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Effects of Apilarnil on Semen Parameters During Ram Semen Short-Term Storage

Koç Spermasının Kısa Süreli Saklanması Sırasında Apilarnil'in Sperma Parametreleri Üzerindeki Etkileri

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ABSTRACT

This study aimed to investigate the effects of different concentrations of apilarnil (APL) added to semen diluent on the short-term storage of ram semen. For this purpose, six Akkaraman rams aged 1.5 years were used in the study outside the breeding season. Semen was collected from the rams twice a week with the help of an artificial vagina. Semen was first analyzed and ejaculates with a total motility score of 70% and above were pooled. The pooled ejaculates were divided into 5 equal parts and reconstituted with tris-egg yolk diluent containing 0%, 0.5%, 1%, 1%.5% and 2% APL. Diluted semen samples were stored at 4°C for 72 hours. As a result of the analyses, no statistically significant difference was observed between the groups in terms of total motility at 0 hour, while progressive motility was significantly higher in the 0.5% APL group ($P < .005$). While rapid spermatozoa rate, curvilinear velocity (VCL), and linear velocity (VSL) were high in the control group in all time periods, medium, and slow spermatozoa rates were high in 1% and 1.5% APL groups ($P < .05$). At 0 and 72 hours, the control group had the highest malondialdehyde (MDA) level ($P < .001$). Glutathione (GSH) level and glutathione peroxidase (GSH-Px) and catalase (CAT) activities were significantly lower in the control group at 0 and 72 hours. In conclusion, 0.5-1.5% APL added to semen diluent for short-term storage of ram semen has a positive effect on semen quality and oxidant status.

Keywords: Apilarnil, CASA, cooling, ram, sperm

ÖZ

Bu çalışma, sperma sulandırıcısına eklenen farklı konsantrasyonlardaki apilarnilin (APL) koç spermasının kısa süreli saklanması üzerindeki etkilerini araştırmayı amaçlamıştır. Bu amaçla, çalışmada üreme mevsimi dışında 1,5 yaşında altı Akkaraman koçu kullanıldı. Koçlardan haftada iki kez suni vajina yardımıyla sperma toplandı. Sperma önce analiz edildi ve toplam motilite skoru %70 ve üzerinde olan ejakülatlar pooling yapıldı. Pooling yapılan ejakülatlar 5 eşit parçaya bölündü ve içerisine %0, %0,5, %1, %1,5 ve %2 oranında APL ilave edilmiş tris-yumurta sarısı sulandırıcısı ile sulandırıldı. Seyreltilmiş sperma örnekleri 4°C'de 72 saat boyunca saklandı. Analizler sonucunda, 0. saatte total motilite açısından gruplar arasında istatistiksel olarak anlamlı bir fark gözlenmezken, progresif motilite %0,5 APL grubunda anlamlı olarak daha yüksek bulunmuştur ($P < ,005$). Hızlı spermatozoa oranı, eğrisel hız (VCL) ve doğrusal hız (VSL) tüm zaman dilimlerinde kontrol grubunda yüksekken, orta ve yavaş spermatozoa oranları %1 ve %1,5 APL gruplarında yüksekti ($P < ,05$). 0 ve 72. saatlerde kontrol grubu en yüksek malondialdehit (MDA) seviyesine sahipti ($P < ,001$). Glutatyon (GSH) seviyesi ile glutatyon peroksidaz (GSH-Px) ve katalaz (CAT) aktiviteleri 0 ve 72. saatlerde kontrol grubunda anlamlı derecede düşüktü. Sonuç olarak, koç spermasının kısa süreli saklanmasında sperma sulandırıcısına eklenen %0,5-1,5 APL, sperma kalitesi ve oksidan durumu üzerinde olumlu bir etkiye sahiptir.

