

## EFFICIENCY OF SKIM MILK WITH AND WITHOUT EGG YOLK EXTENDERS FOR DOG SEMEN PRESERVATION AT REFRIGERATION TEMPERATURE

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### ABSTRACT

The aim of study was to examine the efficiency of skim milk with egg yolk (SMEY) and skim milk without egg yolk (SM) extender in preserving semen of dogs (n=6) at different time slots (0, 24, 48, and 72 hours) at refrigeration temperature (4 °C). Total 24 semen ejaculates were collected by digital manipulation at weekly intervals. The fresh semen was examined for macroscopic (volume, colour, consistency and pH) and microscopic parameters (mass motility, individual motility, sperm concentration, sperm abnormalities, live sperm count and HOST) immediately after collection. Extended samples were evaluated for individual sperm motility, live sperm count, abnormal sperm count, and sperm function test (HOST). The average individual sperm motility, live sperm percentage and HOST was reduced significantly ( $p < 0.05$ ) in SMEY and SM from 0 to 72 hours. When individual sperm motility, abnormal spermatozoa and HOST percentage was compared between SMEY and SM at 0 hour and 24 hour there was non-significant difference but at 48 and 72 hours significant difference was observed. The percentage of live spermatozoa differed significantly at 24, 48, and 72 hours ( $P < 0.05$ ) between extenders. The percentage of abnormal spermatozoa significantly increased from 0 to 72 hours of preservation both in SMEY and SM. In conclusion, the SMEY outperformed the SM extender in terms of preserving the motility, viability and membrane integrity of refrigerated canine semen for up to 72 hours, suggesting it a simple, economical and efficient extender for canine semen refrigeration.

**Keywords:** Dog semen, HOST, Semen extender, Skim milk with egg yolk, Skim milk without egg yolk

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Due to lower costs and simplified rules for import and export compared to frozen semen, chilled, extended semen is becoming more popular in the dog breeding industry. This is because it may be used and shipped internationally more frequently (Ponglowhapan *et al.*, 2004). As chilling process is less expensive and requires limited resources therefore, veterinarians can handle the chilling of dog sperm in their own practices. Chilled canine semen can be deposited in the vagina with a high fertility rate as compared to frozen-thawed semen (Linde-Forsberg, 1991). The insurgence of bioterrorism and avian bird flu the usage of egg yolk in semen extenders for international shipping has decreased. Skimmed milk and egg yolk fulfill the same purpose, which is to preserve the spermatozoa's membranes stable. Skim milk proteins buffer semen pH and may also chelate any heavy metal ions (Batellier *et al.*, 2001). Our study aimed to compare the effects a skim milk with egg yolk and skim milk without egg yolk had on the quality of canine semen at 0, 24, 48, and 72 hours of preservation under refrigeration temperature (4 °C).

### MATERIALS AND METHODS

Semen was collected from 6 dogs (4 German shepherd, 1 Labrador and 1 golden retriever) at weekly interval, for a

total of 24 ejaculates by digital manipulation. The fresh sperm-rich fraction was examined for macroscopic examination included volume, colour, consistency and pH while the microscopic examination included mass motility, individual motility, sperm concentration, sperm abnormalities, live count and hypo-osmotic swelling test (HOST). Following the preliminary evaluations, the sperm-rich fraction of the sperm sample was divided into two equal aliquots; each aliquot was diluted 1:4 in both extender groups; Group I-skim milk with egg yolk (SMEY; Table 1) and Group II-skim milk without egg yolk (SM; Table 1) at room temperature. The extended semen samples were kept in a beaker with water at 37 °C before being chilled to 4 °C in a refrigerator. Diluted samples were examined for individual motility, sperm abnormalities, live & dead count, and HOST at 0, 24, 48, and 72 hours.

Mass motility was observed by drop of fresh semen on a pre-warmed glass slide under a microscope (10x). Individual sperm motility and sperm concentration were evaluated as per the standard procedures described Payan-Carreira *et al.* (2011). On nigrosin/eosin-stained slides, the number of live spermatozoa (%) and abnormal sperm (%) were counted using an oil immersion objective microscope (100x). The spermatozoa (%) with intact plasma membrane

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were determined using the HOST (Jeyendran 1984).

