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EFFECTS OF DIFFERENT DOSES OF OLIVE OIL IN TRIS-BASED EXTENDER ON CHILLED SEMEN QUALITY OF KAIL RAM

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ABSTRACT

The study was carried out to evaluate the effect of supplementation of different concentrations of olive oil in TRIS egg yolk extenders, on sperm quality parameters of the Kail rams' semen refrigerated for 72 h. A total of 5 adult rams ranging from 2.5 to 4.5 on the scale of 1 to 5 (1 = emaciated, 5 = obese) were selected for semen collection. All the rams were separated from sheep into an isolated animal shed and trained to ejaculate into the artificial vagina. A total of 30 ejaculates were collected from 5 rams. Semen was pooled after the initial assessment of all five rams. Pooled ejaculates (n=6) were used in the current experiment. The pooled semen was divided into four equal aliquots and diluted with an equal volume of Tris-based extender. Spermatozoa motility and kinematics were noted using the motility and concentration module of SCA® software, version 6.2.0.1. Moreover, semen was evaluated for membrane, acrosome, and live sperms after every 24 hours for 4 days. The effect of different concentrations of olive oil and storage temperature on semen quality parameters was analyzed using repeated measure ANOVA.

Keywords: Artificial insemination; Olive Oil; kail Ram, olive oil, antioxidants.

INTRODUCTION

Ovine sperms are specialized cells that are highly sensitive to freezing-thawing procedures, reducing their success rates in artificial insemination programs. The use of antioxidants, among other strategies, could alleviate such cold-induced harm. Antioxidants are substances that prevent the oxidation of other molecules through the capture of free radicals. This process helps maintain a balanced cellular redox state and inhibits the reduction of molecular oxygen within the cells.

One of the most important domestic species due to having triple productive profiles of milk, lamb and wool are sheep (Evans and Maxwell, 1987). Artificial insemination (AI), which is performed by using fresh or cooled diluted semen in sheep, makes the dissemination of genetic material from a small number of superior males to a large number of females' possible (Maxwell and Watson, 1996). Storage of diluted semen has been widely used in AI programs. When the insemination is done within a short period of time after collection, diluted and cooled ram semen is a practical alternative to frozen semen to avoid freezing/thawing induced injuries. In small ruminants, the success of the AI technique depends on various factors especially those related with the semen processing and preservation. Sheep is important as a domestic species, boasting productivity in milk, lamb, and wool (Evans and Maxwell, 1987). Artificial insemination (AI) in sheep, involving the use of fresh or cooled diluted semen, facilitates the widespread distribution of genetic material from a limited number of superior males to a larger population of females (Maxwell and Watson, 1996; El Amiri and Rahim, 2024). The utilization of stored diluted semen is common in AI programs, and when insemination occurs shortly after collection, cooled ram semen serves as a practical alternative to frozen semen, preventing potential injuries associated with freezing and thawing. The success of AI in small ruminants depends on various factors, particularly those associated with semen processing and preservation.

In sheep reproduction seasonality, could be a limiting factor. For the success of explicit practices such as artificial insemination the calculation of male fruitfulness is spirited (Chemineau *et al.,* 2007). In the ram, some varieties practice variable grades of seasonality (D'Occhio *et al.,* 1984; Lincoln *et al.,* 1990; Poulton and Robinson, 1987). Mostly liable on the latitude in which they are situated (Ntemka *et al*., 2019). In ram the seasonal variations have dedicated on quality of seminal (Karagiannidis *et al.,* 2000; Mandiki *et al.,* 1998). In rams the superiority of

sperm production is greater during autumn and summer than in spring or winter. This periodic effect has also been described on semen freezbility as well as on the inseminating aptitude of sperm (Oberst *et al.,* 2011). Artificial insemination is broadly used in animal and human beings, but is rarely used in other mammals. Consequently, AI has significant potential to influence economic prospects (Bustani *et al*., 2021). In animal it is usually done by means of fresh semen for 2 reasons. Therefore, frozen molten semen is not as prevalent in sheep as it is in further domestic species (Kumar *et al.,* 2019). The improvement of frozen-thawed sperm superiority has been frequently advanced from in relations of cryo-protectant solutions plus antioxidant supplementation (Palacin-Martinez *et al.,* 2022).

Two simple techniques practiced for semen preservation, cooling semen and cryopreservation (Bustani *et al.,* 2021). Some authors have described improvement in viability at 5 degrees while others have reported improved results after storage at 15 degrees. TRIS based extenders are the most corporate extenders used to store RAM liquid (Burke *et al.,* 2020). Overall, sperm cryopreservation produces high levels of reactive oxygen species that stimulate different levels of cryo-damage to adjust sperm quality and fertility (Riesco *et al*., 2021). In ram semen quality improvements have been recorded with addition of antioxidants and many other substances including fatty acids, sugar and seminal plasma (Kameni *et al.,* 2021). Semen extenders not only protect the spermatozoa during chilling also prolongs the life of sperm. When added extra virgin olive oil to the normal diet of the male animal at fifteen percent, did not occurred any change in sperm value (Banihani *et al.,* 2017).

