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RESEARCH ARTICLE

Effect of Apilarnil Addition to Semen Extender on Post-thaw Sperm Quality Parameters of Rams

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ABSTRACT

Apilarnil is a bee product obtained after the collection of drone larvae aged 3-7 days. It contains water, proteins, carbohydrates, fatty acids, minerals, and is rich in essential amino acids, antioxidants, as well as reproductive hormones. It contributes to the maintenance of normal metabolism due to its amino acids, vitamins, minerals, glycerophosphate calcium and folic acid contents. This study was designed to investigate the effects of different levels of apilarnil supplementation to tris-egg yolk glycerol extender on post-thaw semen quality in 6 Akkaraman rams aged around 2 years. Semen was collected by artificial vagina and diluted at 37ºC using five semen diluents with or without Apilarnil including 0% (Control), 0.5%, 1.0%, 1.5% and 2.0% apilarnil. Diluted semen was cooled to 5ºC; after glycerolisation and equilibration, it was frozen in liquid nitrogen, stored at -196ºC and evaluated for post-thaw sperm quality. According to our results, total sperm motility, progressive sperm motility and curvi-linear velocity (VCL) values were higher in 0.5, 1.0 and 1.5% apilarnil supplemented groups compared to the control group ($p<0.05$). Values for sperm acrosomal damage were decreased in all apilarnil-containing groups than in the control group ($p<0.05$). Plasma membrane integrity was higher in the 0.5, 1.0, and 1.5% apilarnil-containing groups than in the control group ($p<0.05$). Malondialdehyde levels were lower in all apilarnil-treated groups compared to the control group ($p<0.05$). In conclusion, supplementation of apilarnil at 0.5, 1.0 and 1.5% to tris-egg yolk glycerol diluent increased sperm motility and protected plasma membrane integrity and acrosomal membrane integrity of post-thaw ram spermatozoa.

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INTRODUCTION

Artificial insemination is one of the first techniques in the reproduction of domestic animals (Maxwell and Watson, 1996). In this regard, cryopreservation of semen and the development of reproductive technology are at the forefront of artificial insemination (Alcay *et al.*, 2015). However, during freezing and long-term storage of semen in liquid nitrogen, sperm may suffer physical, chemical, and oxidative damage due to overproduction or imbalance in the production of reactive oxygen species (Liu *et al.*, 2020). In farm animals, especially in rams, cold shock sensitivity of sperm is higher than in other species due to the low intra-membrane cholesterol/phospholipid ratio (Saha *et al.*, 2022). In order to eliminate these harmful effects on ram sperm during freezing, different substances are added to the semen diluent as antioxidants (Sönmez *et al.*, 2005; Kaya *et al.*, 2020; Kaya *et al.*, 2021).

Apilarnil is a bee product that is obtained after the collection of drone larvae aged 3-7 days. It contains water, proteins, carbohydrates, fatty acids, lipids, minerals and is rich in essential amino acids, as well as reproductive hormones (Erdem and Özkök, 2017). It is a cheap food source that contains many biologically active compounds and has high nutritional value (Erdem and Özkök, 2017; Doğanyiğit *et al.*, 2019; Doğanyiğit *et al.*, 2020). Apilarnil

is beneficial for the body due to its nourishing, energizing, revitalizing and psychotonic effects. In addition, it contributes to the maintenance of normal metabolism due to its amino acids, vitamins, minerals, glycerophosphate calcium and folic acid contents (Gavrila-Ardelean and Gavrila-Ardelean, 2017). Therefore, Apilarnil has many beneficial effects on male secondary sex characteristics. It has a high level of antioxidant properties due to the presence of rich polyphenols in its structure.

However, there is relatively little information available in the literature regarding the beneficial effects of supplementation of apilarnil into the semen extender on the post-thaw quality of frozen ram spermatozoa. Therefore, this study was planned to investigate the possible effects of supplementation of different levels of apilarnil to tris-egg yolk glycerol extender on post-thaw semen quality parameters in rams.

MATERIALS AND METHODS

Ethics, animals, and semen collection: The approval for the study protocol was taken from Fırat University Animal Experiments Local Ethics Committee (Protocol No: 2022/18-08), Turkey. The animals were housed in Fırat University Animal Hospital Hospitalization Unit during the study. Before and during the study, the rams were fed with concentrate feed, hay, crushed barley and dried grape and drinking water was given *ad libitum.* In our study, six adult Akkaraman rams aged about 2 years were used. The study was carried out in autumn during the breeding season (September–December, 2022). Semen was collected by artificial vagina twice a week for 3 weeks from each ram. Thus, a total of 36 ejaculates, with six ejaculates per ram, were available for further processing.

