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Preservation Efficacy of Tris-egg Yolk-glucose Extender for Motility, Viability, Morphology and Membrane Integrity of Canine Semen during Refrigerated Storage at 4°C

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ABSTRACT

The experiment was conducted during the months from July to September, 2022 at the Department of Veterinary Gynaecology and Obstetrics, Post Graduate Institute of Veterinary Education and Research (PGIVER), Jaipur. The objective of this study was to determine the effect of a Tris-Egg Yolk-Glucose Extender on the quality parameters of dog semen (n=24 ejaculates) during refrigeration (4°C) preservation up to 72 hours. Semen was collected from six male dogs, with four ejaculates obtained per animal using digital manipulation. Each fresh ejaculate underwent thorough macroscopic assessments (including volume, colour, consistency and pH) and microscopic analyses to evaluate sperm motility, morphology, viability and Membrane Integrity. The semen samples were then extended in a Tris-Egg Yolk-Glucose using a split sample technique. The diluted semen was kept in a refrigerator for preservation at 4°C and evaluated at 0, 24, 48, and 72 hours for sperm quality parameters. There was a significant difference (p<0.05) in sperm motility percentage at different time slots of preservation. The sperm motility significantly reduced over a period of time from 0 to 72 hours within Tris-Egg Yolk-Glucose Extender. The live sperm percentage was numerically higher in the TRIS-Egg yolk-glucose dilutor at 24, 48, and 72 hours but lower at 0 hours. The percentages of morphologically abnormal spermatozoa were numerically lower at 0, 24, 48, and 72 hours of storage. There was no significant difference in intact plasma membrane percentage at 0, 24 hours and a significant difference in intact plasma membrane percentage at 48, 72 hours was computed.

KEYWORDS: Canine semen, refrigerated storage, tris-egg yolk-glucose extender

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Data Availability Statement: Legal restrictions are imposed on the public sharing of raw data. However, authors have full right to transfer or share the data in raw form upon request subject to either meeting the conditions of the original consents and the original research study. Further, access of data needs to meet whether the user complies with the ethical and legal obligations as data controllers to allow for secondary use of the data outside of the original study.

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1. INTRODUCTION

The earliest recorded case of artificial insemination in dogs was achieved by Lazzaro Spallanzani in 1780 (Bozzini et al., 2016). Despite this historical milestone, focused scientific interest in companion animal reproduction has only gained momentum in recent decades (Martínez-Barbitta and Rivera Salinas, 2022). Canine spermatozoa possess a remarkable capacity to survive within the female reproductive tract for more than 10 days. This extended viability is closely linked to the unique physiology of the canine reproductive system: bitches exhibit a relatively prolonged oestrous period of up to nine days, and sexual receptivity does not necessarily coincide with ovulation. Consequently, the interval from ejaculation to fertilization in dogs can extend to around 10 days (Bucci et al., 2023). For the successful application of the AI technique, the most important step is the preservation of semen. 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). Refrigerated semen storage offers advantages over freezing, including lower costs, simpler equipment requirements, and higher conception rates in AI (Linde-Forsberg and Forsberg, 1993). The sperm's metabolism peaks at body temperature and falls off at room temperature (24-29°C). The sperm plasma membrane undergoes a shift from the cooled crystalline to the gel phase when semen is stored at 4–8°C. Cellular metabolism decreases by 50% for every 10°C drop in temperature; at 5°C, sperm metabolic activity is only 10% of what it would be at body temperature (McKinnon, 2019). Effective extenders protect spermatozoa from cold shock, supply energy substrates, and stabilize pH and osmolarity, with 20% egg yolk commonly used for this purpose (England, 1993). Tris-egg yolk-glucose extender remains the most widely used medium for preserving canine semen at refrigeration temperature (4°C) during short-term storage (Litvinchuk et al., 2025). In this formulation, egg yolk supplies phospholipids and low-density lipoproteins that help stabilize the sperm plasma membrane, protecting it from cold shock during chilling (Elbehiry et al., 2024). Glucose serves as an essential energy source, supporting sperm motility, maintaining membrane integrity, and thereby extending sperm viability under cooled conditions (Kamal et al., 2023). Recent studies have shown that adding antioxidants such as crocin to Tris-based extenders can further enhance motility and viability of canine spermatozoa for up to 96 hours after collection (Calabria et al., 2023). However, the use of egg yolk also raises concerns regarding microbial contamination, which remains a critical issue during storage and transport of chilled dog semen (Bustani et al., 2021). Fructose and glucose are commonly used in canine semen extenders, serving as energy sources and being metabolized independently by ejaculated spermatozoa (Ponglowhapan et al., 2004). Cheema and Kaur (2021) demonstrated that the addition of catalase to tris egg yolk glucose citrate extender further improved the quality and viability of canine semen up to 72 hours at refrigeration temperature. Tris-egg yolk-glucose provided significantly better motility, lower abnormal sperm percentage, and improved plasma membrane integrity at 48 and 72 hours compared to the coconut water-based extender (Meena et al., 2024). Therefore, the present study aimed to evaluate the effect of Tris-egg yolk-glucose extender on semen motility, viability, morphology, and membrane integrity during short-term storage at 4°C.