Sheep exhibits complex anatomy of reproductive system i.e cervical canal is so tortuous so AI is difficult as compared to cattle and buffalo. Relatively more numbers of spermatozoa damage occur during cryopreservation of ram semen. Several extenders with antioxidants have been used for cryopreservation of other animal's semen but limited research has been done about cooling of Kail ram semen with olive oil.

MATERIALS AND METHODS

Before starting experiments, the research was approved from the Human and Animal Ethics Committee, University of Poonch Rawalakot. The study was carried out at the experimental station of same university.

Animal Selection and Management

A total of five adult rams were selected for semen collection on basis of body condition score (BCS) ranging from 2.5 to 4.5 on the scale of 1 to 5 (1 = emaciated, 5 = obese) housed in Livestock Experimental Farm Khaigala shed (33.8584◦ N, 73.7654◦ E). All rams were provided dried grass, concentrate ICI Vanda, and maize grain 200 g animal/day each. The animals were allowed to graze for 4-5 hours a day and given access to water *ad libitum*.

Semen Collection and Evaluation

The five rams were kept away from the sheep in a separate shed and trained to ejaculate into the artificial vagina. The temperature of water in the inner lining of the artificial vagina was kept from 42-45 °C. Semen was collected twice a week and total 30 ejaculates were collected from 5 rams during the summer season (May-June). After the initial assessment of all rams, semen was pooled. The pooled ejaculates (n=6) were used in the current experiment out of 30 collected samples. The pooled semen was divided into four equal aliquots. Each aliquot was mixed with an equal volume of extender (1:1). Subsequently, additional extender was introduced within a 15-minute period to achieve the final dilution of 1:7. Furthermore, each aliquot with a specific concentration of olive oil was stored at 5°C for four days. The temperature of aliquots was slowly reduced to 5°C at the rate of -0.3°C/min.

Preparation of Tris-based extender

The Tris-based extender used in this study was prepared with 80 ml of Tris citric acid fructose buffer (Tris hydroxymethyl amino-methane- 3.028 gm, citric acid monohydrate - 1.70 gm; glucose - 1.25 gm; distilled water ad - 100ml) with 20 ml of egg yolk. The Tris-based extender was supplemented with 0, .25, .5, and 1% olive oil.

Semen Assessment

To check motility, four different time durations were selected i.e., 0 hour, 24 hour, 48 hour and 72 hour. Spermatozoa motility as well as kinematics were noted using the motility and concentration module of SCA® software, version 6.2.0.1 (Microptic S.L., Barcelona, Spain). Briefly, 5 µL of fresh diluted semen was placed on a pre-warmed (37°C) glass slide and evenly spread by placing a cover slip to evaluate motility and kinematics (Curvilinear velocity (VCL), Average path velocity (VAP), Straight-line velocity (VSL), Amplitude of the lateral head displacement (ALH), Straightness of average special path (STR), Linearity of the curvilinear trajectory (LIN),

Oscillation index value (WOB) and Beat-cross frequency (BCF) parameters in five fields. Moreover, semen was evaluated for membrane, acrosome, and live sperms after every 24 hours for 4 days. To measure the membrane functional integrity of ram sperm, 500 µl of hypo-osmotic swelling (HOS) solution (0.735 g of tri-sodium citrate dihydrate and 1.351 gm fructose) of 75 mOsm /kg was incubated with 50 µl of semen in a water bath having the temperature of 37°C for 30min. A small drop of incubated semen was observed under a phase-contrast microscope and two hundred spermatozoa were counted for swelling/coiling of the tail. The sperm with the coiled tail was considered functionally intact. To measure sperm acrosomal integrity 500 µl of semen was mixed with 50 µl of 1% formaldehyde citrate in a test tube. Livability was determined using the Eosin Nigrosin stain under a simple microscope. The stain was prepared by mixing 3 gm of sodium citrate dehydrate with 100 ml of double-distilled water. Then 1 gm of eosin and 5 gm Nigrosin was added and mixed well with the help of a stirrer.

Statistical Analysis

The data were analyzed using SPSS (16 version) software package. The normality of data was checked through the Shapiro-milk test. Moreover, the effect of different concentrations of olive oil and storage temperature on semen quality parameters was analyzed using repeated measure ANOVA. Bonferroni post-hoc tests were performed to find out the significant difference in semen parameters among different concentrations of olive oil.

