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Comparative study on effects of soy lecithin and egg yolk-based extenders on canine semen preservation at refrigeration temperature

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ABSTRACT

The objective of the study was to evaluate the effect of addition of Tris soy lecithin (TSL) and Tris egg yolk-based (TEY) extenders on canine seminal attributes during 7 days storage at 5°C. Semen was manually collected from fifteen apparently healthy male dogs, which then underwent semen evaluation. Only semen sample with spermatozoa motility >80% and spermatozoa concentration $>200\times10^6$ spermatozoa/mL were used in the present study. After initial evaluation, sperm-rich fractions were divided into two equal parts and extended with 20% TEY and 1% TSL extenders at 1:4 dilution, then stored at a refrigeration temperature of 5°C for 7 days. Chilled semen samples were assessed for *p*H, motility, viability, abnormality, and plasma membrane integrity for a week on daily basis. DNA integrity was assessed on days 0, 1, 3, and 5 of storage. Significant differences were observed in all seminal attributes except DNA integrity between fresh and extended semen. The *p*H of the semen did not differ significantly between the extenders up to day 6, indicating almost equal buffering capacity of the extenders. A significant difference in sperm motility, viability, morphological abnormality, and plasma membrane integrity was observed between the extenders from storage days 4, 3, 1, and 5, respectively. No significant difference in sperm DNA integrity was observed between the extenders. In conclusion, while both extenders were equally effective in preserving sperm DNA integrity, Tris egg yolk extender outperformed Tris soy lecithin extender in all other seminal attributes assessed in this study.

Keywords: Canine semen, Chilling, Semen extenders, Seminal attributes

Natural mating is the most common method of dog breeding; however, there are challenges like transport issues, size differences between male and female, inability of the male to mount due to musculo-skeletal diseases and lack of stud male availability at the appropriate mating time, which results in the loss of valuable breeding season.

The dog semen can be stored at room temperature (25-28°C) (Batista *et al*. 2012), refrigeration temperature (4-5°C) (Dalmazzo *et al*. 2019) and at ultralow temperature (-196°C) (Axner and Lagerson 2016). Several factors like storage temperature, extender composition, sperm count, and number of inseminations influence the keeping quality of semen. The quality of the semen stored at room temperature is said to be poorer when compared to chilled semen (Batista *et al*. 2012). Artificial insemination with chilled canine semen has reported pregnancy rates of 45.4 to 65% according to Linde-Forsberg (1991). It is easier to handle and transport but has a shorter lifespan of about 4.9 days (Ponglowhapan *et al*. 2004). While chilling semen is a simple practice, freezing requires expensive equipment and skilled personnel, which are not always available.

Most popular extender for chilled dog semen was based on an egg yolk−Tris−fructose medium (Lopes *et al*. 2009). Egg yolk is an animal product, therefore choosing it as a component has several drawbacks, including the difficulty of standardization due to its fluctuating composition and the possibility of contamination like salmonellosis (Beccaglia *et al*. 2009 and Kmenta *et al*. 2011). Due to its easy component standardization, and lower potential risk of contamination, soy lecithin is a suitable alternative to egg yolk (Fukui *et al*. 2008). Lecithin is comparable to egg yolk in composition (low-density lipoprotein), and it may protect the sperm plasma membrane from damage caused by cold shock (Forouzanfar *et al*. 2010).

Therefore, the present study was designed to compare the effect of soy lecithin and egg yolk-based extenders on storage of canine semen at refrigeration temperature of 5°C and to evaluate the keeping quality of chilled canine semen based on seminal attributes.

MATERIALS AND METHODS

Selection of animals: The study involved 15 male dogs aged between 2-5 years presented to Department of Gynaecology and Obstetrics, Veterinary College, Hebbal,

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Bengaluru for routine semen evaluation. Healthy dogs of various breeds underwent physical, clinico-andrological, ultrasonographic examinations, and semen was collected for evaluation. Dogs identified as fertile were included in the study.

Preparation of extenders: All chemicals utilized in this research were procured from HIMEDIA. TEY (20%) and TSL (1%) were prepared according to the procedures described by Verstegen *et al*. (2005) and Mingkun *et al*. (2022), respectively.