2. MATERIALS AND METHODS

The present experiment was carried out in the Department of Veterinary Gynaecology and Obstetrics, Post Graduate Institute of Veterinary Education and Research (PGIVER), Jaipur. The work was carried out for the period of three months from July to September, 2022. Semen was collected from 6 dogs (4 German shepherd, 1 Labrador, and 1 Siberian Husky) at weekly intervals for a total of 24 ejaculates by digital manipulation (Figure 1). The fresh sperm-rich fraction was examined for macroscopic examination, including volume, colour, consistency, and pH, while the microscopic examination included mass motility, individual sperm motility, sperm concentration, sperm abnormalities, live count and hypo-osmotic swelling test (HOST). Following the preliminary evaluation, the sperm-rich fraction of the sperm sample was diluted 1:4 in Tris-Egg Yolk-Glucose Extender at room temperature. The composition of extender contained Tris hydroxymethyl aminomethane 2.44 g, Citric acid 1.36 g, D-Glucose 0.82 g, Sodium Penicillin 100 IU and Streptomycin 100 mg in 100

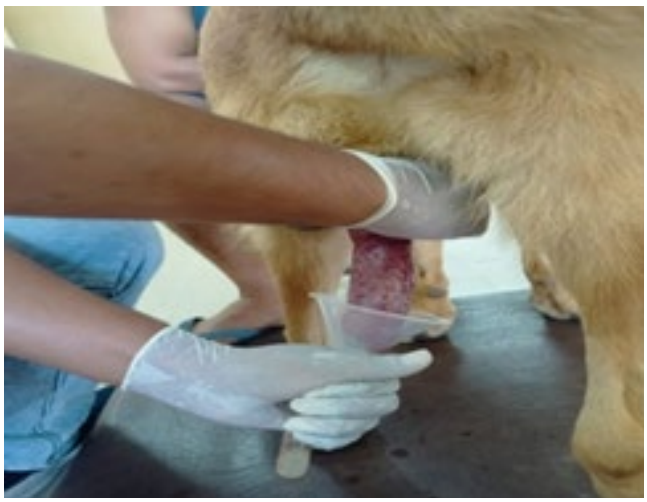

Figure 1: Semen collection (rotation of penis and collection of sperm rich fraction)

mL DW, to which 20 ml Egg yolk was added (Picket et al., 1975). Extended semen samples were equilibrated in a 37°C water bath, then gradually cooled to 4°C in a refrigerator. At 0, 24, 48, and 72h, diluted samples were tested for individual sperm motility, viability, and abnormalities (Payan-Carreira et al., 2011), and the spermatozoa (%) with intact plasma membrane were determined using the host (Jayendran et al., 1984). Data were subjected to analysis by a completely randomized design (CRD) using by one-way analysis of variance technique (Snedecor and Cochran, 1994) using the statistical package SPSS software version 20. The means of different experimental groups were tested for statistical significance by Duncan's multiple range test (Figure 1).