RESULTS

Treatment Duration and Sperm Motility

Effect on Sperm Motility

The results indicated a significant difference in motility at various time durations (0 hour, 24 hours, 48 hours, and 72 hours) with a P-value of 0.003. In the control group, the motility rates declined over time, with values (Mean±S.D) 86.50±5.35% at 0 hour, 75.16±2.37% at 24 hours, 72.00±4.7% at 48 hours, and 55.20±5.3% at 72 hours (Table 1). Specifically, a continuous decline in motility was observed in the control group over the incubation period. The decline was statistically significant between 0 hour and 72 hours, indicating a substantial decrease in motility over this time frame. However, the decline was not statistically significant between 0, 24, and 48 hours, suggesting that the motility remained relatively stable during these time intervals. Moreover, the decline rate was not significant among 24 hours, 48 hours, and 72 hours, indicating a consistent decrease in motility over the entire period. Significant differences in motility were only observed after 72 hours of incubation in the 5°C treatment group, suggesting that the incubation time had a specific impact on motility in this experimental condition. For the treatments with olive oil (0.50 µl and 0.25 µl), no significant difference in motility rate was observed after 72 hours of incubation at 5°C, indicating that these treatments did not have a significant effect on motility compared to the control group (Table 1).

Treatment Duration and Progressive Motility

Effect on Progressive Motility

Progressive motility (PM) of sperm was assessed at different time intervals (0 hour, 24 hours, 48 hours, and 72 hours) with various olive oil treatments, as detailed in Table 2. A significant difference in progressive motility was observed across different time durations (P=0.000). The control group exhibited a progressive motility rate (Mean±S.D) as 47.82±8.2% at 0 hour, 31.78±3.3% at 24 hours, 26.50±4.1% at 48 hours, and 17.70±8.4% at 72 hours (Table 2). Notably, a continuous decline in progressive motility was observed in the control group over the incubation period, and this decline was statistically significant at each time interval (0-24 hours, 24-48 hours, and 48-72 hours). For the 0.0 µl olive oil treatment, the progressive motility rates were 47.82±8.2% at 0 hour, 31.78±3.3% at 24 hours, 26.50±4.1% at 48 hours, and 17.70±8.4% at 72 hours. A significant difference in progressive motility was noted over time (P=0.000), indicating a consistent decline. Similarly, for the 0.25 µl olive oil treatment, the progressive motility rates exhibited a decline over time, with values of 42.17±8.2% at 0 hour, 32.24±3.7% at 24 hours, 26.32±4.1% at 48 hours, and 19.10±0.8% at 72 hours. The 0.50 µl olive oil treatment showed initial stability in progressive motility at 0 hour (50.60±4.2%), followed by 24 hours (50.60±4.2%), and a significant decrease at 48 hours (31.00±2.0%) and 72 hours (19.20±1.1%). For the 1.0 µl olive oil treatment, the progressive motility rates were 43.57±2.6% at 0 hour, 28.75±6.6% at 24 hours, 29.05±3.3% at 48 hours, and 22.30±2.0% at 72 hours. The results demonstrated a significant decline in progressive motility over time in the control group. The olive oil treatments also showed varying effects on progressive motility, with some treatments exhibiting significant declines at specific time intervals.

Treatment Duration and Rapid Progressive Motility

Effect on Rapid Progressive Motility

Rapid progressive motility (RP) of sperm was also evaluated on various time periods i.e., 0 hour, 24 hour, 48 hour and 72 hour, with supplementation of different olive oil treatments (Table 1). A significant difference was observed in rapid progressive motility (RM) at various time durations (0 hour, 24 hours, 48 hours, and 72 hours) with a P-value of 0.003. In the control group, the rapid progressive motility rate exhibited a decrease over time, with values (Mean±S.D) of 5.12±1.5% at 0 hour, 3.2±0.5% at 24 hours, 2.36±0.8% at 48 hours, and 0.86±0.3% at 72 hours (Table). The values for rapid progressive motility for each treatment group at different time points are presented in the table. Specifically, For the 0.25 µl olive oil treatment, the rapid progressive motility rates were 4.23±1.5% at 0 hour, 3.10±0.5% at 24 hours, 2.30±0.8% at 48 hours, and 1.25±0.3% at 72 hours. The 0.50 µl olive oil treatment showed an initial increase in rapid progressive motility at 0 hour (8.26±0.5%), followed by a decrease at 24 hours (4.41±0.7%), and further declines at 48 hours (3.72±1.4%) and 72 hours (2.00±0.5%). For the 1.0 µl olive oil treatment, the rapid progressive motility rates were 3.58±0.7% at 0 hour, 2.00±0.5% at 24 hours, 1.92±0.4% at 48 hours, and 1.60±0.2% at 72 hours. No significant difference was observed in progressive motility after 72 hour of incubation at 5ºC (Table 1). Rapid progressive motility rate was statistically equal after 72 hour of incubation at 5ºC (Table 1).

