner mitochondrial membrane potential, ATP and LDHC in freshly extended semen*

HIMMP, MIMMP, ATP and LDHC were in the range of 38.7±4.4-77.1±1.5%, 20.2±7.2-57.7±8.1, 6.6±1.4 -36.5±3.3 nM /10^9 spermatozoa and 5.2±0.8-30.9±1.3 μg/ 10^9 spermatozoa among the dogs of four breeds (Table 2). Mitochondrial membrane potential is another important indicator of sperm functionality. It is an indicator of sperm functionality that can be assessed using specific fluorescent markers (Volpe et al. 2009). IMMP showed marked variation (53-87%) in ejaculates of ten dogs (Volpe et al. 2009). We evaluated HIMMP and MIMMP separately in four breeds, a total of IMMP was 93.6%, 89.9%, 76.4 % and 95.6% in Labrador, Pug, Pomeranian and German Shepherd breeds, respectively. High inner mitochondrial membrane potential (80.9±17%) was also detected in pooled fresh spermatozoa of three breeds[6].

ATP content (nmol/10^8 spermatozoa) was in the range of 3.09±0.42-3.70±0.31 nmol/ 10^8 spermatozoa) in 30 ejaculates of 5 crossbreed dogs during four seasons of a year. However, ATP content measured separately in dogs of four breeds ranged from 0.66 nM-3.65 nM/ 10^9 spermatozoa (Chequema et al. 2011).

c) *Analysis of motility, viability and PMI and AI in post-thaw semen*

Values for motility, viability, PMI and AI ranged from 40.7±10.4-56.7±1.7%, 45.8±10.1-63.6±.8%, 17.6±7.3-60.9±1.6% and 32.9±11.0-68.3±1.9% in frozen thaw semen of 13 dogs irrespective of the breeds (Table 1). There was a significant ($P\leq0.05$) difference in motility, viability, PMI and AI among the dogs. Comparison of sperm attributes among the breeds also revealed a significant ($P\leq0.05$) difference though the number of dogs was not similar in four breeds. Motility, AI and PMI were 34.8±7.4% 49.7±4.7% in frozen thaw semen of two Shar-pei, one Labrador-Retriever, one Barret-Hound and one cross breed dogs cryopreserved in Tris-citric acid-fructose-egg yolk-Ethylene glycol extender (Oliviera et al., 2006). Pena et al. (1998) also observed post - thaw motility, viability and AI as 60.0±8.2%, 57.0±12.8% and 69.0±16% in pooled semen, cryopreserved in Tris-citric acid-fructose buffer-20 % egg yolk and 8% glycerol of different dog breeds. The difference in post - thaw sperm attributes in our study and previous studies may be due to the difference in extender used. Moreover, our study was focused on semen cryopreservation of individual dogs rather than pooled semen of different dog breeds.

d) *Analysis of inner mitochondrial membrane potential, ATP and LDHC in post - thaw semen*

HIMMP, MIMMP, ATP and LDHC were in the range of 17.8±8.3% – 45.0±1.6%) 30.4±54-52.2±3.3%, 2.9-32.5 nM/ 10^9 spermatozoa and 6.0±0.1-30.1±2.4 μg/10^9 spermatozoa in post – thaw semen of 13 dogs irrespective of the breed (Table 2). Difference in HIMMP, MIMMP, ATP and LD|HC were significant ($P<0.05$) among the dogs. A significant ($P<0.05$) difference was also evident among the breeds.

e) Effect of cryopreservation on sperm attributes

It has been indicated that even with optimized protocols, 40-50% of the sperm do not survive after cryopreservation due to irreversible damage[46]. Significant ($P<0.05$) difference was observed in motility, viability PMI, AI and HIMMP between FE and FT semen in all dogs, whereas in ATP, LDHC and MIMMP difference was non-significant ($P>0.05$). There was a loss of 21.7-36.7%, 20.9-36.6%, 20.3-53.6%, 18.9-43.9%, 16.1-36% in motility, viability, PMI, AI and HIMMP in post-thaw semen of 13 dogs (Table 1 & 2). Loss in PMI and AI was more in Pug breed than Labrador, Pomeranian and German Shepherd breeds. Percentage of spermatozoa that did not survive after freezing-thawing was <30% in post - thaw semen of Labrador dog breed (Cheema et al. 2020). A loss in ATP (0.6-35.2 nM/10^9 spermatozoa) and LDHC (0.3 – 16.8 μg/10^9 spermatozoa) content was also observed in post - thaw semen. A decline in HIMMP, motility and increase in MIMMP may be due to loss of ATP in the frozen thaw semen. Sperm motility is dependent on intracellular ATP content (Ford, 2006). Impairment of mitochondrial function is also associated with reduced sperm motility (Thomas et al. 1998 and Fraser et al.2002).

Recently Sicherle et al. (2020) observed the effect of cryopreservation on semen of five dogs of different breeds and concluded that total and progressive motility, PMI and IMMP suffered from the deleterious effects caused by cryopreservation.

f) In vitro capacitation and acrosome reaction in freshly extended semen

Three stages of acrosome reaction were observed i.e., swollen, vesiculated, partially sheded and completely sheded acrosome during incubation in canine TALP from zero to six hrs of incubation (Fig 3). Most of the altered spermatozoa were with swollen heads after two hrs of incubation. Spermatozoa with vesiculated, partially and completely sheded acrosome were observed only after 4 hrs of incubation. Hyperactivation (spermatozoa with progressive movement) started after 2 hrs of incubation. There was a significant percentage of cells with progressive movement at 2 - 4 hrs of incubation. Incubation of washed spermatozoa of 13 dogs in canine TALP medium for 6 hrs induced agradual average decline in motility, viability and MIMMP of Labrador, Pug, Pomeranian and German Shepherd breeds, respectively (Table 3 &4, Fig 4). Contrary to MIMMP, there was an increase in the percentage of HIMMP from 0 to 6 hrs incubation in Labrador, Pug, Pomeranian and German Shepherd breeds (Table 4, Fig5). The change in motility, viability, MIMMP and HIMMP were accompanied by an increase in the percentage of altered acrosome from 15.6/ 24.4/ 16.7/ 15.1% to 61.7/ 62.6/ 72.6/ 63.4% after 6 hrs of incubation in Labrador, Pug, Pomeranian and German shepherd breeds, respectively (Table 5, Fig6). But the percentage of average induced acrosome reaction (IAR) was 46.1 ± 2.7%, 37.3 ± 2.3%, 55.9 ± 7.2% and 48.3 ± 2.1% after six hrs of incubation (Fig 7). Significant ($P<0.05$) variation in change in motility, viability, HIMMP, MIMMP and IAR during incubation of spermatozoa at 37°C for 6 hrs was observed among the dogs and breeds. Similar to our observations, a decline in viability and increase in altered acrosome during incubation of dog spermatozoa in I-CCM medium for 4 hrs was also observed (Albracin et al. 2004). Percentage of acrosome reacted spermatozoa was higher at 6 hrs of incubation than 4 hrs. Significant increase in the percentage of acrosome reacted spermatozoa was induced by incubation with solubilized zona pellucida (Kawakami et al. 1993). They also believed that the percentage of acrosome reacted spermatozoa was higher at 7 hrs of incubation than 4 hrs. It suggested that more sperm become capacitated and could respond to induction of acrosome reaction as the incubation time increases. Since alteration in acrosome and motility pattern was observed at 2 hrs of incubation, therefore, it indicated that capacitation starts at this time in some of the spermatozoa. Increase in HIMMP and decline in MIMMP observed during the present study may be related to the hyperactivity of spermatozoa during incubation. Mitochondrial membrane potential has been described as one of the most sensitive parameters for evaluating sperm function. Its reduction indicated an imminent loss of sperm motility, fertility and survival in the female reproductive tract (Kasai et al. 2002 and Grunurald et al. 2008). The loss of potential is well known marker in somatic cells and related to an uncoupling of the electron transport chain for ATP synthesis and an increased generation of reactive oxygen species (ROS, Rajender et al. 2010). Spermatozoa themselves produce a small amount of ROS, which are essential to many physiological processes i.e., capacitation, hyperactivation and oocyte fusion. Low levels of ROS are also required for fertilization. Therefore, gradual increase in HIMMP during induction of acrosome reaction may be related to the maintenance of an adequate level of ROS.