Semen collection: Dog semen was collected by digital manipulation of penis (Ray *et al*. 2019). The pre-sperm, sperm rich, and post-sperm fractions were collected separately in prewarmed self-made sterile collection vials (Fig. 1).

Semen evaluation: Only sperm-rich fraction was assessed for volume, colour and *p*H. The *p*H of the semen sample was estimated using a digital *p*H meter with range of 0.0 to 14 *p*H, resolution 0.1, accuracy ±0.1 (eco Testr pH2, Thermo Fisher Scientific Inc.). Aliquots of freshly ejaculated semen was separated and transferred immediately to a water bath at 37°C for evaluation of sperm motility, concentration, viability, abnormal sperm count, plasma membrane and DNA integrity. Sperm progressive motility was assessed according to the procedures of Puja *et al.* (2018). Sperm concentration was determined with Neubauer haemocytometer (Johnston *et al*. 2001). The percentage of live spermatozoa was measured by eosin-nigrosin stain (Agarwal *et al*. 2016) and morphologically abnormal spermatozoa was evaluated by Rose Bengal stain (Sousa *et al*. 2013). Sperm plasma membrane integrity was evaluated by hypo-osmotic swelling test (HOST) (Ramu and Jeyendran 2013). DNA integrity was evaluated by fluorescent microscopy after staining with Acridine Orange (Fig. 2) (Shamsi *et al*. 2011). Only semen samples with spermatozoa motility >80% and concentration >200 $\times 10^6$ spermatozoa/mL were used in the present study.

Semen processing and dilution: After initial evaluation,

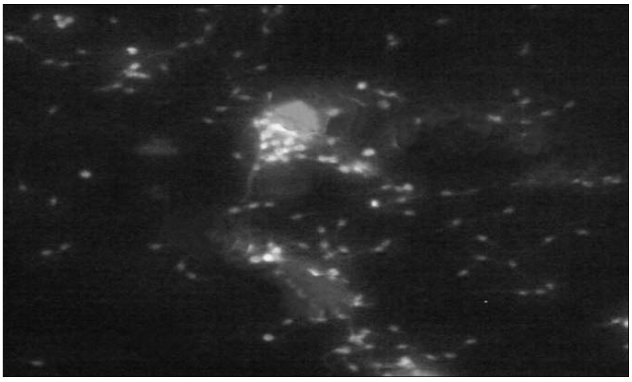

Fig. 2. Sperm DNA integrity evaluation by Acridine Orange stain under fluorescent microscope (400× magnification) (Green coloured sperm- Sperm with intact DNA, Orange coloured sperm- Sperm with denatured DNA).