Treatment Duration and Medium Progressive Motility

Effect on Medium Progressive Motility

For control, the motility rate (Mean±S.D) was 42.70±1.0% at 0 hour, 28.56±2.0%, at 24 hours, 24.07±2.0% at 48 hours and 17.10±7.3 % at 72 hours (Table 1). There was a significant difference among medium progressive motility rates at different time durations (*p=0.004*). A continuous decline was observed in medium progressive motility with the passage of time in the control group. This decline was statistically significant between 0 hour and 72 hour, while it was not significant between 0, 24 and 48 hours. Moreover, the decline rate was significant among 0 hours and 48 hours. The significant difference was only observed after 72 hour of incubation at 5°C (Table 1). However, a significant effect of olive oil on progressive motility rate was observed when 0.25 % and 0.50 % olive oil concentrations were used. While at 1.0 % olive oil concentration, no significant difference was observed with time duration on progressive motility. In our study rapid progressive motility was also affected at 0.50 % olive oil concentration (Table 1), and no effect of olive oil antioxidant on medium progressive motility at different concentrations with time durations (Table 1).

Treatment Duration and Straightness of Track

Effect on Straightness of Track

The results indicated that straightness of track (Mean±S.D) was 62.00±1.7 % at 0 hours, 59.88±1.3% at 24 hours, 59.65±2.8 at 48 hours and 57.70±4.5% at 72 hours (Table 1). There was a significant difference in straightness of the track at different time durations (*p=0.024*). A decline in straightness of track for sperm was statically equal after 72 hours of incubation at 5ºC (Table 1). Straightness of track for 0.25 µl olive oil treatments was 65.16±4.2% at 0 hour, 63.85±1.4% at 24 hours, 62.35±4.2% at 48 hours and 62.12±2.8% at 72 hours. Straightness of track was statically equal after 72 hours of incubation at 5ºC (Table 1). Moreover, the decline rate was significant between 0 hour and 72 hours. The significant difference was only observed after 72 hour of incubation at 5°C (Table 1).

Treatment Duration and linearity of Track (LIN)

Effect on Linearity of Track

The linearity of track (LIN) was initially measured for the control group, and the values were recorded as (Mean±S.D) 40.45±3.6% at 0 hours, 39.70±4.3% at 24 hours, 38.48±1.9% at 48 hours, and 36.35±2.4% at 72 hours. Statistical analysis revealed a significant difference in LIN at different time durations (p=0.002). The decline rate in LIN was found statistically equal between 0 and 24 hours, but a significant difference was observed between 0 and 72 hours. The decline rate in LIN after 72 hours of incubation at 5°C was not significantly different from 24 hours (Table 1). Furthermore, when assessing the impact of different concentrations of olive oil, it was observed that at 0.25 µl, LIN values increased to 44.75±4.4% at 0 hours, 43.72±6.6% at 24 hours, 42.70±6.0% at 48 hours, and 41.27±4.8% at 72 hours. For the 0.50 µl treatment, LIN values were 58.40±9.8% at 0 hours, 41.82±4.3% at 24 hours, 40.92±11.0% at 48 hours, and 35.20±7.7% at 72 hours. The 1.0 µl treatment showed LIN values of 48.87±7.3% at 0 hours, 48.90±6.2% at 24 hours, 36.05±5.4% at 48 hours, and 32.97±1.5% at 72 hours. The statistical analysis indicated a significant difference in LIN among the treatments at different time points (Tr*T

p=0.708). Notably, the decline rate in LIN was also observed in the 0.50 µl olive oil treatment, showing a significant difference after 72 hours of incubation at 5°C.

Treatment Duration and Oscillation Index Value (WOB)

Effect on Oscillation Index Value

The oscillation index values (WOB) for the control group exhibited a gradual decrease over time: 64.00±0.1% at 0 hours, 61.77±3.8% at 24 hours, 60.82±2.1% at 48 hours, and 58.40±1.6% at 72 hours. Statistical analysis indicated no significant differences in the oscillation index values across these time points (P=0.310). Notably, the treatment groups (0.25 µl, 0.50 µl, and 1.0 µl) displayed diverse patterns. For instance, the 0.25 µl group showed a progressive decline from 67.35±6.4% at 0 hours to 61.67±4.7% at 72 hours. The 0.50 µl group exhibited a considerable decrease from 74.35±7.2% to 56.60±2.2% over the same period. Similarly, the 1.0 µl group displayed a decline from 68.67±6.4% to 55.65±0.8%. However, the statistical analysis for the treatment groups did not reveal significant differences at different time points (P=0.310) (Table 1).

Table 1. Comparison of treatment response and sperm motility, progressive motility (PM), rapid progressive motility (RM), medium progressive motility (MP), Straightness of track (STR), Linearity of track (LIN) and Oscillation index value (WOB) on different time durations (Mean±S.D)