Dilution, cooling and freezing: Two different diluents were prepared for semen dilution and glycerolisation. Tris (3.63g), fructose (0.5g), citric acid (1.99g), egg yolk (15 ml), penicillin (100,000 IU) and streptomycin (100 mg) were added to 100 ml of distilled water to dilute the semen. The diluent prepared for glycerolisation was made up of Tris (3.63g), fructose (0.5g), citric acid (1.99g), egg yolk (15 ml), glycerol (10 ml) and antibiotics with distilled water to 100 ml. In the study, five semen diluent groups (w/v) with or without Apilarnil (Harşena®, Amasya, Turkey, Product No: 32) were prepared as 0% (Control), 0.5%, 1.0%, 1.5% and 2.0%.

Semen samples collected from six rams on each collection day were initially pooled. The pooled semen samples were analyzed for sperm motility (at least 70%) and concentration (at least 2.0 billion), divided into five equal parts and each part was supplemented with respective amount of Apilarnil. After diluting the semen samples with respective experimental diluents (1:5) at 37ºC, they were gradually cooled to 5ºC. So sperm concentration following dilution was adjusted to 400 million sperm per mL. Following glycerolisation and equilibration, semen samples were manually filled into 0.25 ml straws (100 million sperm per straw). The straws were frozen in liquid nitrogen vapors using an automatic semen freezing tool (Microdigitcool, IMV, France) and stored at -196ºC in liquid nitrogen for at least 24 hours. Before spermatological analysis, frozen semen samples were thawed in a water bath at 37ºC for 25 seconds (Türk *et al.,* 2022).

Spermatological analysis: Post-thaw sperm motility (total motility, progressive motility, rapid motile sperm, medium motile sperm and slow motile sperm) determination was performed with CASA (Computer Assisted Semen Analyzer) (ISASv1, Proiser, Spain). The analysis with CASA was performed, as described by Kaya *et al.* (2021). Sperm motility kinetics in terms of Curvi-linear velocity (VCL), Straight line velocity (VSL), Average path velocity (VAP), Linearity (LIN), Straightness (STR), Wobble (WOB), Amplitude of lateral head displacement (ALH) and Frequency of head displacement (BCF) were also recorded with CASA.

Plasma membrane integrity (Hypoosmotic swelling test): Frozen thawed semen was diluted 1:5 with Tris Buffer Solution and 50 µL of this mixture was taken and mixed with 500 µL hypoosmotic swelling solution. HOS analysis was performed as described by Söderquist *et al.* (1997). The results of sperm with plasma membrane integrity (with swollen or coiled tails) were calculated in percentage.

Flow cytometric analyses: In order to determine the mitochondrial membrane potential (MMP) in frozen-thawed semen, JC-1/PI (Invitrogen, T3168, Canada) was used. The MMP analysis was performed as described by İnanç *et al.* (2018). Semen was analyzed by flow cytometry (Cytoflex, Beckman Coulter, Fullerton, USA). The results of the analysis were then expressed as percentage.

A commercial kit SYBR-14/ PI (Invitrogen, Canada) was used to determine live sperm ratio in frozen-thawed semen by flow cytometry. This analysis was performed as described by Alvarez *et al.* (2012). Live sperm ratio was expressed as percentage.

For acrosomal integrity analysis, commercial kit Lectin-PNA (peanut agglutinin; PNA-Alexa 488, Invitrogen, UK) was used for analysis of frozen-thawed semen by flow cytometry, as described by Standerholen *et al.* (2014). Sperm with damaged acrosome were expressed in percentage of total sperm examined (Standerholen *et al.*, 2014).

Malondialdehyde determination: Malondialdehyde (MDA) was assumed as a direct measure of lipid peroxidation. A total of 50 µL of semen was taken for each sample and MDA activity was detected by Abnova ELISA Kit (Cat. No. KA3736, Abnova Corporation, Taiwan) at a detection range of 0.125–2 mM (Jannatifar *et al.*, 2019).

Statistical analysis: The data obtained from the study were analysed using SPSS (Version 26.0, USA) software. The results are reported as mean±SEM. In the study, nonparametric Kruskal-Wallis analysis of variance was performed to assess differences between the experimental groups, and pairwise comparisons were made using the nonparametric Mann-Whitney-U test. The $p<0.05$ was considered statistically significant.

Table 1: Motility and kinematic values in post-thaw ram semen with/without apilarnil supplementation of extender.