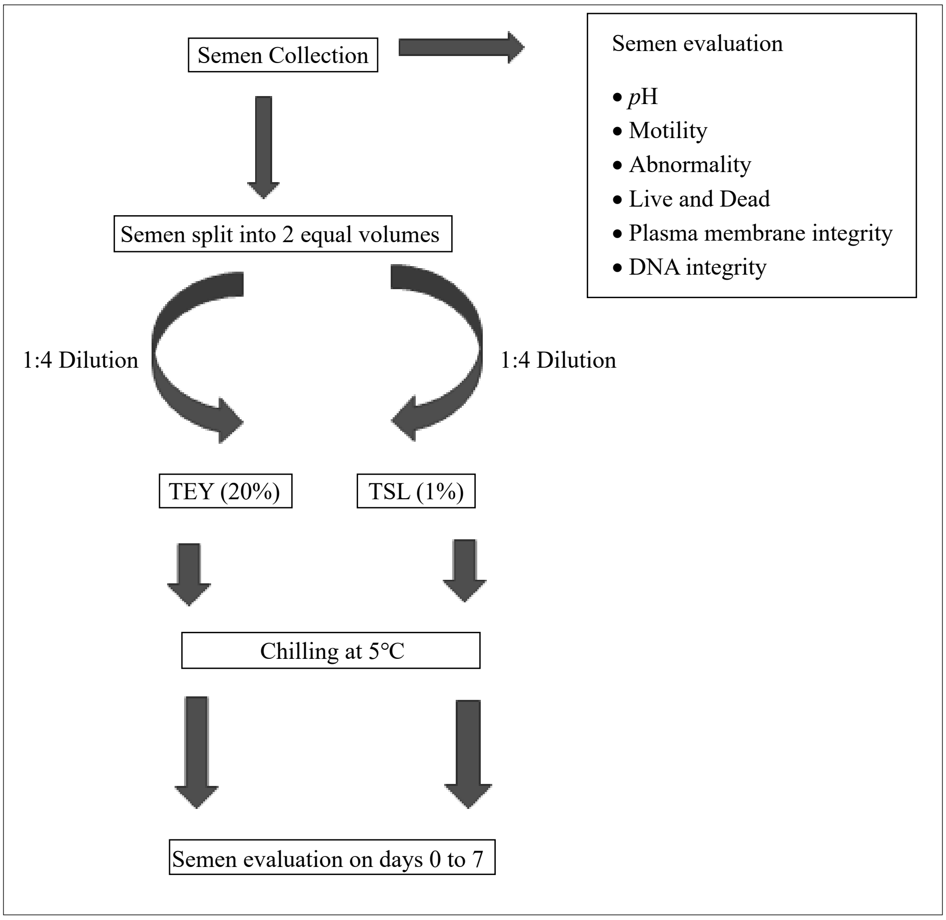

Fig. 1. Schematic presentation of experimental design

the semen was split into two equal volumes and taken in sterile tubes. Then centrifuged at 700 g for 5 min, discarded the supernatants to obtain sperm pellets devoid of seminal plasma. These pellets were then diluted at 1:4 in TEY and TSL extenders and stored at 5°C for 7 days. Seminal attributes were evaluated daily for a week, except DNA integrity, which was assessed on days 0, 1, 3, and 5 of storage.

Statistical analysis: Data comparing the effects of TEY and TSL semen extenders with fresh semen at 0 hours were analysed using paired t-tests in SPSS version 16.0. The effects of semen extenders and duration of preservation on seminal attributes in refrigerated semen were analysed through two-way ANOVA using the R project for statistical computing.

RESULTS AND DISCUSSION

Effects of tris egg yolk and tris soy lecithin semen extenders on seminal attributes at '0' h in comparison with fresh semen: The dilution of semen with extenders significantly increased (P<0.001) the semen *p*H compared to fresh, unextended semen, attributed to buffer addition in the extenders (Table 1). Sperm progressive motility exhibited a significant difference (P<0.001) between fresh and extended semen aligning with Beccaglia *et al*. (2009), who noted higher motility in fresh semen than in semen extended with both TEY and TSL extenders. Significant differences (P<0.001) in sperm viability were observed in semen extended with TEY and TSL at '0' h compared to fresh semen, consistent with Pradana *et al*. (2016) who found significant differences in the percentage of viable sperm between fresh and diluted canine semen in both TEY and TSL extenders. Significant increase (P<0.001) in sperm abnormalities were noted in extended semen compared to fresh semen agreeing with findings of Srinivas *et al.* (2022). The HOST reacted spermatozoa were significantly reduced (P<0.001) in semen extended with TEY and TSL compared to fresh, unextended semen at '0' h, consistent with Rota *et al*. (1995), who found fresh semen had a significantly higher HOST value (93.60±3.70) compared to extended semen in TEY extender (91.20±4.10) immediately after dilution. However, sperm DNA integrity showed no significant difference (P>0.001) between fresh and extended semen at '0' h, consistent with Pradana *et al*. (2016), who

Table 1. Characteristics of sperm-rich fractions of fresh semen samples collected from 15 dogs

Parameter	Mean ± standard deviation
Colour	Milky white to creamy
Volume (mL)	2.55±0.10
*p*H	6.41±0.04
Concentration (10^6/mL)	321.33±2.89
Progressive motility (%)	83.67±0.74
Live and dead (%)	88.50±0.75
Abnormalities (%)	7.33±0.41
Plasma membrane integrity (%)	87.40±0.94
DNA integrity (%)	92.26±0.60

reported no significant difference in the percentage of sperm with intact DNA between fresh and semen extended in TEY and TSL extenders. The observed differences in sperm motility, viability, morphologically abnormality and sperm plasma membrane integrity between fresh and extended semen could be attributed to semen handling and processing, extender *p*H and microenvironmental changes. Sperm DNA, being compact and stable, is relatively resistant to changes induced by semen extension.

