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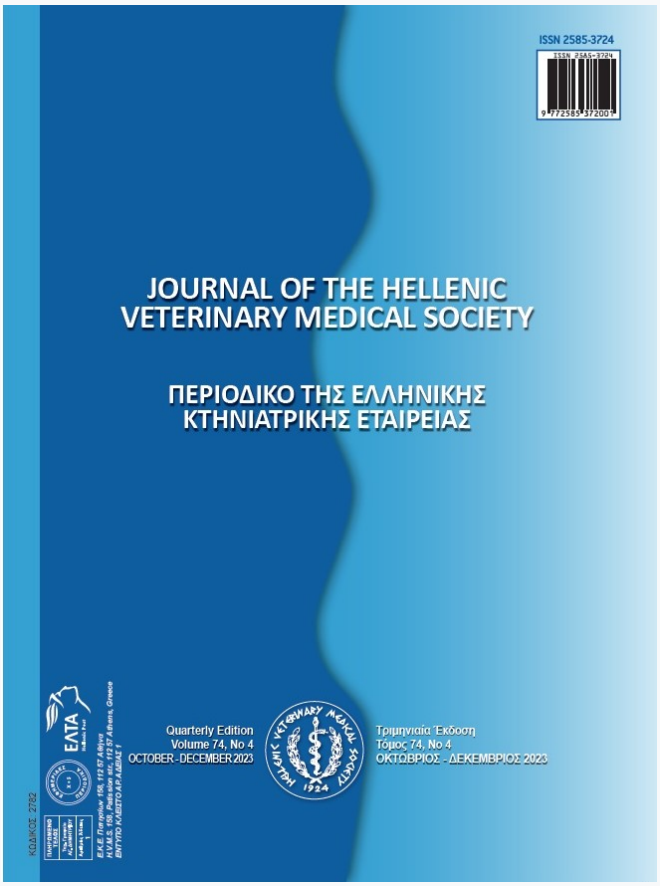

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Research article
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Effects of the addition of flower honey and pine honey to extenders on spermatological characteristics in ram semen

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ABSTRACT: In the current study, the effectiveness of flower and pine honey added to the semen extender at different proportions in freezing ram semen was investigated. Semen samples were collected from 5 Kivircik rams by electroejaculation method. Semen samples were pooled and divided into five equal fractions to form the study groups (FH1.0: 1.0% flower honey (v/v); FH2.5:2.5% flower honey (v/v); PH1.0: 1.0% pine honey (v/v); PH2.5: 2.5% pine honey (v/v); control: no honey addition). The semen samples were diluted with Tris-egg yolk-based semen extenders containing flower and pine honey at different rates. After cooling to 5°C and equilibration at the same temperature for 1 hour, samples were frozen in liquid nitrogen vapor and stored in liquid nitrogen. During the post-equilibration period, total motility, progressive motility, velocity, and kinetic movement parameters were determined by computer-assisted sperm analysis (CASA) method. Acrosomal and total morphological damage rates were determined by phase contrast microscopy. After thawing, CASA and morphological damage assessments were repeated, sperm viability, mitochondrial activity, plasma membrane integrity and acrosomal integrity were also examined by flow cytometry. The study was repeated ten times.

Comparing with the control group, morphological damage rates were lower in all groups containing honey after equilibration ($p < 0.05$). On the other hand, 1.0% (v/v) flower honey added to the semen extender reduced the rate of acrosomal damage (phase-contrastmicroscopy examination) and damaged membrane ratio (florescent microscopy examination) ($p < 0.05$). Meantime, 2.5% (v/v) flower honey increased the rate of viable spermatozoa ($p < 0.05$), the rate of spermatozoa with high mitochondrial activity ($p < 0.05$) and successfully preserved plasma membrane integrity (florescent microscopy examination) after thawing ($p < 0.05$).

It is concluded at the end of the study that, addition of flower honey to extender could be used in cryopreservation of ram semen. Especially the adding of flower honey at the rate of 2.5% (v/v) is recommended for freezing ram semen.

***Keywords*:** Ram semen; semen cryopreservation; extender; flower honey; pine honey.

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INTRODUCTION

The stages of cryopreservation, particularly freezing and thawing processes, have deleterious effects on spermatozoa (Watson, 2000). Especially, ram spermatozoa are very sensitive to temperature changes during the cryopreservation stages due to high ratio of unsaturated/saturated fatty acids (Bacinoğlu et al., 2007; Valente et al., 2010) and a low molar ratio of cholesterol/phospholipids (Motamedi-Mojdehi et al., 2014) in their plasma membranes. Researchers agree that, species-specific semen extenders and freezing protocols should be developed to minimize the factors that adversely affect the potential fertility of spermatozoa due to cryopreservation stages and/or to increase the resistance of spermatozoa from the aforementioned damages (Salamon & Maxwell, 2000). Up to this point, many ram semen extender compositions have been produced, and new ingredients are being studied. However, pregnancy rates obtained with frozen-thawed semen in sheep are not comparable with fresh semen (Özmen et al., 2020). For this reason, intensive studies are carried out on developing species-specific extenders for freezing ram semen (Aisen et al., 2000).