Anahtar Kelimeler: Apilarnil, CASA, koç, soğutma, sperm

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INTRODUCTION

Semen is stored at +5°C for insemination because frozen and thawed semen has a low success rate in sheep inseminations.[1] Chilled ram semen exhibits reduced motility and morphological integrity, a shorter time to survive in the female genital canal, and a higher rate of embryonic losses when compared to fresh semen.[2] One of the most important disadvantages of chilled semen is its short fertile life.[3] It is important that the semen is preserved and stored under ideal conditions in order to attain an appropriate fertility rate.[4] Although various diluents have been developed for fresh storage of semen in sheep, semen can maintain its viability for up to 72 hours.[5]

Free radicals are reactive molecules and are spontaneously produced as many by-products during daily metabolic activities.[6] Although free radicals are needed in many biological processes, excessive increase in the amount of free radicals causes oxidative stress.[7] The potential of sperm to fertilize is decreased by oxidative damage.[8] Spermatozoa are highly sensitive to free radicals due to the unsaturated fatty acids present in their cell membranes.[9] Oxidative damage leads to loss of sperm membrane integrity and decreased sperm quality. Antioxidant compounds should therefore be added to the sperm diluent.[10]

The chemical composition of bee products ensures both the nutrition of living organisms and their widespread use in apitherapy.[11,12] Drone brood homogenate (apilarnil (APL)) is obtained from drone larvae between three and eleven days after hatching.[13] APL is produced by homogenizing, filtering, and lyophilizing the drone larvae after they are removed from the comb before pupation.[12] APL has a high protein content and includes essential amino acids.[14] APL contains high antioxidant properties and it has beneficial health effects.[15,16] APL has been reported to be used for the treatment of male infertility as well as systemic problems such as gastrointestinal and respiratory diseases.[17] APL administration has been observed to enhance sperm quality in male rats exposed to BPA-induced toxicity.[18] In the literature review, it is seen that the studies showing the effect of APL in semen diluents are limited. The present study aimed to investigate the effects of APL on the short-term storage of ram semen.

MATERIALS AND METHODS

Ethical approval

The approval certificate (Date: 16.11.2022, Number of Sessions: 2022/18-08) was obtained from Fırat University Animal Experiments Local Ethics Committee.

Study area and design

The animals were housed at Fırat University, Faculty of Veterinary Medicine, Animal Hospital during the study period. They were fed daily with roughage and concentrate feed, and water was provided ad libitum.

Semen was collected from six, 1.5 years old Akkaraman rams during the non-breeding season twice a week through an artificial vagina. Each ejaculate was evaluated by phase-contrast microscope with heating plate (Nikon) for motility, wave motion and concentration. During the wave motion examination, the evaluation is based on the presence or absence of fluctuating motion in the area and, if present, its intensity and velocity. A score between 0-5 is given taking into account the given characteristics. Computer Assisted Semen Analyzer (CASA) was used to determine sperm motility, and concentration. Semen was first analyzed and ejaculates with a total motility score of 70% and above were pooled. The pooled semen was diluted at 35°C in a 1:1 ratio with tris + egg yolk-based semen extender [297.58 mM tris (hydroxymethyl) aminomethane + 96.32 mM citric acid + 82.66 mM fructose + 100.000 IU penicillin + 100 mg streptomycin + 15% egg yolk] and APL dissolved in water. Semen samples divided into 5 groups (control (no additive), 0.5% APL, 1% APL, 1.5% APL, 2% APL). After the semen is diluted, it is placed in a container of water at 35°C and the temperature is reduced to 4°C over a period of 3 hours and the temperature was stabilized. After different doses of APL (Harşena, Amasya, Turkey, Cat. No: 38) were added to the semen, spermatological examinations were performed on semen samples every 24 hours and biochemical examinations were carried out on semen samples 0 and 72. hours.

Oxidative Stress Analysis

The lipid peroxidation (LPO) level was measured according to the concentration of thiobarbituric acid-reactive substances. The amount of malondialdehyde (MDA) was used as the LPO index.[19] Glutathione (GSH) levels were measured using the method described by Sedlak and Lindsay.[20] Glutathione peroxidase (GSH-Px) activities were determined according to the method of Lawrence and Burk.[21] Catalase (CAT) activities were determined by measuring the decomposition of hydrogen peroxide (H_2O_2).[22]Protein concentration was determined by the method used by Lowry et al.[23] Our results were analyzed at the beginning and end of the experiment at 0 and 72 hours.