Effect of semen extenders and duration of preservation on seminal attributes of semen preserved at refrigeration temperature for a period of 7 days

Semen pH: No significant difference was there in mean *p*H between the extenders up to day 6 and significant difference was observed only on day 7 of storage, suggesting similar buffering capacities for both diluents (Tables 2 and 3; Supplementary Fig. 1). This agrees with Ubah *et al*. (2019), who noted that tris buffer maintains a stable *p*H despite external factors. In TEY and TSL extenders, no significant differences found up to day 3 and day 2 respectively. This finding is similar to Rota *et al*. (1995), who reported a significant *p*H decrease by day 3 to 4 in TEY.

Sperm progressive motility: Significantly lower (P<0.05) percentage of sperm motility was observed from day 4 and day 3 onwards in TEY and TSL extenders respectively (Fig. 3). Motility was found to decrease significantly between the extenders from day 4. The primary increase in sperm metabolism, leading to elevated oxygen and energy absorption, lowered *p*H, and increased lactic acid concentration, likely contributed to this decline in sperm motility (Verstegen *et al*. 2005). Kasimanickam *et al*. (2012)

Table 2. Effect of TEY extender on seminal attributes at '0' h in comparison with fresh unextended semen (mean±S.EM)

	*p*H	Motility	Live and dead	Abnormalities	HOST	DNA integrity
Fresh	6.41±0.04^a	83.67± 0.74^a	88.50±0.75^a	7.33±0.41^a	87.40±0.94^a	92.26 ±0.60^a
TEY	6.75±0.04^b	79.73±0.69^b	85.42±0.79^b	8.93±0.51^b	83.33±1.13^b	90.46 ± 0.55^a

Means with different superscripts (a,b) within a column indicates significant difference at P<0.05 between extenders.

Table 3. Effect of TSL extender on seminal attributes at '0' h in comparison with fresh unextended semen (mean±S.EM)

	*p*H	Motility	Live and dead	Abnormalities	HOST	DNA integrity
Fresh	6.41±0.04^a	83.67±0.74^a	88.50±0.75^a	7.33±0.41^a	87.40±0.94^a	92.26 ±0.60^a
TSL	6.76±0.03^b	72.40±1.02^b	76.00±1.09^b	10.33±0.60^b	79.60±1.04^b	89.73 ± 0.56^b

Means with different superscripts (a,b) within a column indicates significant difference at P<0.05 between extender.

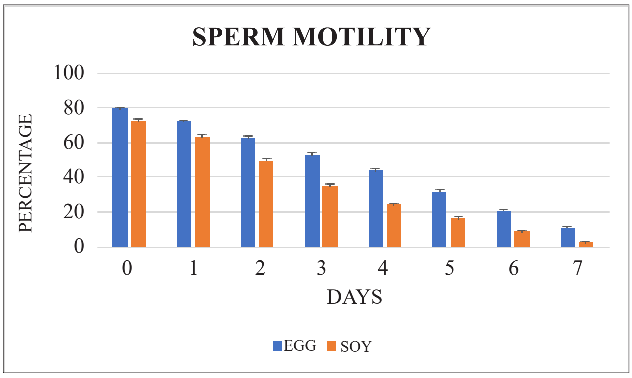

Fig. 3. Progressive motility of semen in egg yolk and soy lecithin-based extender at different days of interval (N=15) preserved at (5°C).