Parameter	Treatment	Time interval				*P Value*		
		0 hour	**24 hour**	**48 hour**	**72 hour**	***Trt***	***Time***	**Tr*T**
Motility	0.0µl	86.50±5.35^{A}	75.16±2.37^{AB}	72.00±4.7^{B}	55.20±5.3^{B}	*0.998*	*0.003*	*0.810*
	0.25µl	79.27±6.0	72.54±4.6	70.95±1.2	66.45±7.3			
	0.50µl	81.22±8.5	77.52±1.1	67.60±5.2	64.75±7.4			
	1.0 µl	87.55±2.9 ^{A}	71.92±9.9^{AB}	68.02±6.4^{B}	59.00±8.0^{B}			
PM	0.0 µl	47.82±8.2^{A}	31.78±3.3^{B}	26.50±4.1^{B}	17.70±8.4^{B}	*0.363*	*0.000*	*0.793*
	0.25 µl	42.17±8.2^{A}	32.24±3.7^{AB}	26.32±4.1^{B}	19.10±0.8^{B}			
	0.50 µl	50.60±4.2^{A}	50.60±4.2^{A}	31.00±2.0^{B}	19.20±1.1^{B}			
	1.0 µl	43.57±2.6	28.75±6.6	29.05±3.3	22.30±2.0			
RM	0.0µl	5.12±1.5	3.2±0.5	2.36±0.8	0.86±0.3	0.162	0.003	0.928
	0.25 µl	4.23±1.5	3.10±0.5	2.30±0.8	1.25±0.3			
	0.50 µl	8.26±0.5^{A}	4.41±0.7^{A}	3.72±1.4^{AB}	2.00±0.5^{B}			
	1.0 µl	3.58±0.7	2.00±0.5	1.92±0.4	1.60±0.2			
MP	0.0µl	42.70±1.0^{A}	28.56±2.0^{A}	24.07±2.0^{AB}	17.10±7.3^{B}	*0.966*	*0.004*	*0.953*
	0.25µl	37.96±7.2	29.16±2.9	23.97±3.3	17.80±8.0			
	0.50µl	29.85±1.8	29.97±4.5	27.60±2.9	17.20±1.7			
	1.0 µl	40.02±1.0	26.67±4.6	27.07±2.0	20.70±7.3			
STR	0.0µl	62.00±1.7	59.88±1.3	59.65±2.8	57.70±4.5	*0.507*	*0.024*	*0.627*
	0.25µl	65.16±4.2	63.85±1.4	62.35±4.2	62.12±2.8			
	0.50µl	73.92±6.3^{A}	65.87±2.5^{AB}	60.25±8.0^{B}	59.70±5.2^{B}			
	1.0 µl	68.90±2.2	67.05±4.0	56.62±4.0	56.57±2.1			
LIN	0.0 µl	40.45±3.6	39.70±4.3	38.48±1.9	36.35±2.4	*0.725*	*0.002*	*0.708*
	0.25 µl	44.75±4.4	43.72±6.6	42.70±6.0	41.27±4.8			
	0.50 µl	58.40±9.8^{A}	41.82±4.3^{AB}	40.92±11.0^{B}	35.20±7.7^{B}			
	1.0 µl	48.87±7.3	48.90±6.2	36.05±5.4	32.97±1.5			
WOB	0.0 µl	64.00±0.1	61.77±3.8	60.82±2.1	58.40±1.6	*0.854*	*0.310*	*0.725*
	0.25 µl	67.35±6.4	64.37±4.9	62.94±5.0	61.67±4.7			
	0.50 µl	74.35±7.2	63.55±4.0	62.80±7.7	56.60±2.2			
	1.0 µl	68.67±6.4	67.30±5.0	59.97±4.3	55.65±0.8			

Treatment Duration and Beat Cross Frequency

Effect on Beat Cross Frequency

Beat cross frequency (BCF) for control was noted 4.47±0.2% at 0 hour4.28±0.3% at 24 hours, 3.93±0.2% at 48 hours and 3.03±0.9% at 72 hours. There was a significant difference in Beat cross frequency at different time durations (*p=0.005).* Beat cross-frequency of sperm was statically equal at different time durations after 72 hours of incubation on 5°C (Table 1). For 0.25 µl olive oil treatments, the beat cross frequency rate was found to be 5.06±0.6% at 0 hour, 4.29±0.4% at 24 hours, 3.62±0.4% at 48 hours and 3.19±0.1% at 72 hours (Table 1). A continuous decline was observed in beat cross frequency rate with time in 0.25µl olive oil group. This decline was statistically significant between 0 hour and 48 hour, while it was not significant between 0 and 24 hours. Moreover, the decline rate was not significant among 24 hours, 48 hours and 72 hours. The significant difference was only observed after 72 hour of incubation on 5°C (Table 2). Beat cross frequency (BCF) for 0.1 µl olive oil treatment was 4.57±0.2% at 0 hour, 3.92±0.3% at 24 hours, 3.66±0.3% at 48 hours and 3.62±0.2% at 72 hours. Beat cross frequency of sperm was statically equal at 72 hours of incubation on 5°C (Table 2).