	Apilarnil levels (%)				
Parameters	Control	0.5	1.0	1.5	2.0
Total motility (%)	27.98±1.85^{A}	53.00±1.59^{B}	68.00±1.21^{C}	62.33±1.52^{C}	46.16±9.55^{AB}
Progressive motility (%)	11.86±1.61^{A}	26.13±1.84^{B}	27.10±1.44^{B}	28.66±2.53^{B}	15.71±2.79^{A}
Rapid spermatozoa (%)	17.25±1.71^{A}	36.51±2.14^{B}	37.88±2.75^{B}	34.62±1.87^{B}	24.15±4.66^{AB}
Medium spermatozoa (%)	5.13±0.66^{A}	7.25±0.51^{B}	10.66±1.91^{BC}	12.26±0.95^{C}	6.15±0.97^{AB}
Slow spermatozoa (%)	5.57±0.58^{A}	9.30±1.68^{AC}	19.60±1.91^{B}	15.42±1.52^{BC}	16.03±4.02^{BC}
VCL (µm/s)	101.45±6.01^{A}	125.97±6.51^{B}	124.22±8.86^{BC}	118.98±6.45^{BC}	102.58±6.49^{AC}
VSL (µm/s)	48.68±7.97^{A}	66.85±2.76^{A}	65.88±5.26^{A}	59.38±6.09^{A}	51.85±2.52^{A}
VAP (µm/s)	60.90±6.90^{A}	81.93±3.68^{B}	79.80±6.45^{AB}	73.25±6.83^{AB}	64.18±3.64^{A}
LIN (%)	46.83±4.93^{A}	53.67±3.11^{A}	53.18±2.47^{A}	49.81±3.82^{A}	50.88±1.95^{A}
STR (%)	77.98±3.91^{A}	81.78±2.21^{A}	82.68±1.28^{A}	80.98±2.54^{A}	80.97±0.93^{A}
WOB (%)	59.37±3.27^{A}	65.43±2.68^{A}	64.30±2.66^{A}	61.25±3.61^{A}	62.83±2.18^{A}
ALH (µm)	3.62±0.18^{A}	4.22±0.23^{AB}	4.25±0.20^{B}	4.30±0.17^{B}	3.95±0.18^{A}
BCF (Hertz)	9.15±0.41^{A}	8.81±0.61^{A}	8.60±0.46^{A}	8.20±0.42^{A}	8.57±0.59^{A}

VCL=Curvi-linear velocity; VSL=Straight line velocity; VAP=Average path velocity; LIN= Linearity; STR=Straightness; WOB=Wobble; ALH=Amplitude of lateral head displacement; BCF=Frequency of head displacement.
Different superscript letters (A, B, C) in the same row indicate statistically significant difference ($p<0.05$).

Table 2: Mitochondrial membrane integrity, acrosomal damage, viability, and plasma membrane integrity values in post-thaw ram sperm with/without apilarnil supplementation of extender.

	Apilarnil levels (%)				
Parameters (%)	Control	0.5	1.0	1.5	2.0
HMMP	41.38±3.16^{A}	41.59±2.37^{A}	40.16±1.61^{A}	29.48±6.98^{A}	43.86±3.18^{A}
LMMP	44.99±2.21^{A}	52.98±2.80^{B}	49.93±3.98^{AB}	64.26±8.74^{B}	52.17±4.39^{B}
AD	50.06±2.22^{A}	36.43±3.39^{B}	33.16±2.63^{B}	31.52±2.31^{B}	35.05±1.61^{B}
Viability	32.82±2.88^{A}	30.13±1.98^{A}	29.83±1.57^{A}	31.97±3.19^{A}	28.90±3.44^{A}
PMI	21.83±1.66^{A}	43.50±1.72^{B}	53.33±2.55^{C}	50.50±2.11^{C}	28.33±6.82^{A}

HMMP=High mitochondrial membrane potential; LMMP=Low mitochondrial membrane potential; AD=Acrosomal damage; PMI=Plasma membrane integrity. Different superscript letters (A, B, C) in the same row indicate statistically significant difference ($p<0.05$).

RESULTS

Total motility, progressive motility, and kinematic parameters of the post-thaw sperm from control group and apilarnil supplemented groups are presented in Table 1. When total and progressive sperm motility, rapid moving spermatozoa, medium moving spermatozoa and VCL values were analysed, the values were higher in 0.5, 1.0 and 1.5% apilarnil supplemented groups compared to the control group ($p<0.05$). However, VSL, LIN, STR, WOB and BCF values did not differ among control and supplemented groups. The value for VAP was higher in 0.5% group, while for ALH it was higher in 1.0 and 1.5% groups ($p<0.05$) compared to control group.