g) Pearson correlation among the sperm attributes of freshly extended and frozen - thaw semen

Pearson's correlation coefficient among the sperm attributes of FE and FT semen is given in Tables 6. Positive and negative correlations were observed among the functional sperm attributes. Negative correlation is used to measure the amount that changes in one variable can affect an opposite change in another variable. A positive correlation is a relationship between two variables in which both variables move in the same direction. Therefore, positive correlation between motility, viability and AI/HIMMP/IAR in FE as well as FT demonstrated semen with a higher motility will have higher percentage of viable spermatozoa and

spermatozoa with intact acrosome, high membrane potential and induced *in vitro* acrosome reaction. A correlation of motility is also reported with acrosome reaction in dog (Cheuquema et al. 2011). PMI and good motility are also known to be highly correlated (Schafer Somi and Aurich, 2007). Several reports had also suggested that sperm mitochondrial function of sperm may also be a means to assess sperm motility and changes in IMM could be a good indicator of a functional status of spermatozoa (Volpe et al. 2009). A highly significant correlation between HIMM and motility is described in stallion (Love et al. 2003) and humans[25]. A negative correlation between motility/viability and MIMMP indicated that motility and viability of spermatozoa are more dependent of HIMMP. A strong correlation between PMI and HIMMP in FE and FT is supported by the finding of Volpe et al. 2009) Higher the percentage of combined HIMMP and MIMMP than motility and viability in FE and FT spermatozoa (Table 1,2), indicated that even immotile and dead spermatozoa have shown a HIMMP or MIMMP. These results are in accordance with the findings of Volpe et al. 2009). Contrary to our observations, there was no correlation between mitochondrial potential and motility in dog[27]. They used a DiCO2 probe to measure potential and suggested inhibition of ATP production would not be sufficient to reduce sperm motility.

A negative correlation between LDHC/ATP concentration and motility/viability/PMI/AI/HIMMP revealed that variation in LDHC/ATP concentration in the spermatozoa of different dogs has impacts on sperm function. Laudat et al. (1997) did not observe a correlation between seminal LDHC-4 concentration and motility. It is suggested that mature spermatozoa could oxidize lactate for energy source through LDHC-4 (Laudat et al. 1997).Energy produced by spermatozoa is necessary for motility. It may be perceived from this that although seminal LDHC-4 is not an indicator[22], but sperm LDHC4 may be a good indicator of sperm function. However, positive correlation between LDHC-4 and IAR revealed its relationship with capacitation. This observation is supported by the fact that disruption of LDHC gene results in sperm which cannot reach the capacitation site (Gavella et al. 1985). Negative correlation between ATP content and motility, viability, PMI, AI and HIMMP in FE and FT semen may be due to variation in sperm function among the dogs. Motility, viability, PMI, AI in post - thaw semen were> 45% in most of the dogs. Although, freezing-thawing process resulted in a significant ($P<0.05$) decline in sperm attributes, but decline in ATP content was non-significant ($P>0.05$). It may be due to the statement given by Calemera et al. (2010) that almost consistent level of ATP in FT semen represents the balance between biosynthesis and use of ATP. Although they thaw human semen at 40°C but thawing of dog semen was done at 37°C due to its more sensitivity for> 37°C temperature. ATP content of FE and FT spermatozoa is positively correlated with LDHC and IAR. It may be explained as only motile sperm may undergo capacitation and ATP is required to sustain the motility. A fully active glycolytic pathway is required for multiple steps in the fertilization cascade including capacitation-dependent tyrosine phosphorylation, hyperactivated motility and oocyte penetration (Travis et al. 2001, Goodson et al.2012; Odet et al., 2013; Tang et al., 2013). It is also established that hyperactive sperm (a distinct pattern of capacitated sperm) display an increase in energy demand requiring ATP ((Mujica et al., 1994). LDHC-4 is localized in the sperm principal piece, involved in sperm capacitation and facilitates the reversible conversion of pyruvate to lactate in glycolysis (Duan and Goldberg, 2003).

A positive correlation between motility x viability (0.96, $P\leq0.01$); motility x HIMMP (0.35); motility x IAR (0.045) and viability x HIMMP (0.22); viability x IAR (0.80) and HIMMP x IAR (0.080) was also detected during induced acrosome reaction in canine TALP medium. However, there was a negative correlation between motility x MIMMP (-0.287); viability x MIMMP (-0.265); HIMMP x MIMMP (-0.652, $P\leq0.05$) and MIMMP x IAR (-0.208) during induction of acrosome reaction. It indicated that rate of capacitation/ AR is associated to motility, viability and mitochondrial potential of spermatozoa. This study concluded that TCFEYPG extender was suitable for semen freezing of Labrador, Pug, German Shephard and Pomeranian dog breeds. It is suggested to perform multiple functional tests of FE/FT semen before selecting the dogs for breeding. Freezing of semen of individual dogs rather than pooled semen to achieve higher fertility rate is advised. Further, induced acrosome reaction may be an indicator of semen freezability.