CASA Analysis

Semen motility analyses were performed using a computer-assisted sperm analyzer (CASA, ISASv1, Proiser, Spain). Semen samples were diluted with tris buffer solution [3.63 g tris, 1.99 g citric acid, 0.50 g glucose in 100 mL distilled water] in an Eppendorf tube at 37°C. 3.5 µL of

the mixture was placed on a Spermtrack20 slide and covered with a coverslip. Total, progressive, rapid, medium and slow motility ratios (%), static sperm ratio (%) and kinematic parameters [VCL: curvilinear velocity (µm/s); VSL: straight line velocity (µm/s); VAP: mean path velocity (µm/s); LIN: linearity index (%); STR: straightness index (%), WOB: wobble index (%); ALH: mean amplitude of lateral head movement (µm); BCF: beat cross frequency (Hz)] were analyzed.[24] All analyses were performed after post equilibration and at 24, 48, 72 hours.

Morphological Analysis

A small amount of semen-tris mixture was placed on a slide, froth was taken and allowed to dry. They were immersed in Diff - Quick staining set solutions for 30 seconds, 20 seconds, and 30 seconds respectively, washed with distilled water and air dried. The smears were then examined under 400x magnification of a phase-contrast microscope. A total of 200 spermatozoa were analyzed in each smear and the results were presented as percentages. Our results were analyzed at the beginning and end of the experiment at 0 and 72 hours.

Statistical Analysis

Before significance tests, the data obtained were analyzed by Shapiro Wilks test for normality and Levene's test for homogeneity of variances. The statistical control of the difference between normally distributed variables was performed by ANOVA, and the control between non-normally distributed variables was performed by Kruskal Wallis test. Tukey's test was used as a post-hoc test for variables in which the difference between groups was significant. Descriptive statistics were calculated for each variable and presented as "Mean ± Standard Error of Mean" (Mean ± SEM). Data were evaluated by two-way mixed design ANOVA (analysis of variance) using the General Linear Model for repeated measures procedure to examine the effect of "group" and "time" for the measurements obtained. Post-hoc testing for significant interactions was performed using simple effects analysis with Bonferroni adjustment. Where interaction terms were not statistically significant, contrasts were used to analyze main effects. All statistical analyses were analyzed with a minimum margin of error of 5%. SPSS 26.0 (IBM SPSS Corp., Armonk, NY, USA) package program was used. $P < .05$ was considered significant.[25]

RESULTS

CASA Evaluation

CASA parameters analysis results are shown in Tables 1 and 2. At the post equilibration, the lowest total motility and progressive motility values were observed in 1.5% and 2% APL groups. Progressive motility values at 24, 48, and 72 hours were highest in 0.5% APL group. At 24, 48, and 72 hours, the highest rate of rapid spermatozoa was seen in the 0.5% APL group and medium spermatozoa was seen in the 0.5% and 1% APL group. VCL and VSL values were highest in the control and 0.5% group at post equilibration and decreased in parallel with the following time periods. LIN, STR, and WOB were higher in the 0.5% APL group compared to the other groups. In general, ALH value was higher in the control group and BCF value was higher in the 0.5% APL group compared to the other groups ($P < .05$).

Table 1. Total motility and progressive motility values (mean±sem) of the experimental groups.

	TM 0	TM 24	TM 48	TM 72	PM 0	PM 24	PM 48	PM 72
Control	80.33±2.53^{Aa}	65.08±2.75^{Ab}	48.18±5.43^{Ab}	33.18±1.43^{Ac}	35.65±3.10^{ABa}	32.9±3.65^{ABa}	14.28±2.36^{Ab}	11.72±1.74^{ABb}
%0.5	83.63±3.07^{Aa}	69.04±4.69^{Ab}	46.13±3.08^{Ac}	35.5±2.44^{Ad}	45.18±2.08^{Ba}	35.72±2.99^{Ab}	16.10±2.81^{Ac}	13.32±0.91^{Ac}
%1	71.72±2.52^{ABa}	64.1±3.14^{Aa}	39.48±3.56^{ABb}	34.43±6.96^{Ab}	30.58±2.73^{ACa}	24.72±3.36^{ABa}	11.08±1.11^{ABb}	11.63±2.37^{ABb}
%1.5	63.48±2.68^{Ba}	53.06±5.41^{Aba}	32.72±0.98^{Bb}	22.97±2.05^{Bc}	24.37±2.41^{CDa}	23.4±2.77^{ABa}	9.52±0.78^{ABb}	6.68±1.10^{BCb}
%2	45.92±5.1^{Ca}	42.00±4.76^{Bb}	25.32±3.63^{Cc}	15.82±0.6^{Cd}	13.37±3.00^{Da}	19.32±2.93^{Ba}	5.05±0.85^{Bb}	3.62±0.55^{Cb}