**Statistical analysis:** Data obtained were subjected to analysis by completely randomized design (CRD) by one-way analysis of variance technique (Snedecor and Cochran, 1989) using the statistical package SPSS software 20 version. The mean of different experimental groups was tested for statistical significance by Duncan's multiple range test (Duncan, 1995).

## RESULTS AND DISCUSSION

The volume, pH, sperm concentration, mass motility, individual sperm motility, live sperm, abnormal sperm, and hypo-osmotic swelling test results for fresh sperm samples were  $1.77 \pm 0.06$  ml,  $6.24 \pm 0.02$ ,  $368.75 \pm 13.50$  million per milliliter,  $4.25 \pm 0.14$ ,  $92.92 \pm 0.27\%$ ,  $92.33 \pm 0.41\%$ ,  $6.08 \pm 0.27\%$  and  $92.92 \pm 0.27\%$ , respectively. The colour and Consistency of semen samples was observed as creamy to milky and thin to thick, respectively. The variations in semen volume in present findings may caused by differences in dog size, age, body weight and breeds and frequency of semen collection. However, Srinivas *et al.* (2022) reported lower while Khye *et al.* (2021) and Patti *et al.* (2021) reported higher volume of sperm rich fraction than the present work. Observations of colour of different semen samples were in accordance with the Barve (2014) and Srinivas *et al.* (2022). The consistency of sperm rich fraction and pH with sperm concentration values in the present study was similar to the reported by Barve (2014) and Shalini and Antoine (2018), respectively. Moreover, Khye *et al.* (2021) and Martinez-Barbitta *et al.* (2022) reported lower pH while Srinivas *et al.* (2022) observed higher pH than our observed value. However, Martinez-Barbitta and Rivera (2022) reported higher while Srinivas *et al.* (2022) reported lower sperm concentration than present findings. These differences in sperm concentration may be due to the number of spermatozoa per ejaculate varies with age, testicular weight, sexual activity, and dog size.

The mass motility observed during the present study was higher than reported by Shalini and Antoine (2018) and Srinivas *et al.* (2022) while Dostal *et al.* (2001) reported lower side also. The individual sperm motility (Fig. 1) in the present study was in accordance with Kawakami *et al.* (2005) where as higher and lower sperm motility was reported by Silva *et al.* (2009) and Khye *et al.* (2021), Srinivas *et al.* (2022), respectively. The live spermatozoa count (Fig. 2) in present study was in agreement with Michael *et al.* (2009) whereas Puja *et al.* (2018) reported higher while Srinivas *et al.* (2022) and Martinez-Barbitta and Rivera (2022) reported lower live sperm percentages. The average HOST (Fig. 4) of fresh semen observed in the present study is comparable with the prior findings reported by Patti *et al.* (2021). Average HOST of canine semen was recorded by Barve (2014) which are comparatively lower than the present study values.

The present study aimed to compare the effects of SMEY and SM extenders on sperm motility, live sperm count, and sperm abnormalities in canine semen preservation. The findings of this study were compared with previous research conducted by Rota *et al.* (1995), Diaz *et al.* (2013), Sánchez *et al.* (2006), Barve (2014), Das *et al.* (2018), Allai *et al.* (2015), Allai *et al.* (2017) and Ubah *et al.* (2019) to gain a comprehensive understanding of the results.

Regarding sperm motility, Rota *et al.* (1995) and Barve (2014) reported a decrease in individual sperm motility when using SM and SMEY extenders, respectively, which contrasted with the observations of the present **Table 1. Composition of extenders**

Ingredient	SMEY	SM
Skim milk	100ml	80%
Sodium Penicillin	100IU	1mg/ml
Streptomycin	100mg	1mg/ml
Egg yolk	20%(v/v)	-
Total volume		

**Table 2. Extended semen parameters (Mean±SE) during preservation (4°C) in skim milk with egg yolk (SMEY) and Skim milk without egg yolk (SM)**