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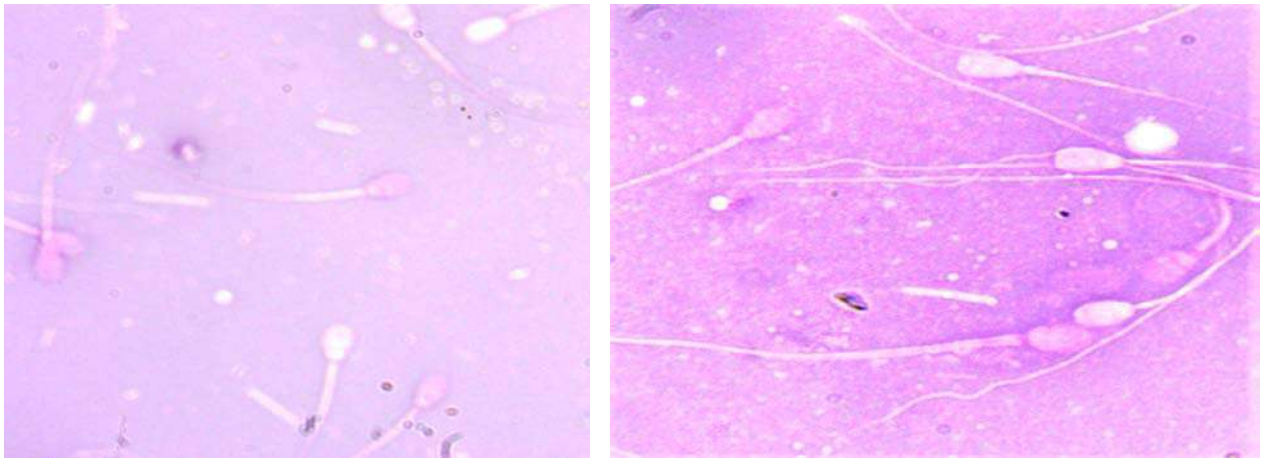
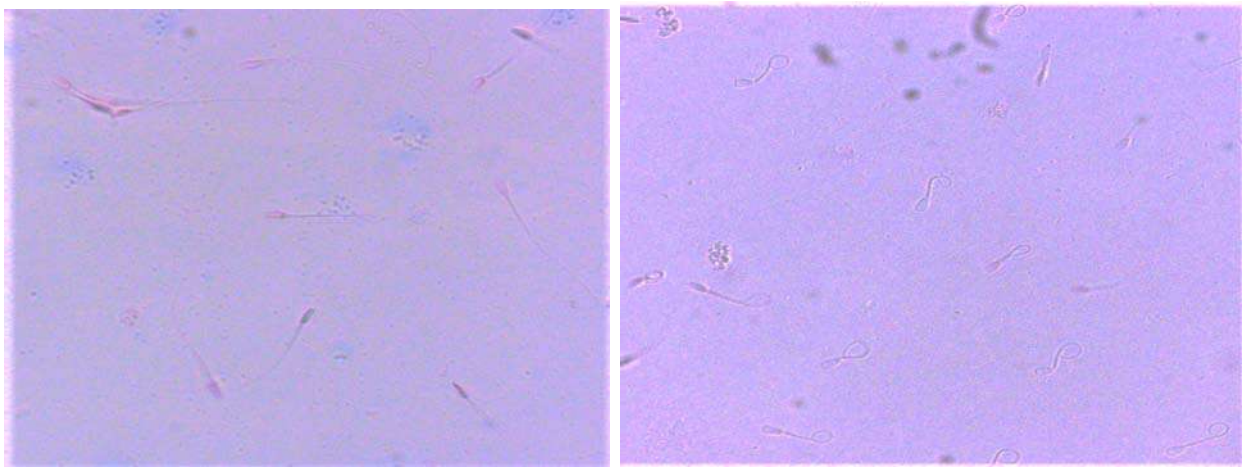
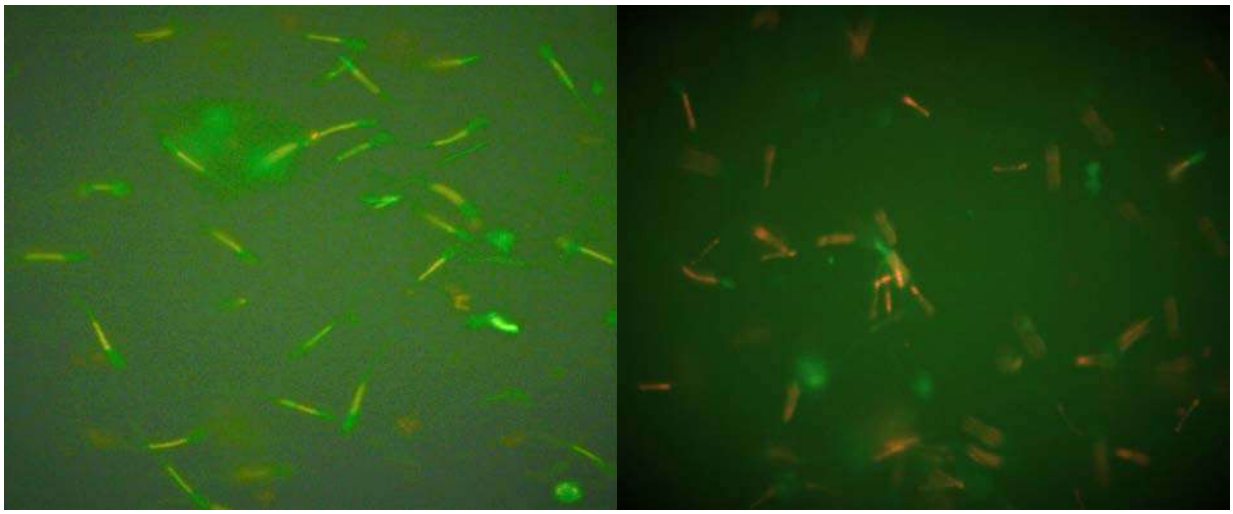


(A) Live (white) and Dead (pink) spermatozoa

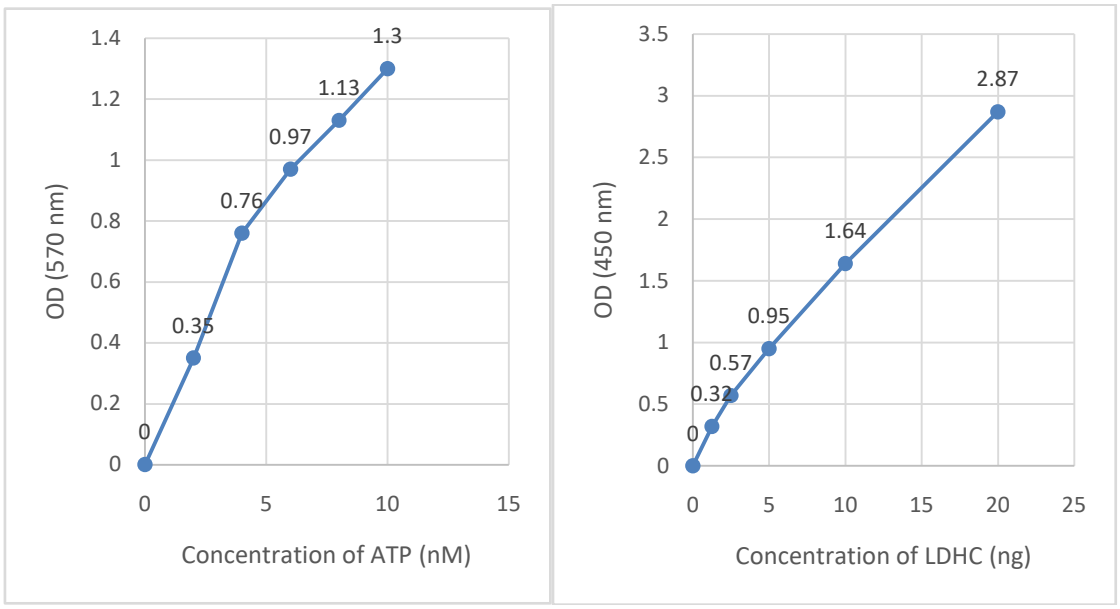


(B)Spermatozoa with straight tail (a) andcoiled tail (b)



(C) Green, yellow, orange-red fluorescence on mid piece indicate low, medium and high inner mitochondrial membrane potential.