reported no change in progressive motility percentage between egg yolk and 0.04% soy lecithin extenders until day 3. Overall, these findings suggest that egg yolk outperforms soybean lecithin in maintaining motility. However, soy lecithin showed statistical similarity to egg yolk up to day 3. Axner and Lagerson (2016) and Hermansson *et al*. (2021) similarly observed superior motility in egg yolk-based extenders compared to soybean lecithin-based extenders. Egg yolk stabilizes sperm membranes through interactions with seminal plasma proteins, preventing phospholipid efflux, controlling cation loss and scavenging reactive oxygen species (ROS), thereby inhibiting cold shock-induced pre-term capacitation (Manjunath *et al*. 2002). In contrast to our findings, Kmenta *et al*. (2011) reported that a 0.8% lecithin extender with added antioxidant catalase preserved motility better than 20% egg yolk extender for 8 days. Discrepancies with our results may be due to differences in soybean lecithin sources, extender preparations, and lecithin concentrations. Dalmazzo *et al*. (2018) suggested that elevated phosphatidylcholine levels in soybean lecithin may disrupt intracellular and extracellular phosphatidylcholine balance, reducing sperm motility and mitochondrial activity. Accordingly, reduced sperm motility in TSL may be because of the higher concentrations (1%) of soy lecithin used in the present study compared to Dalmazzo *et al.* (2018) who used lower soy lecithin concentrations (0.01, 0.05 and 0.1%) in their study.

Percentage of live spermatozoa: In this study, the mean percentage of live sperm exceeded that of motile spermatozoa (Supplementary Fig. 2), consistent with Puja *et al*. (2018). This could be because viable spermatozoa are not always motile; occasionally, non-motile spermatozoa may still be alive. Significant reductions (P<0.05) in spermatozoa viability were observed on day 4 in TEY, day 3 in TSL and from day 3 onwards between the extenders. Throughout semen storage at refrigeration temperature, the percentage of viable sperm gradually decreased, consistent with Verstegen *et al.* (2005), who noted a steady decline in live spermatozoa in chilled dog semen stored at 4°C. Damage induced by reactive oxygen species (ROS) to lipids, proteins, and DNA can lead to decreased motility and viability of spermatozoa (Saraswat *et al*. 2014).

Percentage of morphologically abnormal spermatozoa: The morphological abnormalities observed were mid-piece defects, abnormal heads and tails (Supplementary Fig. 3). There was a significant increase in the percentage of morphologically abnormal spermatozoa over the 7-days storage within and between the two extenders. According to Hori *et al.* (2014), sperm metabolites lower semen *p*H and oxidative stress damages the sperm cell membrane and inhibits enzyme activity. This results in abnormal sperm morphology, particularly abnormal tails, and reduced sperm motility, which aligns with findings of the present study.

Sperm plasma membrane integrity: There was a gradual reduction in the mean percentage of HOST-responsive spermatozoa over seven days in both extenders (Supplementary Fig. 4). Ponglowhapan (2004) reported that extension, chilling, and storage deteriorate plasma membrane integrity over time. In this study, a positive correlation between sperm plasma membrane integrity and sperm progressive motility was noted. A significantly lower (P<0.05) percentage of HOST-responsive spermatozoa was observed from day 4 in TEY, from day 3 in TSL and from day 5 onwards between the extenders. This indicates that TEY is superior in protecting sperm plasma membrane integrity compared to TSL. These findings are consistent with Hermansson *et al*. (2021), who reported better plasma membrane integrity in canine sperm with TEY than with TSL.

Sperm DNA integrity: After storing extended canine semen at refrigeration temperature, minimal and mostly non-significant sperm DNA denaturation was observed. In the TEY extender, significant difference was found between day 0 and 5, but not on other days (Fig. 4). This aligns with Puja *et al*. (2018), who also found no significant differences within the egg yolk extender. Similarly, in the TSL extender, significant differences were noted between day 0 and day 1 compared to day 5, but not on other days. This is consistent with Dalmazzo *et al*. (2018), who observed no significant differences in DNA integrity within soy lecithin (0.1%) extender over 5 days of refrigeration. No significant differences were detected between the extenders on days 0, 1, 3, and 5 of evaluation. Low-density

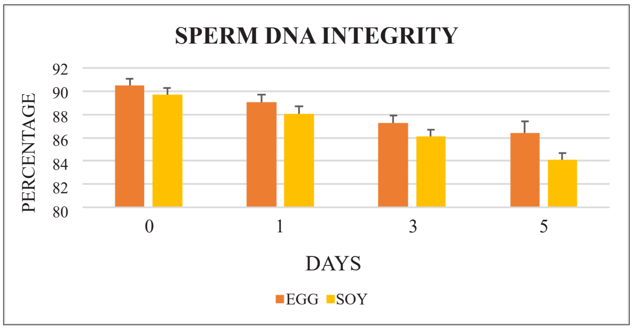

Fig. 4. Percentage of sperm with intact DNA in egg yolk and soy lecithin-based extender at different days of interval (N=15) preserved at (5°C).