Parameter	Extender	0 hour	24 hours	48 hours	72 hours
Individual sperm motility	SMEY	$90.67 \pm 0.60^{Ad}$	$83.38 \pm 0.56^{Ac}$	$76.13 \pm 0.93^{Ab}$	$67.21 \pm 1.00^{Aa}$
	SM	$90.84 \pm 0.83^{Ad}$	$84.59 \pm 0.90^{Ac}$	$66.46 \pm 1.43^{Bb}$	$41.05 \pm 1.34^{Ba}$
Live Sperm	SMEY	$92.38 \pm 0.33^{Ad}$	$86.25 \pm 0.84^{Ac}$	$83.5 \pm 0.68^{Ab}$	$81.17 \pm 0.61^{Aa}$
	SM	$93.34 \pm 0.34^{Ad}$	$81.96 \pm 0.71^{Bc}$	$77.34 \pm 0.93^{Bb}$	$69.09 \pm 1.19^{Ba}$
Abnormal Sperm	SMEY	$5.96 \pm 0.26^{Aa}$	$7.25 \pm 0.27^{Ab}$	$8.67 \pm 0.24^{Ac}$	$9.92 \pm 0.27^{Ad}$
	SM	$6.38 \pm 0.25^{Aa}$	$7.84 \pm 0.27^{Ab}$	$9.92 \pm 0.31^{Bc}$	$11.42 \pm 0.27^{Bd}$
Hypo-Osmotic Swelling Test	SMEY	$92.88 \pm 0.35^{Ad}$	$89.92 \pm 0.51^{Ac}$	$87.55 \pm 0.70^{Ab}$	$81.25 \pm 0.76^{Aa}$
	SM	$92.21 \pm 0.28^{Ad}$	$90.05 \pm 0.63^{Ac}$	$78.09 \pm 0.89^{Bb}$	$70.17 \pm 1.12^{Ba}$

Mean values having different superscripts in a row (a, b, c, d) and in a column (A, B) differ significantly ( $P \leq 0.05$ )

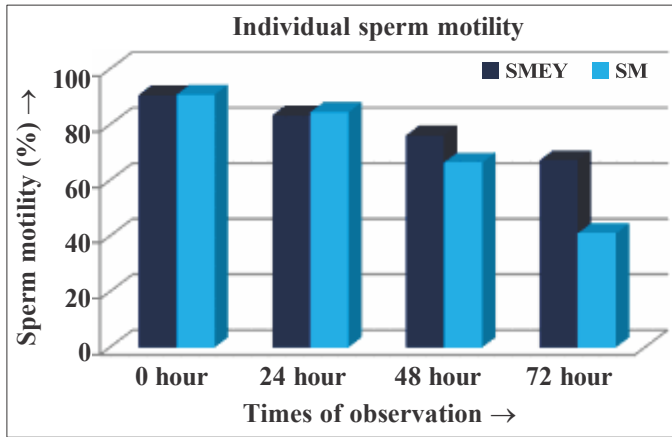


Fig. 1. Individual sperm motility of refrigerated aliquots (n=24)

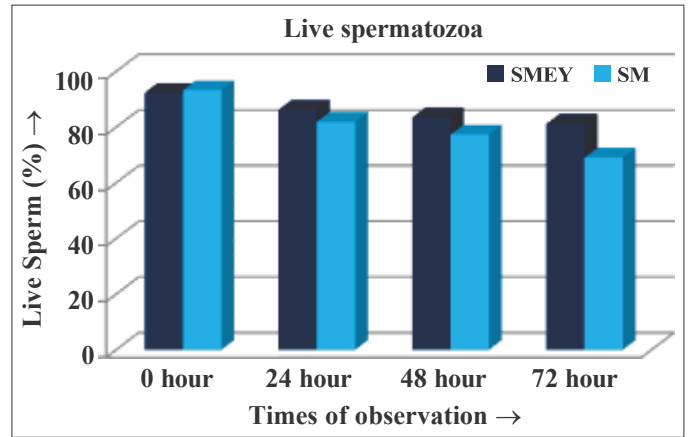


Fig. 2. Percentage of live spermatozoa of refrigerated aliquots (n=24)

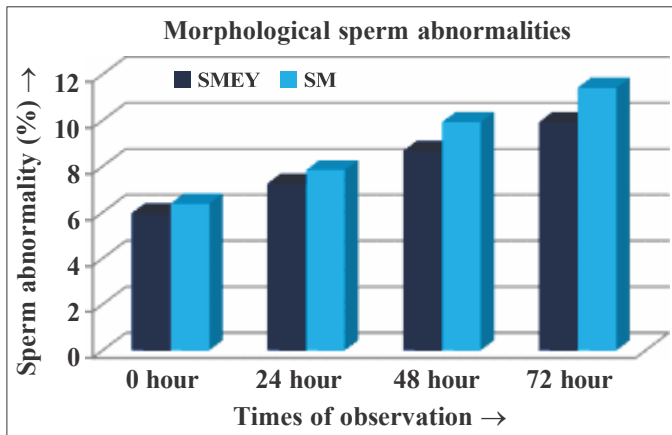


Fig. 3. Morphological sperm abnormalities of refrigerated aliquots (n=24)