Fig. 1: Viability: Eosin-nigrosine staining (A), plasma membrane integrity: Hypo-osmotic swelling test (B)and Inner mitochondrial potential: JC-1 (C) in canine spermatozoa.



(A) (B)

Fig. 2: Standard curve for estimation of ATP (A) and LDHC (B)

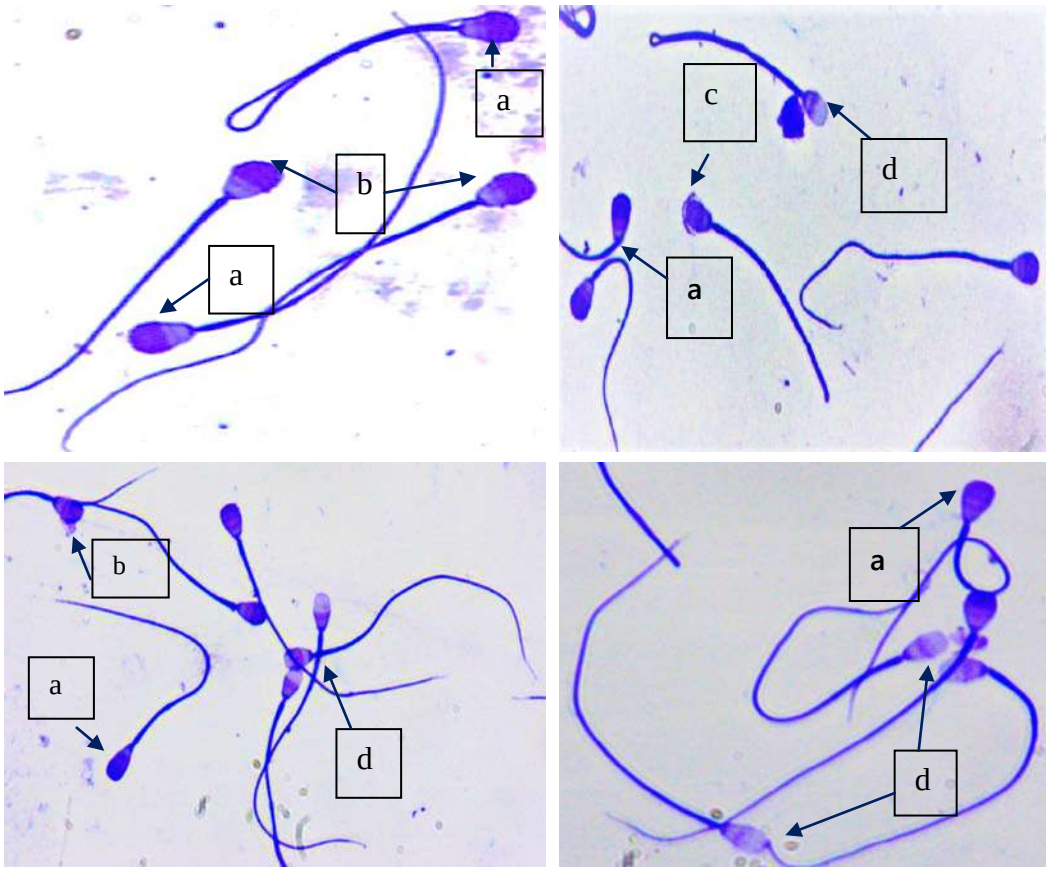


Fig. 3: Different stages of acrosome reaction in canine spermatozoa during incubation at 37°C in canine – TALP. a) Normal sperm, b) swollen and vesiculated acrosome, c) partially sheded acrosome, d) completely sheded acrosome.

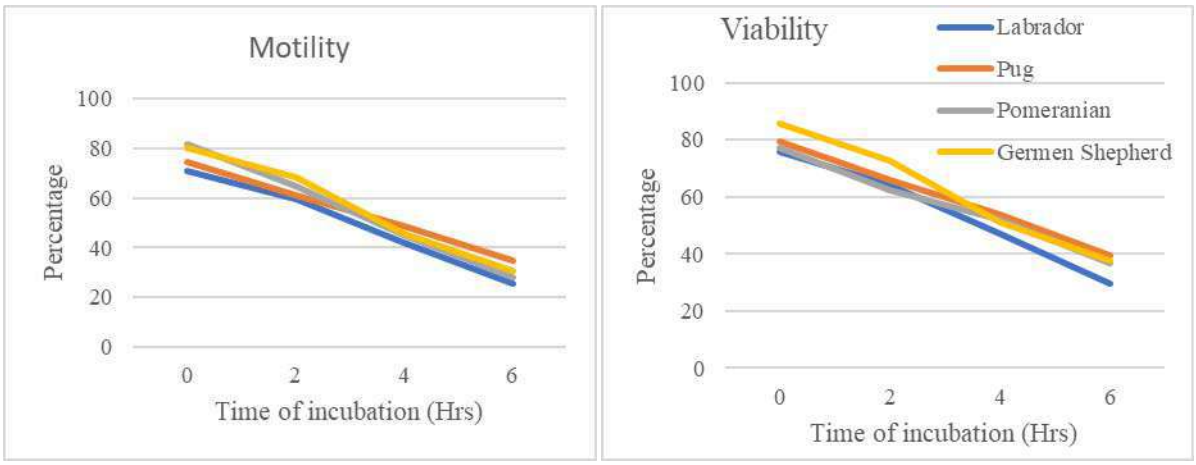


Fig. 5: Effect of incubation of spermatozoa in canine TALP medium on motility and viability.