Semen extenders used in cryopreservation should be able to provide necessary conditions for continuity of the metabolic functions in spermatozoa, maintain the pH and osmotic pressure balance of extracellular environment, reduce cryogenic damage, and control the development of reactive oxygen species and bacterial contamination (Yimer et al., 2015). It is known that honey contains high amounts of simple sugars (Fuller, 2004). By adding honey to the extender, these sugars can be used as an energy source for spermatozoa. They can reduce the harmful effects of freezing by acting as a non-penetrating cryoprotectant (Yimer et al., 2015). In addition, honey has antibacterial activity (Kwakman & Zaat, 2012) and contains many antioxidant compounds such as chrysin, vitamin C, catalase, pinobanksin, and pinocembrin (Bogdanov et al., 2008; Fakhrildin & Alsaadi, 2014). Studies conducted by adding various types of honey to semen extenders have shown that honey preserves spermatological values in humans (Fakhrildin & Alsaadi, 2014; Abdullah et al., 2015), buck (Maidin et al., 2018), fish (Ögretmen& İnanan, 2014; Arif et al., 2023), ram (Jerez-Ebensperger et al., 2015; Khatun et al., 2022), bull (El-Sheshtawy et al., 2014; Yimer at al., 2015), stallion (El-Sheshtawy et al., 2016) and boar (Balogun et al., 2023) semen.

The physicochemical structure of honey changes depending on the water content, the type of flora from where it is produced, the temperature, and the ratio of sugars it contains (Fakhrildin et al., 2014).In nature,production of pine honey is different from flower honey.The primary source of pine honey is an insect called *Marchalina hellenica*, which lives on *Pinus brutia* (Turkish red pine) (Akbulut et al., 2009; Özkök et al., 2016). These insects use the essential nutrients in the plants sugar solution and discard the remainder. Bees use this residual material in honey production (Özkök et al., 2016).

Compared to classical flower honey, pine honey contains higher amounts of oligosaccharides such as melezitose and raffinose (Bogdanov et al., 2008) and phenolic compounds with antioxidant properties (Ouchemoukh et al., 2007; Akbulut et al., 2009; Özkök et al., 2016). To our knowledge, there is no study to determine the effectiveness of pine honey in cryopreservation of ram semen. The present study aimed to comparatively determine the effects of adding flower and pine honey in different concentrations (1.0% and 2.5%) to media in freezing of Kivircik ram semen on spermatological parameters.

MATERIALS AND METHODS

Necessary permission was obtained for this study from the Istanbul University Animal Experiments Local Ethics Committee (13.11.2017-425005)

Preparation of extenders

Chemicals, except the honey samples were purchased from Sigma Chemical Co. (Saint Louis, MO). Commercial flower (batch number: BPC17.0075, Balparmak®) and pine honeys (batch number: BPC17.0021, Balparmak®) were used in this study. A tris-egg yolk diluent (tris 27.1 g/l, fructose 10 g/l, citric acid 14 g/l, egg yolk 15%, pH 6,8) was used as the base sperm extender (Cirit et al., 2013).Extenders were prepared by adding various amounts of flower and pine honey to the 4 study groups as follows: Tris-egg yolk (control), FH1.0 (1.0% flower honey, v/v), FH2.5 (2.5% flower honey, v/v), PH1.0 (1.0% pine honey, v/v) and PH2.5 (2.5% pine honey, v/v).

Semen collection and dilution

This study was performed outside of the breeding season (April-June). Two to five years old Kivircik rams (n=5) were hosted at the Faculty of Veterinary Medicine in Istanbul University-Cerrahpasa (28°S, 41°W) and kept under standard environmental, man-

agement, and nutritional programs throughout the study. Semen samples were collected by an electro-ejaculator (P-T Electronics, Model 302, Boring, Oregon, USA). The ejaculate of each ram was examined individually, and only good quality (volume: ≥ 0.5 ml; mass motility: ≥ 4; motility: ≥ 70%; sperm concentration: ≥ 2×10^9/ml) samples were pooled to eliminate individual differences (Cirit et al., 2013). Sperm motion parameters of the pooled semen sample were then evaluated by a computer-assisted semen analysis system (CASA 12.3 IVOS, Hamilton - Thorne Biosciences, Beverly, MA, USA; Amann & Waberski, 2014). The pooled semen sample was divided into five equal parts (study groups) and diluted with five different extenders.