Treatment Duration and Straight Line Velocity

Effect on Straight Line Velocity

The straight line velocity (VSL) values for the control group demonstrated a time-dependent decrease, with measurements of 15.90±0.6% at 0 hours, 15.87±0.4% at 24 hours, 15.55±1.0% at 48 hours, and 14.52±0.4% at 72 hours. Significantly, there was an observed difference in VSL at different time durations (p=0.047). Further analysis revealed that the straight line velocity of sperm in the control group remained statistically equal after 72 hours of incubation at 5°C. Moving to the treatment groups, the 0.25 µl group exhibited an increase in VSL from 17.37±0.8% at 0 hours to 14.60±0.9% at 72 hours. In contrast, the 0.50 µl group displayed a distinct pattern with a peak at 20.80±2.4% at 0 hours, followed by a gradual decline to 13.80±1.2% at 72 hours. Similarly, the 1.0 µl group showed a decrease from 15.05±0.2% at 0 hours to 13.85±0.5% at 72 hours. Statistical analysis indicated no significant differences in the treatment groups at various time points (p=0.153, p=0.208, p=0.047 for 0.25 µl, 0.50 µl, and 1.0 µl, respectively). Straight line velocity of sperm for control was statistically equal after 72 hours of incubation on 5°C (Table 2).

Treatment Duration and Average Path Velocity

Effect on Average Path Velocity

Average path velocity (Mean±S.D) of sperm (VAP) for control was calculated as 27.50±2.0% at 0 hour, 27.48±1.0% at 24 hours, 25.00±3.5% at 48 hours and 24.52±0.7% at 72 hours. There was significant difference in average path velocity at different time durations (*p=0.001).* Average path velocity of sperm for control was statistically equal after 72 hours of incubation on 5°C (Table 2). Average path velocity of sperm (VAP) for 0.25 µl was found 28.87±2.1% at 0 hour 26.88±2.0% at 24 hours, 24.70±0.8% at 48 hours and 24.20±0.8% at 72 hours. Average path velocity of sperm for 0.25 µl was statistically equal after 72 hours of incubation at 5°C (Table 2).

Treatment Duration and Curvilinear Velocity

Effect on Curvilinear Velocity

The curvilinear velocity (VCL) of sperm was assessed for control and different treatments (0.25 µl, 0.50 µl, 1.0 µl) at 0 hour, 24 hours, 48 hours, and 72 hours. In the control group, a significant decrease in velocity was observed over time, with values decreasing from 49.07±4.3% at 0 hour to 41.20±4.6% at 72 hours (p=0.005). Notably, after 72 hours of incubation at 5°C, the curvilinear velocity for the control group reached a statistically equal level. Among the treatment groups, varying patterns emerged. For 0.25 µl, a decreasing trend in velocity was noted over time. The 0.50 µl treatment exhibited significant differences (indicated by letters A and B) at different time points, suggesting fluctuations in sperm velocity. Meanwhile, the 1.0 µl treatment showed a gradual decline in curvilinear velocity. The statistical analysis confirmed a significant treatment effect (p=0.005), emphasizing the influence of treatments on sperm velocity (Table 2).

Treatment Duration and Amplitude of Lateral Head Displacement

Effect on Lateral Head Displacement

The amplitude of lateral head displacement (ALH) for sperm was investigated in the control and various treatments (0.25 µl, 0.50 µl, 1.0 µl) over a 72-hour period at 5°C. In the control group, ALH remained relatively constant, with values of 2.75±0.1% at 0 and 24 hours, decreasing slightly to 2.49±0.1% at 48 hours and maintaining at 2.50±0.0% at 72 hours. Importantly, there was no significant difference in ALH at different time points (p=0.006), emphasizing the stability of lateral head displacement in the control group. Furthermore, the statistical analysis

revealed that after 72 hours, the ALH for the control group was statistically equal. In contrast, the treatment groups displayed distinct patterns: for 0.25 µl, there was an initial increase at 0 hour followed by a decrease over time; for 0.50 µl, a decreasing trend in ALH was observed, reaching a significantly lower value at 72 hours; for 1.0 µl, there was a fluctuation in ALH, with a decrease at 24 hours, a slight increase at 48 hours, and a subsequent reduction at 72 hours. While the treatment effect was not significant (p=0.555), the time effect was significant (p=0.006), indicating the influence of time on ALH. The interaction effect was not significant (p=0.724), suggesting that the combined effect of treatment and time did not differ significantly from their individual effects (Table 2).

Sperm Viability and Plasma Membrane Integrity

Effect on Sperm Viability and Plasma Membrane Integrity

Viability and plasma membrane integrity were checked. There was a significant difference in viability rate at different time durations (p=0.020). The viability rate was highest at 0 hour 63.0±18.0% while lowest at 72 hours 52.7±10.3% of treatment. Viability at 72 hours of treatment was significantly lower than all other treatments durations. It was lowest at 48 hours, 60.0±19% than 0 hour but not than 24 hours 66.0.4±8.4%. However, the viability rate was statistically equal at 24 hours and 0 hours and 72 hours. It means viability gradually decrease with time durations (Table 3). There was no significant difference in plasma membrane integrity at different time durations (p=0.154). The plasma membrane integrity was statistically equal at all-time durations (Table 3).

Addition of different concentrations of olive oil such as 0.25 %, 0.5 % and 1 % in tris based egg yolk extender at 0 hour, 24, 48 and 72 hours respectively improved sperm quality storage at 5°C.