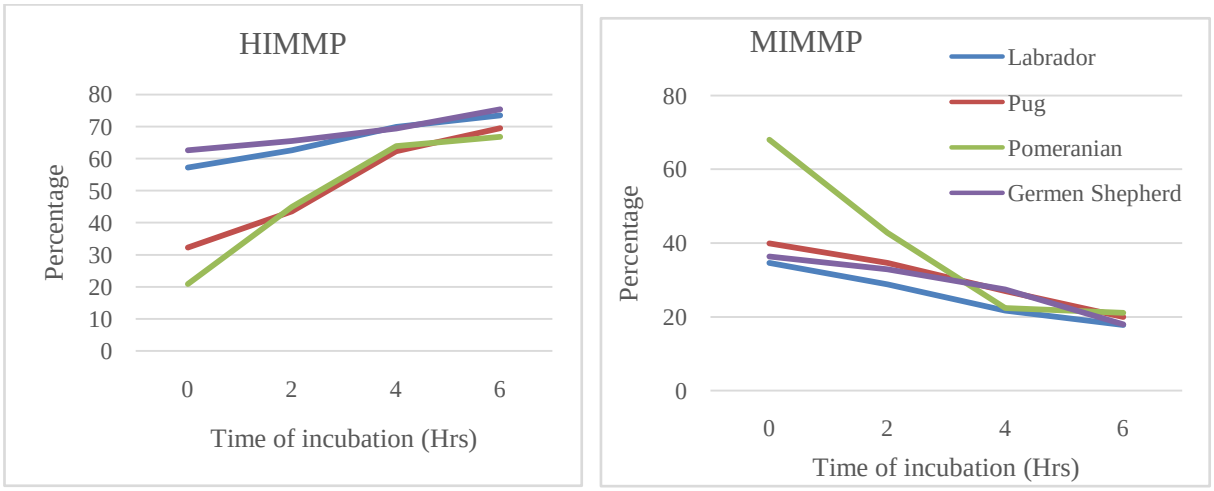


Fig. 6: Inner membrane mitochondrial membrane potential (IMMP). high IMMP (HIMMP) and medium IMMP (MIMMP).

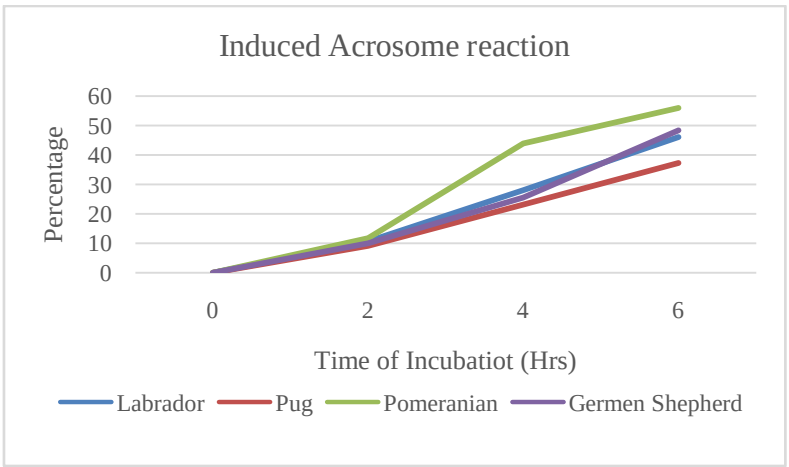


Fig. 7: Effect of incubation of spermatozoa in canine TALP medium on induced *in vitro* acrosome reaction.

Table 1: Motility, Viability, Plasma membrane- and Acrosomal integrity (Mean ± SE) in freshly extended and frozen-thaw semen of different dog breeds.

Dog breed/ No	Motility (%)		Viability (%)		Plasma Membrane Integrity (%)		Acrosome Integrity (%)	
	FE^{A}	FT^{B}	FE^{A}	FT^{B}	FE^{A}	FT^{B}	FE^{A}	FT^{B}
Labrador Retriever								
1	78.3^{ab} ±1.7	45.0^{b} ±5.0	83.6^{ab} ±2.4	50.4^{b} ±4.3	85.7^{a} ±0.5	53.0^{b} ±5.1	81.8^{ab} ±2.3	57.4^{c} ±2.6
2	78.3^{ab} ±1.7	41.7^{c} ±4.4	84.5^{ab} ±1.6	47.9^{ab} ±4.1	86.4^{a} ±1.7	47.2^{b} ±2.6	83.5^{ab} ±1.0	56.1^{c} ±3.3
3	68.3^{b} ±2.9	46.7^{b} ±1.7	72.9^{b} ±1.6	48.9^{ab} ±0.8	62.0 ±4.4	41.7^{c} ±1.6	66.5^{c} ±3.2	43.7 ±1.0
4	83.3^{a} ±2.9	45.0^{b} ±2.9	88.7^{a} ±1.1	50.2^{b} ±3.7	85.8^{a} ±0.7	51.3^{b} ±1.7	88.2^{a} ±1.0	61.8^{b} ±4.6
5	83.3^{a} ±1.7	56.7^{a} ±1.7	89.5^{a} ±1.5	63.6^{a} ±0.8	87.3^{a} ±1.8	60.9^{a} ±1.9	87.2^{a} ±2.2	68.3^{a} ±1.9
6	83.3^{a} ±1.7	55.0^{a} ±2.9	87.0^{a} ±1.5	60.0^{a} ±1.8	87.6^{a} ±1.6	59.3^{a} ±0.7	88.4^{a} ±4.2	64.8^{ab} ±2.7
Average	79.1^{C} ±2.1	48.3^{CD} ±2.6	84.3^{C} ±1.6	53.5^{CD} ±2.3	82.5^{C} ±1.8	52.2^{C} ±2.1	82.6^{C} ±2.3	58.6^{C} ±2.7
Pug								
1	78.3^{ab} ±1.7	53.3^{a} ±1.7	83.5^{ab} ±3.4	57.2^{ab} ±1.2	80.3^{ab} ±1.4	26.7^{e} ±2.1	81.3^{ab} ±4.1	53.8^{c} ±1.0
2	80.0^{a} ±5.0	43.3^{bc} ±1.7	82.2^{ab} ±6.0	47.9^{bc} ±1.0	73.9^{ab} ±1.7	21.3^{f} ±4.6	75.8^{b} ±4.5	40.4^{d} ±1.6
3	75.0^{ab} ±2.9	43.3^{bc} ±1.7	79.6^{ab} ±3.2	49.9^{bc} ±1.9	72.3^{ab} ±4.5	29.4^{e} ±6.5	76.8^{b} ±6.3	32.9^{e} ±11.0
4	66.7^{b} ±4.4	40.0^{c} ±10.4	76.9^{b} ±3.2	45.8^{c} ±10.1	77.6^{ab} ±5.3	17.6^{f} ±7.3	69.3^{c} ±3.8	45.3^{d} ±7.7
Average	75^{D} ±3.5	45.0^{D} ±4.5	80.5^{CD} ±3.9	50.2^{bD} ±3.5	76.0^{D} ±3.2	23.7^{E} ±5.1	75.8^{D} ±4.7	43.1^{D} ±5.3
Pomeranian								
1	72.9^{abD} ±4.3	46.7^{bD} ±1.7	73.4^{bD} ±5.9	49.2^{bcD} ±1.1	69.6^{bE} ±1.1	34.6^{dD} ±4.3	83.3^{abC} ±1.1	62.8^{bC} ±1.9
Germen Shepherd								
1	80.0^{a} ±2.9	53.3^{a} ±7.3	85.6^{ab} ±2.6	60.2^{ab} ±7.7	60.6^{c} ±1.8	35.7^{d} ±8.2	90.0^{a} ±1.4	60.6^{b} ±4.0
2	76.7^{ab} ±8.8	55.0^{a} ±2.9	81.0^{ab} ±8.4	60.1^{ab} ±3.1	65.6^{b} ±3.0	28.8^{e} ±4.4	79.8^{ab} ±3.9	65.2^{ab} ±6.0
Average	78.3^{C} ±5.8	54.1^{C} ±5.1	83.3^{CD} ±5.5	60.1^{C} ±5.4	63.1^{F} ±2.4	32.2^{D} ±6,.3	84.9^{C} ±2.6	62.9^{C} ±5.0