*A, B, C, D: indicates the difference between groups in the same column ($P < .05$). *a, b, c, d: indicates the difference between times in the same row ($P < .05$).
Abbrevations: TM: total motility, PM: progressive motility

Oxidant/Antioxidant Status Assessment

Mean values of oxidant and antioxidant status at 0 and 72 hours are given in Table 3. MDA levels were significantly higher in the control group compared to the other groups at 0 and 72 hours. GSH levels were significantly higher in all APL groups compared to the control group at 0 and 72 hours. At post equilibration and 72 hours, GSH-Px and CAT activities were highest in 1.5% APL group ($P < .001$).

Abnormal Sperm Rate

Abnormal sperm ratio findings are presented in Table 4. According to this, when 0. hour is analyzed, it is seen that the lowest rate is in 0.5% APL and control group, respectively. The highest value was observed in the 2% APL group ($P < .001$). At 72 hours, it was again observed that the lowest value was in the control and 0.5% APL group, and the highest value was in the 2% APL group ($P < .001$).

Table 2. Sperm kinematic parameters values (mean±sem) according to groups.

Parameters		0. Hour	24. Hour	48. Hour	72. Hour
Rapid (%)					
	Control	66.73±2.35^{Aa}	51.00±3.86^{Ab}	23.27±3.54^{Ac}	15.43±1.57^{Ad}
	0.5 %	68.02±1.48^{Aa}	54.02±5.12^{Ab}	23.65±1.69^{Ac}	17.83±2.10^{Ac}
	1%	48.40±3.25^{Ba}	49.76±4.50^{Aa}	17.68±1.03^{ABb}	12.95±2.06^{ABb}
	1.5 %	36.73±3.21^{Ba}	39.94±5.85^{Aba}	13.42±0.96^{BCb}	8.32±1.75^{BCc}
	2%	22.52±4.17^{Ca}	28.36±4.49^{Ba}	6.72±1.26^{Cb}	2.13±0.32^{Cd}
Medium (%)					
	Control	8.68±0.87^{Aab}	11.18±0.83^{a}	9.48±0.77^{ab}	7.85±0.81^{b}
	0.5 %	8.28±0.57^{Aa}	11.82±0.81^{b}	10.32±1.09^{ab}	6.20±0.59^{c}
	1%	10.88±1.36^{AB}	9.48±0.81	10.12±0.89	8.03±1.71
	1.5 %	13.07±0.78^{Ba}	10.56±0.49^{b}	9.02±0.34^{b}	5.88±0.27^{c}
	2%	11.65±1.27^{ABa}	10.74±0.62^{a}	7.57±0.96^{b}	5.08±0.66^{b}
Slow (%)					
	Control	6.47±0.96^{Aa}	3.92±0.45^{b}	12.10±1.75^{c}	9.90±0.88^{c}
	0.5 %	9.02±0.58^{ABa}	2.74±0.34^{b}	12.15±1.20^{c}	11.28±1.42^{c}
	1%	12.55±1.46^{Ba}	2.88±0.31^{b}	13.40±1.52^{a}	7.7±1.93^{c}
	1.5 %	12.32±0.82^{Ba}	2.54±0.24^{b}	10.25±1.08^{a}	8.73±1.21^{a}
	2%	11.73±1.00^{Ba}	2.92±0.39^{b}	9.33±0.99^{a}	8.57±0.54^{a}
VCL (µm/s)					
	Control	129.80±5.24^{Aa}	102.68±5.60^{Ab}	89.28±3.5^{Abc}	81.83±4.20^{Ac}
	0.5 %	118.23±2.84^{Aa}	102.88±4.66^{Ab}	84.75±1.67^{Ac}	85.5±4.85^{Ac}
	1%	100.03±5.14^{Ba}	95.72±2.75^{Aa}	76.93±1.79^{Bb}	77.28±2.68^{Ab}