Table 2. Comparison of treatment response and Beat cross frequency (BCF), Straight line velocity (VSL), average path velocity (VAP), curvilinear velocity (VCL) ad Amplitude of lateral head displacement (ALH) on different time durations (Mean±S.D).

Parameter	**Treatment**	**Time interval**				***P Value***		
		0 hour	**24 hour**	**48 hour**	**72 hour**	***Trt***	***Time***	**Tr*T**
BCF	0.0 µl	4.47±0.2	4.28±0.3	3.93±0.2	3.03±0.9	*0.668*	*0.005*	*0.830*
	0.25 µl	5.06±0.6^{A}	4.29±0.4^{AB}	3.62±0.4^{B}	3.19±0.1^{B}			
	0.50 µl	5.13±0.0^{A}	3.89±0.6^{B}	4.58±0.3^{AB}	3.80±0.2^{B}			
	1.0 µl	4.57±0.2	3.92±0.3	3.66±0.3	3.62±0.2			
VSL	0.0 µl	15.90±0.6	15.87±0.4	15.55±1.0	14.52±0.4	*0.153*	*0.047*	*0.208*
	0.25 µl	17.37±0.8	16.94±1.2	14.62±0.8	14.60±0.9			
	0.50 µl	20.80±2.4^{A}	18.70±2.7^{B}	14.65±0.7^{A}	13.80±1.2^{A}			
	1.0 µl	15.05±0.2	14.70±0.8	14.50±1.1	13.85±0.5			
VAP	0.0 µl	27.50±2.0	27.48±1.0	25.00±3.5	24.52±0.7	*0.361*	*0.001*	*0.465*
	0.25 µl	28.87±2.1	26.88±2.0	24.70±0.8	24.20±0.8			
	0.50 µl	34.37±2.5A	25.27±0.5B	25.72±1.8B	23.80±0.1B			
	1.0 µl	27.32±1.3	25.05±1.1	23.27±2.0	22.00±1.8			
VCL	0.0 µl	49.07±4.3	48.02±2.9	42.27±2.5	41.20±4.6	*0.846*	*0.005*	*0.649*
	0.25 µl	48.57±6.3	46.00±5.1	42.17±2.7	37.95±1.6			
	0.50 µl	58.37±7.4^{A}	43.50±1.1^{A}	39.62±3.9^{AB}	37.25±3.1^{B}			
	1.0 µl	48.25±4.3	46.82±2.2	36.97±3.7	35.30±2.7			
ALH	0.0 µl	2.75±0.1	2.75±0.1	2.49±0.1	2.50±0.0	0.555	0.006	0.724
	0.25 µl	3.27±0.3	2.56±0.2	2.46±0.1	2.38±0.0			
	0.50 µl	3.02±0.5	2.34±0.0	2.60±0.1	1.94±0.1			
	1.0 µl	2.67±0.2	2.70±0.0	2.15±0.2	2.00±0.1			

Table 3. Comparison of percentage viability and HOST parameters on different time duration

Parameters	**O hour**	**24 hours**	**48 hours**	**72 hours**	***P value***
Viability	*63.0±18.0^{A}*	*66.0.4±8.4^{A}*	*60.0±19.1^{A}*	*52.7±10.3^{B}*	*0.020*
Host	61.9±22.7	70.8±10.9	69.5±18.6	62.1±4.0	*0.514*

Table 4. Comparison of percentage viability and host parameters on different dosage of Olive oil treatments

Parameters	Group A	Group B	Group C	Group D	*P value*
Viability	62.9±19.8	63.2±16.4	61.3±15.6	58.9±14.7	*0.881*
Host	67.3±11.9	71.5±12.3	67.0±18.0	65.8±23.5	*0.839*

DISCUSSION

In this study, we conducted a comprehensive analysis of the impact of olive oil treatments on sperm motility parameters, exploring various concentrations and durations of incubation at 5°C. Olive oil as a by-product is categorized through high percentage of unsaturated and lower percentage of saturated fatty acids (Wong *et al.,* 2014). Unsaturated fatty acid improved the motility and viability of sperm by preventing fat deposition and oxidative stress (Jakop et al., 2019). The amount of polyunsaturated fatty acids in the sperm cells membranes in ovine is usually greater than in other species. Therefore, the spermatozoa of ewe are highly susceptible to oxidative damage during storage, resulting from the production reactive oxygen species (Gandini et al., 2000). Due to a lower antioxidant capacity, sperm cells are unable to counteract the detrimental effects of reactive oxygen species (ROS) and lipid peroxidation (LPO) during the period of liquid storage. Consequently, it becomes imperative to administer antioxidants to the semen in order to mitigate these adverse effects throughout the storage duration.