FE: Freshly extended semen and FT: Frozen thawed semen
Superscripts A and B indicate significant difference in sperm attributes in FE and FT semen
Superscripts C, D and E indicate significant difference among the breeds
Superscripts a, b, c, d, e and f indicate significant difference among the dogs irrespective of breed

Table 2: Inner mitochondrial membrane potential, ATP and LDHC concentration (Mean ± SE) in freshly extended and frozen-thaw semen of different dog breeds.

Dog breed/ No	HIMMP (%)		MIMMP (%)		ATP (nM/10^9 spermatozoa)		LDHC (ng/10^9 spwermatozoa)	
	FE ^{A}	FT^B	FE^A	FT^A	FE ^{A}	FT^A	FE ^{A}	FT^A
Labrador Retriever								
1	69.1^a ±1.9	42.8^{ab} ±1.8	24.1^e ±1.5	36.9^c ±2.5	26.8^b ±6.0	23.2^c ±2.5	13.2^c ±1.2	5.2^e ±0.8
2	72.2^a ±3.4	41.4^{ab} ±3.8	22.3^e ±4.6	34.3^c ±5.6	25.3^{bc} ±5.2	18.1^c ±4.6	14.3^c ±1.8	9.8^{de} ±0.2
3	55.3 ±2.1	32.8^{bc} ±1.4	28.3^{cd} ±2.0	34.9^c ±0.8	14.6^c ±1.3	13.6^d ±1.0	22.1^b ±0.4	6.2^e ±1.1
4	55.5 ±2.6	39.4^{ab} ±1.6	38.2^b ±2.8	35.1^c ±3.5	8.3^{de} ±0.6	7.7^d ±3.7	10.9^{de} ±0.6	6.9^d ±0.4
5	77.1^a ±1.5	45.0^a ±1.6	25.0^{de} ±1.9	33.6c ±2.3	13.8^d ±0.3	12.9 ±3.1	9.5^{cd} ±0.1	7.5^e ±0.5
6	74.6^a ±6.1	42.8^{ab} ±1.3	20.2^e ±7.2	34.1^c ±1.3	11.0^d ±2.3	2.9^e ±0.3	16.2^{bc} ±0.5	6.0^d ±0.1
Average	67.3^C ±2.9	40.6^C ±1.6	26.3^E ±3.3	34.8^D ±2.7	16.6^D ±2.6	13.0^D ±2.5	14.3^D ±0.8	6.9^B ±0.6
Pug								
1	71.1^a ±1.3	35.1^b ±3.4	22.0^e ±0.7	52.2^a ±3.3	11.3^c ±0.6	6.6^e ±1.4	7.3^e ±0.8	7.0^d ±0.1
2	48.7 ±6.1	28.1^c ±4.6	42.8^b ±7.3	43.0^b ±10.4	12.6^c ±0.8	11.7^d ±1.5	20.8^b ±0.4	18.7^{bc} ±1.4
3	60.1^b ±7.8	30.2^{cd} ±4.9	29.1^{cd} ±6.2	34.9^c ±2.0	25.9^b ±1.0	21.0^b ±1.0	21.6^b ±1.4	8.7^{cd} ±2.1
4	46.5^d ±7.1	25.9^d ±4.6	39.5^b ±5.1	30.4^c ±5.4	16.7^c ±1.0	12.5^d ±0.6	30.9^a ±1.3	15.1^c ±0.5
Average	56.6^D ±5.6	29.8^D ±4.3	33.3^{DE} ±4.8	40.1^C ±5.3	16.4^D ±0.8	12.9^D ±1.1	20.1^C ±1.0	12.4^B ±0.4
Pomeranian	18.7^{eE} ±4.4	17.8^{eE} ±8.3	57.7^{aC} ±8.1	41.2^{bC} ±4.7	29.4^{bC} ±3.0	24.3^{bCD} ±1.1	16.5^{cB} ±1.7	14.9^{cCD} ±1.3
Germen Shephard								
1	56.5^c ±1.4	31.3^{cd} ±1.1	41.5^b ±1.2	37.7^{bc} ±0.7	28.1^b ±4.6	24.8^b ±1.5	30.1^a ±2.4	14.4^c ±1.3
2	56.9^c ±2.7	39.0^{ab} ±1.0	36.4^c ±1.6	36.3^{bc} ±2.7	36.5^a ±3.3	32.5^a ±0.9	26.5^{ab} ±2.3	19.7^{bc} ±0.7
Average	56.7^D ±2.0	35.1^C ±1.0	38.9^D ±1.4	37.0^{CD} ±1.7	32.3^C ±3.9	28.6^C ±1.2	17.0^{CD} ±1.0	28.3^A ±2.3

FE: Freshly extended semen and FT: Frozen thawed semen
HIMMP: High inner mitochondrial membrane potential, MIMMP: Medium inner mitochondrial membrane potential, ATP: Adenosine triphosphate, LDHC: Lactate dehydrogenase C
Superscripts A and B indicate significant difference in sperm attributes in FE and FT semen
Superscripts C, D and E indicate significant difference among the breeds
Superscripts a, b, c, d, e and f indicate significant difference among the dogs irrespective of breed

Table 3: Effect of incubation time on motility, viability (Mean ± SE) of ejaculated spermatozoa incubated in canine-TALP medium of different dog breeds.