3. RESULTS AND DISCUSSION

3.1. Semen quality of dogs

The overall mean±SE of reaction time and ejaculation time was 90.02±5.73 seconds and 207.30±9.74 respectively recorded during 24 semen collection from 06 dogs on weekly basis (Table 1).

Table 1: Reaction time and ejaculation time

Reaction time (seconds) (Mean±SE)	Ejaculation time (seconds) (Mean±SE)
90.02±5.73 seconds	207.30±9.74

In relation to dogs, fresh semen is analyzed, and the results show that the average canine semen volume ejaculate is about 2.17±0.16 ml. The semen also appears milky white in color, has a thick consistency, and a pH of 6.35±0.03. Standard results for mass motility of sperm, individual motility, live sperm percentage, abnormal sperm percentage, total sperm concentration and HOS-reactive spermatozoa are 3.91±0.15, 86.45±0.97%, 87.37±0.98%, 9.08±0.25%, 353.89±11.1 million ml^{-1}, and 89.0±0.94% respectively (Table 2).

Variations in semen volume were influenced by factors such as dog size, age, breed, body weight, prostate size, collection frequency, and second fraction volume. The observed semen characteristics are consistent with the findings of Srinivas Rao et al. (2022) and Shalini and Antoine (2018), though differences exist across studies. Filho et al. (2011) reported lower values of volume. Shalini and Antoine (2018), in their study on German shepherd dogs, found comparatively lower values of mean mass motility of canine spermatozoa than those obtained in the present investigation. Initial sperm motility was comparable to the values reported by Dobranic et al. (2005), whereas higher motility levels were documented by Kawakami et al. (2005) and Silva et al. (2009). The percentage of live spermatozoa observed in the present study was slightly higher values reported by Alamo et al. (2005), Kawakami et al. (2005), and Khye et al. (2021). Lower percentages of live spermatozoa were reported by

Table 2: Different macroscopic and microscopic parameters of fresh semen

Sl. No.	Parameters	Observations (Mean±SEm)
1.	Pre sperm fraction (ml)	1.958±0.136
2.	Sperm rich fraction (ml)	2.17±0.16
3.	Post sperm fraction (ml)	4.563±0.375
4.	pH	6.35±0.03
5.	Mass activity (Scale 0-5)	3.91±0.15
6.	Individual motility (%)	86.45±0.97
7.	Live count (%)	87.37±0.98
8.	Abnormal count (%)	9.08±0.255
9.	Sperm concentration (10^6 ml^{-1})	353.89±11.1
10.	HOST (%)	89.0±0.94

Shalini and Antoine (2018) as well as Ray et al. (2019). The proportion of abnormal spermatozoa was found to be lower in the study by Kawakami et al. (2005) and higher in that of Gradil et al. (2006). Sperm concentration values in the present study were consistent with those reported by Belala et al. (2016), although higher concentrations were noted by Shalini and Antoine (2018). Variability in sperm abnormalities and concentrations may reflect individual, breed, age, and environmental differences. HOST results corresponded with Rodriguez-Gil (1994) while lower intact membrane percentages were reported by Violeta and Pana (2007) and Ray et al. (2019), and higher values by Michael et al. (2009) and Arunmozhi et al. (2021).

3.2. Effect of refrigeration preservation

Sperm motility percentages at 0, 24, 48, and 72 hours in groups I and II were 85.21±1.14, 72.71±2.12, 59.17±3.4, 47.92±3.95, respectively. At 0 hours, post-dilution sperm motility percentages were 85.21±1.14 in TRIS-egg yolk-glucose. Similar observations were made by Michael et al. (2009). At 24 hours, almost similar observations were made by Ponglowhapan et al. (2004), which also corroborates our findings. However, at 0, 24, 48, and 72 hours, higher observations were reported by Sanchez et al. (2006), Hori et al. (2014), and Das et al. (2018).