Mitochondrial membrane integrity, acrosomal damage and viability values of the control group and apilarnil containing study groups are presented in Table 2. The high mitochondrial membrane potential (HMMP) values in the apilarnil supplemented groups differed non-significantly from the control group. However, low mitochondrial membrane potential (LMMP) values in the groups containing 0.5 1.5 and 2.0% apilarnil were significantly increased compared to the control group ($p<0.05$). The acrosomal damage values were significantly decreased in all apilarnil supplemented groups than the control group ($p<0.05$). However, sperm viability values did not differ among treatment and control groups.

Sperm plasma membrane integrity values for 0.5, 1.0 and 1.5% apilarnil supplemented groups were higher ($p<0.05$) than the control group, while there was no difference between control and 2.0% apilarnil group (Table 2). MDA values for all apilarnil supplemented groups were lower ($p<0.05$) than the control group (Fig. 1).

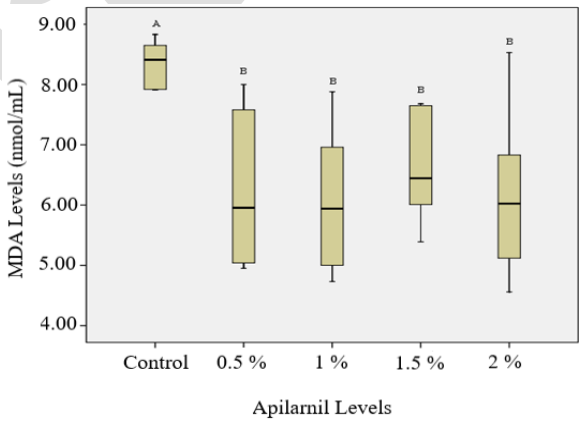

Fig. 1: MDA levels in post-thaw ram semen with or without apilarnil supplementation in extender. Bars with different letters indicate statistically significant difference ($p<0.05$).

DISCUSSION

Maintaining redox balance represents one of the most important challenges to overcome freezing damages during sperm storage. Decreased antioxidant defense, combined with increased ROS leads to redox imbalance. This imbalance can lead to many structural defects, especially sperm motility. Therefore, there are numerous studies showing that the addition of antioxidants to semen extenders in rams (Riesco *et al.*, 2021), stallions (Contreras *et al.*, 2020), pigs (Zhang *et al.*, 2012), bulls (Mousavi *et al.*, 2019) and goats (Anghel *et al.*, 2010) to prevent the ROS concentration from exceeding the physiological limit during sperm cryopreservation can reverse the negative effects of oxidative stress that persists during cryopreservation of sperm of different species.

Our study showed the positive effect of apilarnil at 0.5, 1.0 and 1.5% levels on sperm total motility, progressive motility, rapid moving spermatozoa, medium

moving spermatozoa and VCL values. This may be due to the fact that apilarnil contains minerals and essential amino acids (Erdem and Özkök, 2017). However, the fact that the addition of 2.0% apilarnil showed results similar to the control group suggests that the antioxidants or other preservatives should be added to semen diluents at the optimum level and that additional doses may be ineffective or even toxic (Bucak *et al.*, 2007; Bucak and Tekin, 2007).

Acrosomal integrity is the indicator of the ability of sperm penetration, digestion of the zona pellucida and fusion with the oocyte, so it is related to fertility of frozen semen (Alcay *et al.*, 2017). In our study, a significant decrease in acrosomal damage was observed in all groups containing apilarnil compared to control. In addition, in our study, HMMP level in all treatment groups was similar to the control group, while LMMP was higher in 0.5, 1.5 and 2.0% apilarnil groups than the control group. In addition, a higher rate of sperm plasma membrane integrity was found in 0.5, 1.0 and 1.5% apilarnil groups than the control group. These changes may affect sperm motility and may be due to the membrane protective biostimulator property of apilarnil (Hamamci *et al.*, 2020; Tugalay and Altan, 2020; İnandıklıoğlu *et al.*, 2021).

Our study showed that MDA, which is the end product of lipid peroxidation, decreased with the addition of all levels of apilarnil. This may be due to the polyphenolic antioxidants present in apilarnil (Doğanyiğit *et al.*, 2020).

Conclusions: Our study showed that apilarnil, which is cheap and easily available, can be effectively used as a supplement for semen extender. Supplementation of apilarnil at 0.5, 1.0 and 1.5% to tris-egg yolk glycerol diluent increased total and progressive sperm motility, protected plasma membrane integrity and acrosome membrane integrity and reduced MDA activity of post-thaw ram spermatozoa.

Authors' contribution: All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by RHK, IHG, SAA, TCA, NB, ACC, SOK, GT, MS, and SG. The first draft of the manuscript was written by RHK. All authors read and approved the final manuscript.

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