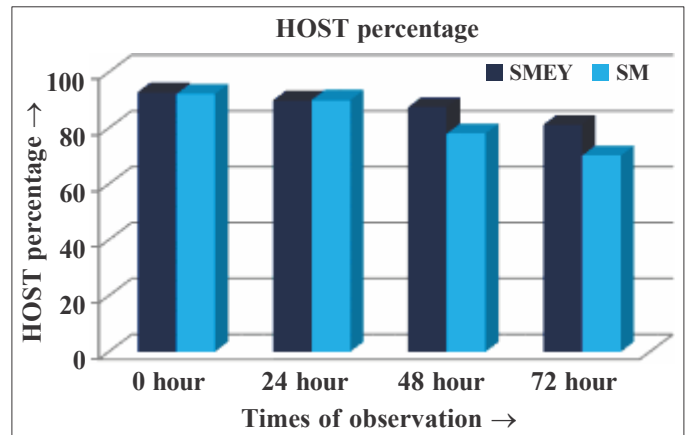


Fig. 4. HOST percentage of refrigerated aliquots (n=24)



Collection of sperm rich fraction



Live spermatozoa with proximal droplet (A) Double headed spermatozoa and dead spermatozoa (B)



Bent tail of spermatozoa



Hypo-osmotic swelling test (HOST) showing coiled sperm tail

study. In the current study, canine semen preserved in the SMEY extender exhibited higher individual sperm motility percentages (86.26%, 83.13%, and 78.13% on days 1, 2, and 3, respectively) compared to the SM extender. However, Diaz *et al.* (2013) found similar sperm motility percentages in SMEY and SM extenders, which aligned with the present study's findings at different preservation time points. Sánchez *et al.* (2006) reported higher sperm motility in SM compared to the present study. These variations in sperm motility among the different studies may be attributed to differences in initial motility before dilution, variations in the composition of the extenders, and environmental factors.

Regarding live sperm count, Diaz *et al.* (2013) reported higher average percentages of live sperm in both SMEY and SM extenders compared to the present study findings at various preservation time points. Conversely, Barve (2014) and Das *et al.* (2018) reported lower and higher percentages of live spermatozoa in SMEY extenders at different time intervals, respectively. Due to the limited literature on canine semen preservation using Skim Milk extender, findings from ram semen were considered. Allai *et al.* (2015) reported live spermatozoa percentages in SM extenders that agreed with the present study findings. Allai *et al.* (2017) also reported similar live spermatozoa percentages in ram semen preserved in SM extender. These contrasting results among studies may be due to variations in extender composition and the specific characteristics of the semen samples used.

Furthermore, Barve (2014) reported higher percentages of abnormal sperm in the SMEY extenders at different time intervals. Ubah *et al.* (2019) reported lower percentages of abnormal spermatozoa in dog semen diluted with the SMEY extenders. Rota *et al.* (1995) reported intact plasma membrane percentages (HOST) for SMEY in line with the present study findings. Diaz *et al.* (2013) found similar percentages of the intact plasma membrane in both SMEY and SM extenders at various time points.

In conclusion, the comparison of SMEY and SM extenders in canine semen preservation revealed varying results among different studies. While the present study demonstrated higher sperm motility percentages in SMEY extenders, previous research reported mixed findings. Live sperm count percentages also differed among studies, with some reporting higher values in both extenders compared to the present study. Similarly, the percentage of abnormal spermatozoa showed discrepancies across studies. These variations may be attributed to differences in initial semen quality, extender composition, and

environmental factors. Further research is needed to understand better the effects of these extenders on canine semen preservation and to optimize the protocols for successful preservation.

## CONCLUSION

SMEY outperformed the SM extender in terms of preserving the motility, viability, and membrane integrity of refrigerated canine semen for up to 72 hours, suggesting it a simple, economical and efficient extender for canine semen refrigeration.

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