	1.5 %	88.22±3.64^{BCa}	85.26±5.42^{Aba}	73.50±2.76^{Bb}	66.28±4.45^{Bb}
	2%	76.90±3.84^{Ca}	72.04±4.36^{Ba}	59.93±3.56^{Cb}	47.47±2.35^{Cc}
VSL (µm/s)					
	Control	52.05±3.27^{Aa}	39.25±5.11^{Ab}	33.60±3.22^{b}	37.50±4.09^{Ab}
	0.5 %	59.18±2.48^{Aa}	40.24±3.76^{Ab}	34.33±4.66^{c}	39.92±1.90^{Abc}
	1%	42.60±3.66^{Ba}	31.78±2.03^{Abb}	26.38±1.76^{b}	33.28±4.00^{ABb}
	1.5 %	36.08±2.23^{Ba}	24.64±1.99^{Bb}	24.70±1.90^{b}	23.85±2.43^{Bb}
	2%	25.03±3.36^{Ca}	21.56±1.42^{Bb}	21.63±2.82^{b}	18.17±0.27^{Bb}
VAP (µm/s)					
	Control	67.12±3.01^{a}	51.12±4.51^{Ab}	43.95±3.16^{Ab}	45.57±3.52^{Ab}
	0.5 %	70.95±2.40^{a}	51.36±3.15^{Ab}	42.60±3.16^{Ac}	47.20±1.24^{Abc}
	1%	69.98±2.49^{a}	43.48±1.25^{Abb}	35.73±1.59^{Abb}	41.23±4.07^{ABb}
	1.5 %	45.96±3.12^{a}	35.38±2.33^{BCb}	33.53±1.55^{Bb}	31.87±2.18^{BCb}
	2%	36.4±2.70^{a}	30.7±1.58^{Cb}	29.47±2.41^{Bbc}	24.78±0.36^{Cc}
LIN (%)					
	Control	40.65±3.21^{AB}	38.35±4.85	37.42±2.73	46.03±5.02
	0.5 %	50.00±1.52^{A}	38.88±3.78	39.17±4.72	46.68±2.73
	1%	42.50±2.61^{AB}	33.68±2.91	34.30±2.14	42.82±4.31
	1.5 %	40.88±1.22^{AB}	29.00±2.01	33.82±2.81	36.07±3.48
	2%	33.13±2.99^{Bab}	29.90±0.73^{a}	35.70±3.42^{ab}	38.77±2.03^{b}
STR (%)					
	Control	77.7±2.15^{AB}	75.58±3.22	75.77±2.02	81.27±3.30
	0.5 %	83.32±1.09^{A}	77.02±3.00	76.50±4.02	69.82±12.05
	1%	78.63±1.94^{AB}	72.84±2.55	73.47±1.67	80.12±2.78
	1.5 %	79.08±0.97^{AB}	190.20±120.22	73.27±2.59	73.98±3.02
	2%	70.03±4.51^{B}	70.00±1.45	63.03±11.64	73.20±0.84
WOB (%)					
	Control	51.98±2.62^{AB}	49.12±4.43	49.08±2.43	55.92±4.10
	0.5 %	59.83±1.24^{Aa}	50.08±2.91^{ab}	49.43±3.24^{b}	56.10±3.43^{ab}
	1%	53.82±2.04^{AB}	45.68±2.34	46.52±1.89	53.02±4.12
	1.5 %	52.46±1.49^{ABa}	41.62±1.71^{b}	45.82±2.17^{ab}	48.47±3.35^{ab}
	2%	46.03±1.60^{Bab}	42.72±0.61^{a}	49.08±2.44^{ab}	52.90±2.47^{ab}
ALH (µm/s)					
	Control	4.90±0.30^{A}	4.28±0.22^{AB}	4.08±0.09^{A}	4.05±0.30^{A}
	0.5 %	4.27±0.12^{AB}	4.32±0.19^{A}	3.93±0.16^{A}	3.97±0.18^{A}
	1%	4.05±0.24^{B}	4.24±0.24^{AB}	4.05±0.07^{A}	3.68±0.14^{A}
	1.5 %	3.86±0.13^{B}	3.96±0.28^{AB}	3.62±0.08^{A}	3.47±0.21^{AB}
	2%	3.72±0.09^{Ba}	3.36±0.15^{Bb}	2.97±0.22^{Bc}	2.83±0.11^{Bc}
BCF (Hz)					
	Control	8.77±0.47^{A}	7.72±0.41^{AB}	7.50±0.27	8.05±0.56
	0.5 %	9.57±0.17^{Aa}	8.04±0.51^{Bab}	7.82±0.65^{b}	8.05±0.38^{b}
	1%	8.43±0.44^{A}	7.30±0.43^{AB}	7.45±0.44	7.87±0.52
	1.5 %	8.46±0.44^{Aa}	6.86±0.36^{ABab}	6.97±0.18^{ab}	7.03±0.45^{b}
	2%	6.02±0.77^{Ba}	6.28±0.26^{Aa}	6.95±0.66^{ab}	7.85±0.25^{b}