lipoproteins in egg yolk have protective effects against reactive oxygen species (Aboagla and Terada 2004). Astuti (2012) reported that isoflavones in soybeans act as antioxidants, reducing reactive oxygen species (ROS) and counteracting free radicals, thus maintaining sperm DNA integrity. Prinosilova *et al*. (2012) proposed that both cryopreservation and refrigeration did not significantly alter DNA integrity. These findings indicate that DNA integrity is not significantly impacted by different extenders and storage at refrigeration temperature.

Based on the findings of the current study, it can be concluded that there is a significant difference in all seminal attributes except DNA integrity between fresh and extended semen in both extenders. The buffering action of both of the extenders were similar throughout the study, except on day 7. Tris egg yolk (20%) was superior to tris soy lecithin (1%) in preserving sperm motility, viability, morphology, and plasma membrane integrity when canine semen was stored at refrigeration temperature for 7 days. DNA integrity was not significantly affected by either extender or refrigeration, indicating that both extenders are equally effective in protecting sperm DNA integrity during the chilling of canine semen. Canine semen qualities can be maintained for up to 4 days when extended with the Tris egg yolk extender and stored at a temperature of 5°C.

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REFERENCES

Aboagla E M E and Terada T. 2004. Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology* **62**(6): 1160–72.

Agarwal A, Gupta S and Sharma R. 2016. Eosin-nigrosin staining procedure, pp. 73-77. *Andrological Evaluation of Male Infertility: A Laboratory Guide*, 1st edn. Springer, USA.

Astuti S. 2012. Isoflavon kedelai dan potensinya sebagai penangkap radikal bebas. *Jurnal Teknologi & Industri Hasil Pertanian* **13**(2): 126–36.

Axner E and Lagerson E. 2016. Cryopreservation of dog semen in a tris extender with 1% or 2% soya bean lecithin as a replacement of egg yolk. *Reproduction in Domestic Animals* **51**(2): 262–68.

Batista M, Santana M, Alamo D, Gonzalez F, Nino T, Cabrera F and Gracia A. 2012. Effects of incubation temperature and semen pooling on the viability of fresh, chilled and freeze-thawed canine semen samples. *Reproduction in Domestic Animals* **47**(6): 1049–55.

Beccaglia M, Anastasi P and Luvoni G C. 2009. Freezing of canine semen in an animal free protein extender. *Veterinary research communications* **33**: 77–80.

Dalmazzo A, Losano J D A, Rocha C C, Tsunoda R H, Angrimani D D S R, Mendes C M, Assumpcao M E O D A, Nichi M and Barnabe V H. 2018. Effects of soy lecithin extender on dog sperm cryopreservation. *Journal of Animal Science and Biotechnology* **29**(3): 174–82.

Forouzanfar M, Sharafi M, Hosseini S M, Ostadhosseini S, Hajian M,Hosseini L, Abedi P, Nili N, Rahmani H R and Nasresfahani M H. 2010. *In vitro* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of ram semen. *Theriogenology* **1**(73): 480–87.

Fukui Y, Kohno H, Togari T, Hiwasa M and Okabe K. 2008. Fertility after artificial insemination using a soybean-based semen extender in sheep. *Journal of Reproduction and Development* **54**(4): 286–89.

Hermansson U, Johannisson A and Axner E. 2021. Cryopreservation of dog semen in a Tris extender with two different 1% soybean preparations compared with a Tris egg yolk extender. *Veterinary Medicine and Science* **7**(3): 812–19.

Hori T, Yoshikuni R, Kobayashi M and Kawakami E. 2014. Effects of storage temperature and semen extender on stored canine semen. *The Journal of Veterinary Medical Science* **76**(2): 259–63.

Johnston S D, Root Kustritz M V and Olson P N S. 2001. Semen collection, evaluation, and preservation, pp.287-306. *Canine and Feline Theriogenology*. 1st edn, W. B. Saunders Company, Philadelphia.