Dog No and Breed/ Time of incubation	Motility (%)				Viability (%)			
	0 H	2 H	4 H	6H	0 H	2 H	4 H	6H
Labrador								
1	78.3^{ab} ±1.7	68.3^{ab} ±1.3	45.0^{cd} ±2.9	30.0^{bc} ±2.9	83.6^{ab} ±2.4	73.9^{ab} ±2.1	51.2^{cd} ±3.1	34.1^{bc} ±3.3
2	63.3^{c} ±1.7	53.3^{c} ±1.3	30.0^{e} ±2.9	20.0^{d} ±2.9	68.5^{d} ±0.7	56.4^{c} ±2.1	34.8^{e} ±3.0	16.5^{e} ±6.6
3	66.7^{c} ±1.7	53.3^{c} ±2.7	38.3^{de} ±3.3	21.7^{d} ±6.7	70.9^{cd} ±1.3	58.7^{c} ±7.5	42.3^{d} ±3.0	25.0^{cd} ±6.9
4	68.3^{c} ±1.7	60.0^{bc} ±2.3	43.3^{cd} ±7.3	21.7^{d} ±7.3	71.9^{cd} ±1.3	64.7^{b} ±2.1	45.4^{d} ±5.0	27.6^{c} ±8.4
5	83.3^{a} ±2.9	73.3^{a} ±1.3	63.3^{a} ±1.7	43.3^{a} ±1.7	89.5^{a} ±1.5	78.9^{a} ±1.5	70.3^{a} ±2.7	49.9^{a} ±2.0
6	66.7^{c} ±1.7	50.0^{c} ±2.3	33.3^{e} ±1.7	18.3^{d} ±3.3	71.7^{cd} ±2.7	53.1^{c} ±2.7	38.0^{e} ±1.1	23.5^{d} ±3.2
Average	71.1^{A} ±1.9	59.7^{B} ±1.9	42.2^{C} ±4.1	25.8^{D} ±4.1	75.9^{A} ±1.4	64.3^{B} ±3.0	47.0^{C} ±3.0	29.4^{D} ±5.1
Pug								
1	78.3^{ab} ±1.7	68.3^{ab} ±4.4	56.7^{b} ±6.0	38.3^{ab} ±7.3	83.4^{ab} ±3.1	73.1^{ab} ±5.3	62.8^{b} ±6.8	45.4^{a} ±7.6
2	73.3^{b} ±4.4	61.7^{bc} ±3.3	48.3^{c} ±1.7	40.0^{ab} ±2.9	79.8^{ab} ±4.7	65.8^{b} ±3.7	53.6^{c} ±1.9	43.2^{ab} ±2.7
3	75.0^{b} ±2.9	53.3^{c} ±6.7	43.3^{cd} ±4.4	26.7^{c} ±6.0	79.6^{ab} ±3.1	60.1^{bc} ±6.7	48.1^{d} ±4.8	30.3^{c} ±6.2
4	71.7^{bc} ±4.4	61.7^{bc} ±4.4	46.6^{c} ±3.3	33.3^{b} ±6.7	75.7^{c} ±3.8	65.8^{b} ±3.6	51.5^{cd} ±3.7	39.6^{b} ±6.6
Average	74.6^{A} ±2.6	61.2^{B} ±4.7	48.7^{C} ±3.8	34.6^{D} ±5.7	79.6^{A} ±2.5	66.2^{B} ±4.8	54.0^{C} ±4.3	39.6^{D} ±5.8
Pomeranian	81.7^{aA} ±1.7	65.0^{bB} ±2.9	45.0^{cC} ±2.9	28.3^{cD} ±1.7	77.1^{cA} ±1.3	62.6^{bB} ±5.6	52.0^{cC} ±3.4	37.0^{bD} ±3.2
Germen Shephard								
1	80.0^{a} ±2.9	66.7^{ab} ±1.7	38.3^{d} ±4.4	28.3^{c} ±6.7	85.5^{ab} ±2.6	70.5^{b} ±1.6	46.9^{d} ±2.8	34.6^{bc} ±5.7
2	80.0^{a} ±5.0	70.0^{ab} ±2.9	53.3^{bc} ±4.4	33.3^{b} ±6.0	86.0^{ab} ±4.3	74.8^{ab} ±1.6	55.2^{c} ±1.5	40.4^{ab} ±5.3
Average	80.0^{A} ±3.9	68.3^{B} ±2.3	45.8^{C} ±4.4	30.8^{D} ±6.3	85.7^{A} ±3.4	72.6^{B} ±1.6	51.0^{C} ±2.1	37.5^{D} ±5.5

Superscripts A, B, C and D indicate significant difference in motility and viability among incubation periods
Superscripts a, b, c, d and e indicate significant difference among the dogs irrespective of breed

Table 4: Effect of incubation time on Inner mitochondrial membrane potential (Mean ± SE) of ejaculated spermatozoa incubated in canine-TALP medium of different dog breeds.

Dog No and Breed/ Time of incubation	HIMMP (%)				MIMMP (%)			
	0 H	2 H	4 H	6H	0 H	2 H	4 H	6H
Labrador Retriever								
1	69.1^{b} ±1.9	72.9^{b} ±2.3	79.2^{ab} ±0.9	81.4^{ab} ±0.8	24.1^{e} ±1.5	18.0^{c} ±1.2	12.8^{d} ±1.2	10.4^{c} ±1.4
2	60.9^{bc} ±3.0	64.8^{c} ±2.3	70.0^{b} ±1.7	72.3^{b} ±1.2	30.8^{d} ±3.8	29.2^{b} ±2.7	27.4^{ab} ±3.9	21.0^{ab} ±0.8
3	56.2^{cd} ±1.4	60.0^{c} ±1.1	65.7^{bc} ±1.6	70.2^{b} ±1.4	25.2^{e} ±1.8	22.1^{bc} ±1.3	19.0^{cd} ±1.0	16.0^{b} ±1.4
4	37.6^{e} ±5.7	41.5^{e} ±5.0	54.7^{d} ±4.5	62.6^{c} ±2.9	46.9^{c} ±3.6	40.3^{a} ±3.7	29.9^{ab} ±4.1	25.3^{a} ±3.2
5	77.1^{a} ±1.5	83.4^{a} ±1.2	85.3^{a} ±1.5	87.5^{a} ±1.0	26.2^{d} ±2.0	22.4^{bc} ±1.3	16.8^{cd} ±1.0	13.9^{bc} ±1.0
6	42.2^{d} ±2.9	53.0^{d} ±2.0	64.8^{bc} ±4.7	67.0^{bc} ±3.3	54.3^{b} ±2.9	40.9^{a} ±3.6	24.4^{b} ±8.0	20.5^{ab} ±5.4
Average	57.2^{D} ±2.7	62.6^{C} ±2.3	$69.^{B}$ ±2.5	73.5^{A} ±1.8	34.6^{A} ±2.6	28.8^{B} ±2.3	21.7^{C} ±3.2	17.8^{C} ±2.2
Pug								
1	57.9^{c} ±9.8	61.3^{c} ±9.4	68.5^{bc} ±7.0	73.6^{b} ±6.9	32.2^{d} ±9.3	28.2^{b} ±8.9	22.5^{b} ±6.2	15.7^{b} ±4.6
2	45.5^{d} ±3.1	52.5^{d} ±4.2	61.0^{c} ±3.9	68.4^{bc} ±3.2	46.1^{c} ±6.4	38.3a ±7.3	29.8^{ab} ±4.5	23.6^{ab} ±6.1
3	49.7^{d} ±9.0	56.1^{d} ±7.7	64.9^{bc} ±4.7	71.8^{b} ±9.7	41.7^{cd} ±9.0	35.8^{ab} ±8.6	28.2^{ab} ±4.9	18.1^{ab} ±4.2
4	40.8^{e} ±2.2	48.7^{e} ±3.6	54.8^{d} ±2.9	64.1^{c} ±3.7	39.8^{cd} ±0.6	36.3^{ab} ±0.7	27.7^{ab} ±2.6	22.7^{ab} ±1.7
Average	48.4^{D} ±6.0	54.6^{C} ±6.2	62.3^{B} ±4.6	69.5^{A} ±4.1	39.9^{A} ±6.3	34.6^{B} ±6.4	27.0^{C} ±4.5	20.0^{D} ±4.1
Pomeranian	20.9^{fC} ±4.5	44.9^{eB} ±8.4	63.9^{bcA} ±2.2	66.8^{bcA} ±2.4	68.0^{aA} ±6.5	42.8^{aB} ±10.0	22.4^{bC} ±7.0	21.1^{abC} ±6.6
Germen Shephard								
1	58.7^{c} ±2.6	61.9^{c} ±1.4	65.3^{bc} ±3.2	74.5^{b} ±2.8	39.1^{cd} ±2.7	36.2^{ab} ±1.4	32.6^{a} ±3.7	20.2^{ab} ±3.1
2	66.5^{b} ±2.9	69.1^{a} ±2.5	73.6^{b} ±3.6	76.3^{b} ±2.9	33.7^{e} ±1.9	29.6^{b} ±2.3	22.4^{b} ±3.2	15.9^{b} ±1.8
Average	62.6^{A} ±2.7	65.5^{A} ±1.9	69.4^{B} ±3.4	75.4^{C} ±2.8	36.4^{A} ±2.3	32.9^{A} ±1.8	27.5^{B} ±3.4	18.0^{C} ±2.4