Velocity Straight Line (VSL), Velocity Curve Linear (VCL), Velocity Average Path (VAP), Wobble (WOB), Linearity (LIN), Straightness (STR), Amplitude of Lateral Head Displacement (ALH) and Beat Cross Frequency (BCF). A, B, C: indicates the difference between groups in the same row ($P < .05$). a, b, c, d: indicates the difference between times in the same column ($P < .05$).

Table 3. Mean oxidative stress levels (mean±sem) between experimental groups.

	MDA (nmol/ml)		GSH (nmol/ml)		GSH-Px (IU/g protein)		CAT(kU/g protein)	
	0. Hour	72. Hour	0. Hour	72. Hour	0. Hour	72. Hour	0. Hour	72. Hour
Control	11.18±0.42^{b}	11.58±0.65^{c}	0.70±0.55^{Aa}	1.38±0.81^{Ba}	7.70±0.32^{a}	7.80±0.34^{a}	176.51±12.48^{a}	193.74±16.92^{a}
0.5 %	8.26±1.26^{a}	8.54±0.79b^{a}	0.86±0.66^{Aba}	2.35±0.16^{Bb}	8.18±0.86^{a}	10.10±0.91^{ba}	194.48±22.74^{a}	263.73±31.39^{ba}
1%	8.52±0.79^{a}	7.31±0.37^{a}	1.06±0.70^{Acb}	2.36±0.14^{Bb}	9.26±0.45^{a}	9.73±0.62^{ba}	205.27±7.30^{a}	264.23±18.46^{ba}
1.5 %	7.53±1.45^{a}	6.94±0.26^{a}	1.1±0.61^{Adc}	2.81±0.11^{Bcb}	12.86±0.72^{b}	10.62±1.05^{b}	262.11±19.00^{b}	287.31±28.60^{b}
2%	9.15±1.2b^{a}	9.14±0.65^{b}	1.30±0.11^{Ad}	2.55±0.54^{Bcb}	8.39±0.71^{a}	9.02±0.54^{ba}	220.50±23.14^{ba}	225.70±39.90^{ba}

Superscript letters (a, b, c) indicate the difference between groups within the same time ($P < .001$). Superscript letters (A, B) shows the difference between times within the same group ($P < .05$).

Table 4. Mean abnormal sperm ratio (mean±sem) between experimental groups.

Groups	0. Hour	72. Hour
Control	7.16±0.98^{a}	3.66±0.42^{a}
0.5%	5.83±1.76^{a}	3.16±1.76^{a}
1%	10.00±2.26^{ab}	12.33±3.56^{b}
1.5%	14.00±2.00^{bc}	14.33±1.75^{b}
2%	16.67±2.00^{c}	20.16±4.40^{c}

Superscript letters (a, b, c) indicate the difference between groups ($P < .001$). There is no statistical difference between times within the same group ($P > .05$).

DISCUSSION

Since the freezing/thawing process of ram semen in sheep breeding causes serious damage to spermatozoa, chilled semen dilution method is used.[26] Cooling semen storage in rams also reduces sperm fertility efficiency, so the semen diluent medium should be supplemented with antioxidants and sperm motility should be increased for spermatozoa to reach the ampulla.[27] In the present study, the effects of APL added to semen diluent during cooling storage of semen on sperm kinematic parameters and oxidant status were investigated in rams.