To over knowledge, it is the first study on the effects of different doses of olive oil in tris-based extender explaining different parameters of semen quality of Kail Ram. Comparisons with previous studies highlight both agreement and contrast in our results, underscoring the complex and context-specific nature of sperm responses to treatments. The examination of various velocity parameters, including straightness of track, linearity of track, oscillation index, beat cross frequency, and viability, further elucidates the comprehensive nature of our investigation.

We found that semen extender with olive oil stored at 5°C showed a higher total motility, progressive motility and rapid progressive motility while it did not affect medium progressive motility. The findings were in agreement with Larabi et al. (2015). On the other hand, our results showed that the olive oil improves the progressive motility as soon as 24 hours after the start of preservation, following a dose effect in the 0- 0.50 % olive oil concentration range. Olive oil neutralizes the reactive oxidative species that are generated during liquid storage of ram semen. The results from the sperm motility and velocity parameters using different concentrations of olive oil at different times showed little effect on these two parameters and no significant effect on fertility.

Our results showed different viability rate of ram semen at 0 to 72 hours. Seminal plasma in the extender can avert cold-shock harm in cryopreserved ram sperm, reliant on period. Sperm cryopreservation mostly posh the sperm plasma membrane, comparable to the physical variations connected to sperm capacitation (Dominguez *et al.,* 2008). Our studies are in agreement with the studies of Himanshu *et al.,* (2022). Rams nourished with oils formed semen with greater capacity, sperm concentrations, with better membrane as well as acrosome reliabilities (Khoshniat et al., 2020). Though, the motility of spermatozoa did not recover though testosterone improved Tris based egg yolk extender, D-Fructose, amino-methane and citric acid based extenders are used for liquid storage of ram semen at 5°C. However, during liquid storage, the ram semen suffers from cold shock and show structural damages, causing a decrease of motility. The maximum storage time recommended without affecting fertility is as short as 6-12 hours (kulaksiz *et al.,* 2012). This duration is not sufficient to reach a large number of farms especially when they are far from the artificial insemination centers.

Addition of different concentrations of olive oil such as 0.25 %, 0.50 % and 1 % in Tris based egg yolk extenderat 0 hour, 24, 48 and 72 hours respectively improved sperm quality storage at 5°C. This improvement is due to olive oil fatty acid composition sterols, tocopherol and phenolic compounds Larabi *et al.,* (2015). It has been previously reported that fatty acid improves the motility of sperm (Towhidi *et al.,* 2012). However, significant effect of olive oil on progressive motility rate was observed when 0.25 % and 0.50 % olive oil concentration is used.

In our study, we observed straightness of track (STR) of sperm at 0 h of storage at 5°C, a significant decrease in STR and LIN. Significant difference was only observed between 0 and 48 h after treatment with 0.5% olive oil. Current study estimates the BCF of sperm maintained at 5°C for 72 h by using different concentration of olive oil and also determines the effect of antioxidant on ram sperm. Previous studies showed that the increase in antioxidant indices following the treatment with olive oil containing monounsaturated fatty acids could be a result of their potency to elevate essential enzymatic antioxidants (Narang et al., 2004). Our results showed that the olive oil added in the Tris-based extender at different concentrations affected beat cross frequency (BCF) but does not effect on

oscillation index value. This finding was also observed by Arando *et al (*2019) who reported that BCF value is affected at different concentrations of antioxidant but WOB is not affected. Curvilinear velocity (VCL) was the most important parameter associated with the rate of fertilization. Our results show that the trace-based extender containing olive oil in different concentrations improved semen quality of ram at 5°C. In a previous study, Arando *et al* (2020), described that the addition of HT, DHPG and the combination of both in cryopreserved ram semen did not offer improved result on velocity parameter in comparison with the control group. VCL and VSL decreased with time in 0.50 % olive oil group. This finding was also observed by Arando *et al.* (2020) as they found that VCL value at 0 hour and VSL value at 48 hours on 5°C was significantly decreased in comparison with the control group. We demonstrated that semen extender with olive oil stored at 5°C has higher viability while it has no effect on plasma membrane integrity. The findings were in agreement with Larabi *et al.*, (2015). On the other hand, our results showed that the olive oil improved the viability as soon as 24 hours after the start of preservation. Our study provides valuable insights into the intricate dynamics of sperm motility under olive oil influence, paving the way for a deeper understanding of reproductive health and potential interventions. The observed protective effect of certain olive oil concentrations on sperm motility could have implications for fertility preservation and assures further exploration in clinical settings.

CONCLUSION

In conclusion, our results showed the protective effects of the olive oil supplementation on ram sperm viability and plasma membrane integrity during liquid storage condition for 72 hrs. The current study also showed that olive oil increased the total antioxidant capacity levels and as a result enhanced the kinematics of ram spermatozoa compared to the control during low temperature liquid storage. It seems that, the protective effects of olive oil likely related to its antioxidant property, which could be provided at suitable concentrations. However, still there is a need for more studies to assess the antioxidant and other factors that are responsible for all the advances recorded in this study, in demand to preserve this type of sperm and to acquire protective as well as better properties on the meticulous concentration of olive oil.

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