Superscripts A, B, C and D indicate significant difference in high inner mitochondrial membrane potential (HIMMP) and medium IMMP (MIMMP)among incubation periods.
Superscripts a, b, c, d and e indicate significant difference among the dogs irrespective of breed

Table 5: Effect of incubation time on induced acrosome reaction (Mean ± SE) of ejaculated spermatozoa incubated in canine-TALP medium of different dog breeds.

Dog No/ Breed	Time of incubation (Hrs)			
	Abnormal acrosome (%)	Induced Acrosome reaction (%) (Total abnormal acrosomes-Abnormal acrosomes at zero hour)		
	0	2	4	6
[ab] Labrador Retriever				
1	$18.2^{bc}\pm2.3$	$9.2^{b}\pm3.6$	$35.1^{b}\pm2.7$	$47.7^{b}\pm1.0$
2	$16.5^{bc}\pm1.0$	$8.0^{b}\pm0.3$	$34.8^{b}\pm5.1$	$51.0^{a}\pm2.4$
3	$34.5^{a}\pm3.2$	$9.4^{b}\pm0.7$	$24.3^{cd}\pm5.2$	$39.9^{c}\pm4.5$
4	$11.8^{c}\pm1.0$	$11.6^{ab}\pm2.1$	$28.5^{c}\pm6.7$	$38.6^{c}\pm4.1$
5	$12.8^{c}\pm2.2$	$11.8^{ab}\pm2.0$	$45.2^{a}\pm1.7$	$55.5^{a}\pm0.6$
6	$11.6^{c}\pm4.2$	$14.2^{a}\pm3.0$	$33.1^{b}\pm4.2$	$43.8^{bc}\pm3.4$
Average	15.6 ± 2.3	$10.7^{C}\pm1.9$	$28.0^{B}\pm4.3$	$46.1^{A}\pm2.7$
Pug				
1	$19.7^{bc}\pm4.1$	$10.8^{ab}\pm3.5$	$20.2^{d}\pm3.0$	$34.9^{cd}\pm2.3$
2	$24.2^{b}\pm4.5$	$11.0^{ab}\pm0.4$	$28.6^{c}\pm1.9$	$44.0^{bc}\pm3.0$
3	$23.2^{b}\pm6.3$	$8.5^{b}\pm2.2$	$23.9^{cd}\pm1.5$	$39.5^{c}\pm2.2$
4	$30.7^{ab}\pm3.8$	$6.2^{c}\pm0.5$	$20.2^{a}\pm2.3$	$30.7^{d}\pm1.8$
Average	24.4 ± 4.7	$9.1^{C}\pm1.6$	$23.2^{B}\pm2.2$	$37.3^{A}\pm2.3$
Pomeranian	$16.7^{bc}\pm1.1$	$11.7^{ab}\pm3.1$	$43.8^{a}\pm3.8$	$55.9^{a}\pm7.2$
Germen Shepherd				
1	$10.0^{c}\pm1.4$	$15.6^{a}\pm6.1$	$24.4^{cd}\pm3.8$	$51.5^{a}\pm1.7$
2	$20.2^{bc}\pm3.9$	$6.5^{c}\pm0.8$	$26.6^{c}\pm7.1$	$45.1^{b}\pm2.5$
Average	15.1 ± 2.6	$10.0^{C}\pm3.4$	$25.5^{B}\pm5.4$	$48.3^{A}\pm2.1$

Superscripts A, B, C and D indicate significant difference in acrosome reaction among incubation periods
Superscripts a, b, c and dindicate significant difference among the dogs irrespective of breed

Table 6: Correlation coefficient among different sperm quality traits of freshly extended and frozen-thaw semen.

Sperm attribute	Mot	Via	PMI	AI	HIMMP	MIMMP	ATP	LDHC	IAR
Mot	1								
Via	0.91 (0.97**)	1							
PMI	0.51 (0.35)	0.62** (0.38)	1						
AI	0.84** (0.66*)	0.77** (0.65*)	.38 (0.60*)	1					
HIMMP	0.52 (0.41)	0.69** (0.50)	0.54 (0.73*)	-0.28 (0.45)	1				
MIMMP	-0.29 (0.22)	-0.44 (0.10)	-0.53 (0.35)	-0.06 (-004)	-.938** (-0.24)	1			
ATP	-0.11 (0.11)	-.193 (0.11)	-0.31 (0.22)	-.001(0.26)	-.05 (-0.16)	).002 (0.05)	1		
LDHC	-0.55 (0.03)	-0.46 (0.01)	-0.71** (0.64)	-0.43 (0.02)	-0.41 (-0.50)	.43 (0.05)	0.001(0.452)	1	
IAR	0.39 (0.33)	0.20 (0.34)	-.010 (0.48)	0.56 (0.55)	-.05 (0.12)	.185 -0.05)	0.02 (0.46)	0.21 (0.15)	1

Figures in parentheses are of frozen thaw semen
**. Correlation is significant at the 0.05 level (2-tailed).*
***. Correlation is significant at the 0.01 level (2-tailed).*
ATP: Adenosine triphosphate, LDHC: Lactate dehydrogenase C, IAR: Induced acrosome reaction (Freshly extended semen), Mot: Motility, Via: Viability, PMI: Plasma membrane integrity, AI: Acrosome integrity, HIMMP: High mitochondrial membrane potential, MIMMP: Medium mitochondrial membrane potential