Kasimanickam V R, Kasimanickam R K, Memon M A and Rogers H A. 2012. Effect of extenders on sperm mitochondrial membrane, plasma membrane and sperm kinetics during liquid storage of canine semen at 5^0C. *Animal Reproduction Science* **36**(1): 139–45.

Kmenta I, Strohmayer C, Muller-Schlosser F and Schafer Somi S. 2011. Effects of a lecithin and catalase containing semen extender and a second dilution with different enhancing buffers on the quality of cold-stored canine spermatozoa. *Theriogenology* **75**(6): 1095–03.

Linde-Forsberg C. 1991. Achieving canine pregnancy by using frozen or chilled extended semen. *Veterinary Clinics of North America: Small Animal Practice* **21**(3): 467–85.

Lopes G, Simoes A, Ferreira P, Martins-Bessa A and Rocha A. 2009. Differences in preservation of canine chilled semen using different transport containers. *Animal Reproduction Science* **112**(2): 158–63.

Manjunath P, Nauc V, Bergeron A and Menard M. 2002. Major proteins of bovine seminal plasma bind to the low-density lipoprotein fraction of hen's egg yolk. 2000. *Biology of Reproduction* **67**(4): 1250–58.

Mingkun Sun, Wangcheng Song, ChangrongLv, Yu Li and Song H. 2022. Soybean lecithin-based extender improves the quality of chilled canine sperm. *Journal of Veterinary Science and Animal Husbandry* **10**(1): 102–13.

Ponglowhapan S, Essen-Gustavsson B and Forsberg C L. 2004. Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. *Theriogenology* **62**(8): 1498–1517.

Pradana M Y W, Bebas W and Puja K. 2016. The effect of soyabean extender on viability and DNA integrity of Kintamani dog sperm on cold storage. *Veterinary Science and Medicine Journal* **4**(1): 11–16.

Puja I K, Sawitri N M, Maharani N, Gunawan I W N F and Heryani L G S S. 2018. A comparative study on the effects of coconut water-based extenders on the quality of Kintamani dog semen preserved at 4^0C. *Advances in Animal and Veterinary Sciences* **6**(5): 192–96.

Ramu S and Jeyendran R S. 2013. The hypo-osmotic swelling test for evaluation of sperm membrane integrity. *Methods in Molecular Biology* **927**: 21–25.

Ray K, Jha A K, Biswas P and Basu S. 2019. Study on physicomorphological seminal characters of German shepherd canine breed. *Indian Journal of Animal Health* **58**(1): 95–100.

Rota A, Strom B and Linde-Forsberg C. 1995. Effects of seminal plasma and three extenders on canine semen stored at 4^{0}C. *Theriogenology* **44**(6): 885–900.

Saraswat S, Kharche S D and Jindal S K. 2014. Impact of reactive oxygen species on spermatozoa: A balancing act between beneficial and detrimental effects. *Iranian Journal of Applied Animal Science* **4**(2): 247–55.

Shamsi M B, Imam S N and Dada R. 2011. Sperm DNA integrity assays: diagnostic and prognostic challenges and implications in the management of infertility. *Journal of Assisted Reproduction and Genetics* **28**(11): 1073–85.

Sousa P C, Santos E A, Souza A L, Lima, G L, Barros F F, Oliveira M F and Silva A R. 2013. Sperm morphological and morphometric evaluation in captive collared peccaries (*Pecari tajacu*). *Pesquisa Veterinaria Brasileira* **33**: 924–30.

Srinivas R T, Reddy K C S, Venkata Ramana K and Nagaraj P. 2022. Studies on extension and preservation of canine semen by addition of catalase at refrigeration temperature. *Journal of pharmaceutical innovation* **11**(6): 655–58.

Ubah S A, Sule M, Chibuogwu I C, Columbus P K, Abah K O, Agbonu O A, Ejiofor C E, Mshelbwala P P and Bankole S A. 2019. Comparative study of chicken egg yolk and quail egg yolk in two chilled canine semen extenders. *Sokoto Journal of Veterinary Sciences* **17**(4): 62–69.

Verstegen J P, Onclin K and Iguer-Ouada M. 2005. Long-term motility and fertility conservation of chilled canine semen using egg yolk added Tris–glucose extender: *In vitro* and *in vivo* studies. *Theriogenology* **64**(3): 720–33.