The percentage of live spermatozoa at 0, 24, 48, and 72 hours was 84.17±1.11, 73.63±0.97, 59.17±1.11 and 47.3±1.21, respectively. Almost similar observations were made by Sanchez et al. (2006). However, higher observations were reported by Michael et al. (2009), Das et al. (2018), and lower observations were reported by Srinivas et al. (2022) (Figure 2).

Abnormal sperm percentage at 0, 24, 48 and 72 hours were 10.59±0.28, 12.25±0.27, 13.63±0.26, 14.96±0.29, respectively. In TRIS-Egg yolk glucose, nearly similar

observations were made by Michael et al. (2009), which also corroborates our findings. However, higher observations were reported by Srinivas et al. (2022), and Lower observations were reported by Das et al. (2018) (Figure 3 and 4).

Intact plasma membrane percentage at 0, 24, 48, and 72 hours was 88.8±0.95, 85.21±1.09, 79.84±1.50, 73.75±1.83, respectively. However, higher observations were reported by Michael et al. (2009) and lower observations were reported by Sanchez et al. (2006) (Figure 5) (Table 3).

Table 3: Comparison of the microscopic evaluated parameters of canine semen in TRIS-egg yolk-glucose at different time interval

Time of storage	Individual sperm motility (%)	Live sperm (%)	Abnormal sperm (%)	Intact plasma membrane (%)
0 h	85.21±1.145^{d}	84.17±1.11^{d}	10.59±0.28^{a}	88.80±0.95^{c}
24 h	72.71±2.12^{c}	73.63±0.97^{c}	12.25±0.27^{b}	85.21±1.09^{c}
48 h	59.17±3.48^{b}	59.17±1.11^{b}	13.63±0.26^{c}	79.84±1.50^{b}
72 h	47.92±3.95^{a}	47.3±1.21^{A}	14.96±0.29^{d}	73.75±1.83^{a}

Values bearing uncommon superscripts (a,b,c) differ significantly between storage periods at $p<0.05$

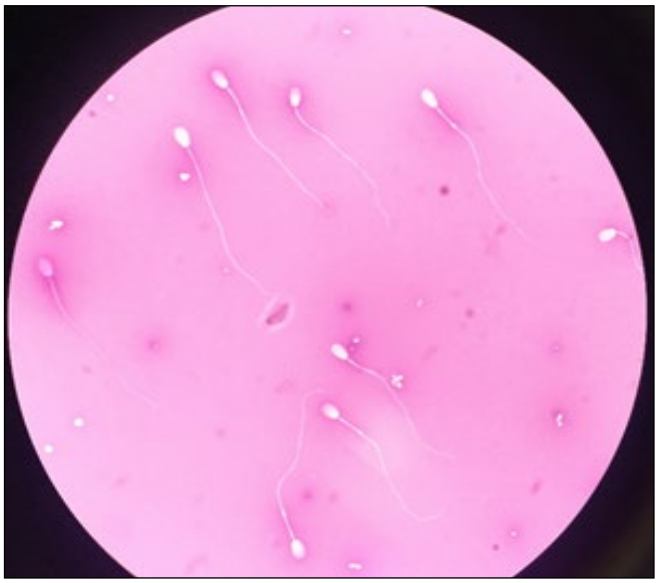

Figure 2: Dead sperms (Pink coloured), live sperms (White coloured)