Antioxidant compounds are used to prevent increased oxidative damages, different antioxidant substances added to the diluent during short-term storage of ram semen are reported to reduce increased LPO and also increase the reduced total antioxidant capacity induced by short time storage.[28,29] APL, a bee product, is a compound with high antioxidant activity.[30] APL has both androgenic activity [12] and a protective effect against toxic agents.[18] In addition mammalian spermatozoa contain a high polyunsaturated fatty acid ratio in the plasma membrane and a low cholesterol-to-phospholipid molar ratio.[31] These membranes are susceptible to peroxidation during aerobic long-term storage.[32] Storage of ram spermatozoa under cooling conditions can produce large amounts of ROS leading to loss of fertility.[29] It has been reported that an increase in oxidative stress may lead to a decrease in important sperm functions such as the acrosome reaction. [33] It is seen that different antioxidant compounds added to ram semen diluent have positive effects on semen quality.[28,34] A proper oxidant/antioxidant balance can improve sperm fertilization capacity.[35] In a study, it was reported that Chlorogenic Acid supplementation added to semen diluent decreased oxidative stress and increased progressive motility in short-term storage of semen in rams.[36] In the present study, progressive motility up to 72 hours was numerically superior in the group treated with 0.5% APL ($P < .05$). Antioxidants have an important role in preserving sperm physiological activities and can counteract the negative effects of the cooling process.[37] Because of its high polyphenol concentration, APL has excellent antioxidant activity.[6] MDA is a byproduct of lipid peroxidation that serves as an indicator of ROS-induced damage in spermatozoa.[38] In the present study, MDA level was found to be lower in all concentrations of APL compared to the control group. This indicates that APL leads to a decrease in MDA level by decreasing LPO. GSH, GSH-Px, and CAT are enzymatic and non-enzymatic antioxidant defense system parts.[39] GSH acts as a co-factor for GSH-Px, a protective enzyme that catalyzes the reduction of harmful H_2O_2 and other hydroperoxides.[40] Normally, semen contains antioxidants such taurine, catalase, glutathione, glutathione peroxidase (GSH-Px), and superoxide dismutase, which inhibit lipid peroxidation (LPO) and excessive ROS production.[41] Different antioxidant compounds are reported to protect sperm quality by increasing antioxidant activity in ram semen.[42] It has been reported that APL protects the semen from testicular toxicity due to its antioxidant properties.[18,43] In the present study, APL caused a increase in GSH level and GSH-Px and CAT activities in ram semen at all time periods.

Semen morphology is used as an important criterion in the evaluation of semen in domestic animals and it is stated

that semen with a high percentage of abnormalities reduces fertility.[44,45] The composition of semen diluents is reported to be effective in sperm abnormalities.[46] In the present study, the lowest abnormal sperm rates were observed in the 0.5% APL and control group at the 0th hour ($P < .001$). At high doses of APL, sperm quality decreased. This is thought to be due to the fact that it is above the physiological antioxidant level.

VCL, VSL and VAP are positively correlated with sperm motility, whereas VCL is highly correlated with sperm fertilization ability. It is reported that spermatozoa progression in cervical mucus is positively correlated with the VCL kinetic parameter.[47] In the present study, it was generally observed that VCL, VSL and VAP values decreased in all groups with the passage of time. In addition, higher VCL, VSL and VAP values were found in the control and 0.5% APL groups compared to the other experimental groups. This suggests that fertilization ability may be higher in control and 0.5% APL groups. In a study, it was reported that antioxidant compounds improved motility and kinematic parameters in ram semen. This is reported to be due to the energy production that occurs as a result of the antioxidant compound used affecting aerobic respiration.[48]

High concentrations of antioxidant compounds can damage spermatozoa and cause a decrease in fertility rate.[49] In the present study, the high abnormal sperm rates in the 1%, 1.5% and 2% APL groups support the literature. In another study, it was reported that gallic acid added to ram semen diluent increased the rate of abnormal semen as the hours progressed.[25] In the present study, it was observed that the rate of abnormal spermatozoa increased as time progressed in all groups except the 0.5 APL group. It is thought that this may be due to the change in the osmotic pressure of the semen diluent of the antioxidant compound used.[50] Moreover abnormal semen is formed during spermatogenesis. It is also thought that abnormalities other than spermatogenesis may be due to osmotic changes in the semen diluent.

As a result the use of APL at doses between 0.5% and 1.5% added to semen diluent for short-term storage of semen in rams has been proven to improve the quality of ram semen for up to 72 hours.

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