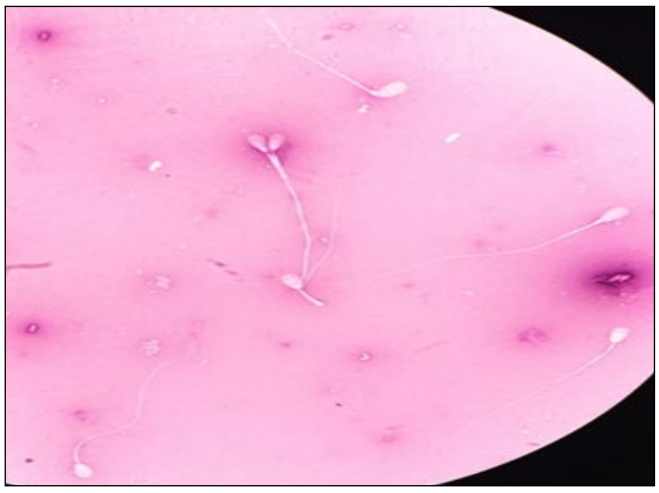

Figure 3: Double heads

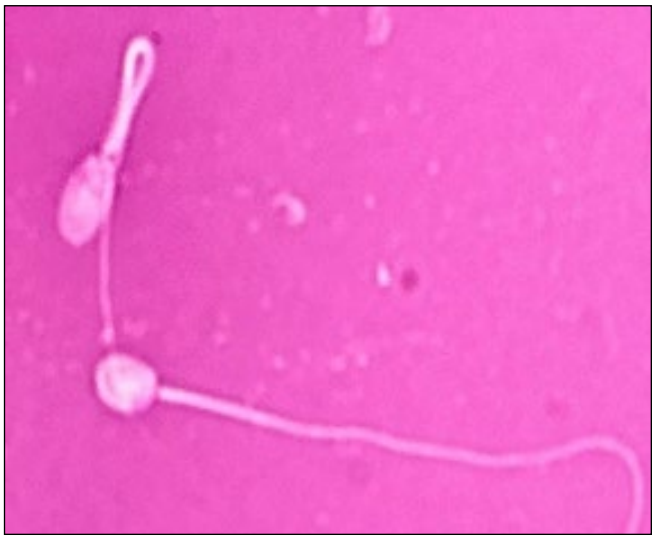

Figure 4: Bent tail

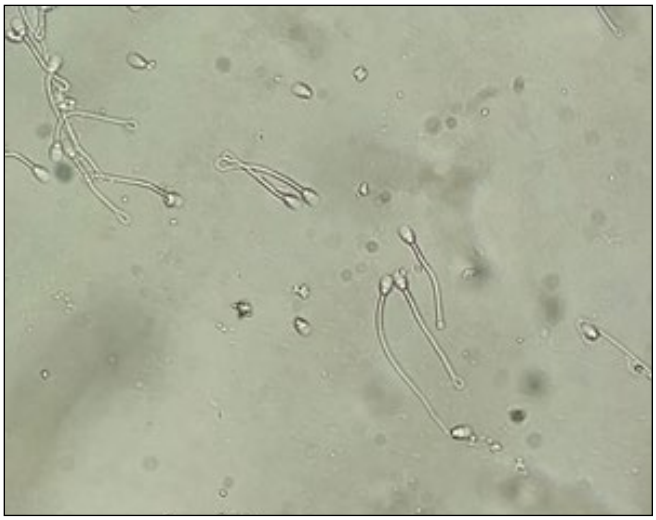

Figure 5: Sperm with curling tail as sperm having intact plasma membrane (40X)

4. CONCLUSION

The current study focused on assessing the TRIS-Egg Yolk-Glucose (TEYG) extender's effectiveness in maintaining the quality of refrigerated canine semen. Findings showed that the TEYG extender preserved semen motility and viability above 50% for 48 hours post-collection. Also, the proportion of sperm with abnormalities was less than 20% for 72 hours, indicating a lag phase in the breakdown of structure. Furthermore, the integrity of the plasma membrane was preserved, remaining above 60% until 72 hours essential for the functionality and fertility potential of the sperm.

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