Dilution of semen was carried out in two steps (with fractions A and B), and fraction B extender containing 10% glycerol was added in the second step (Ak et al., 2010). The pooled semen was diluted gradually with fraction A (without glycerol) to a final concentration of 400×10^6 motile sperm/ml in a water bath at +26°C. Then, extended semen samples were cooled to +5°C with 0.3°C/min cooling rate by controlled rate freezer (Bio-cool III). At the same temperature, dilution with the fraction B extender (glycerolization) was carried out slowly by addition of 10%, 20%, 30%, and 40% of the extender at 8 min intervals (Cirit et al., 2013).

As a result of the dilution of the semen groups, the final sperm concentration was 200×10^6 spermatozoa/ml and contained 5% glycerol. After equilibration at 5°C for 1 hour, sperm motility and kinetic velocity parameters were evaluated by CASA, and sperm morphology was examined under a phase-contrast microscope using 100× objective (Olympus, BX51). Diluted semen samples were packaged in 0.25 ml straws and frozen in nitrogen vapor. The straws were then immersed in liquid nitrogen and stored in containers until spermatological examinations (Ak et al., 2010).

Spermatological examinations

Computer-assisted semen analysis (CASA)

The pooling (for individual evaluation), equilibration, and post-thaw sperm motility (total and progressive motility), velocity (rapid, slow, medium, static sperm rates), and kinetic motion parameters (VAP, VSL, VCL) of the sperm samples were analyzed with CASA system. Before the examinations, the standard values specified for ram semen were defined in the CASA system (Demir et al., 2015). To examine the pooled semen sample, semen was diluted with a Tris-egg yolk extender at a ratio of 1:40 (v/v).

In CASA examinations after thawing,two randomly selected frozen straws for each group were thawed in a 37°C water bath for 30 sec and pooled. A 3 µl sample was placed onto a 37°C pre-heated 10-µm-deep Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). At least six fields were scanned, and the motion characteristics of approximately 600-800 spermatozoa were evaluated.

Assessment of morphological integrity

Morphological integrity assessment with the Hancock fixation technique was used after equilibration and post-thaw period. Ten µl of semen sample was added slowly into 100 µl of Hancock fixation solution. Three µl of the sample was taken, placed on a microscope slide, and covered by a cover slip. The evaluation was carried out by determining the acrosome and total damage (acrosome, head, middle piece, and tail of sperm) ratios of 200 sperm cells at 1000× magnification by phase-contrast microscope (Öztürkler et., 2001; Bacinoglu et al., 2008).

Assessment of acrosomal integrity by flow cytometry

The sperm acrosomal integrity status of the viable population was assessed using a combination of fluorescein isothiocyanate conjugated to Arachis hypogaea (peanut) (FITC-PNA) and propidium iodide (PI) fluorescence probes as described previously by Marco-Jiménez et al. (2005), with modifications. Briefly, 0.5 ml post-thawed semen was diluted to $5\text{-}10\times10^6$ ml^{-1} with Tris base solution, 2 µl of FITC-PNA (100 µg ml^{-1} solution in saline solution), 200 µl Tris base solution, and 2 µl PI (0.5 mg ml^{-1} solution in distilled water) was added to 100 µl of diluted sperm. Samples were incubated at room temperature in a dark room for 10 min. The measurement was performed by evaluating 5000 spermatozoa in 519 nm, and 590 nm emission ranges in flow cytometry (Guava EasyCte™, Guava® Technologies, Hayward CA, USA). Sperm cells that did not receive FITC-PNA and PI stains, in other words, did not show green or red fluorescent reflections, were considered viable sperm with intact acrosome membranes.

Assessment of plasma membrane integrity

This assessment was performed using the carboxy-fluorescein diacetate - propidium iodide (CFDA-PI) staining protocol with fluorescent microscopy and

flow cytometry.

In the evaluation with a fluorescent microscope, CFDA-PI staining protocol was modified by Cámara et al. (2011). For each group, 5 µl of CFDA (0.46 mg/ml stock) and 20 µl of PI (0.5 mg/ml stock) stains were added into 50 µl of thawed semen, respectively. The sample was mixed gently and incubated for 5 min in the dark at room temperature. Then, 10 µl of Hancock solution was added to stop sperm motions. A sample of 5µl was placed on a microscope slide and covered by a cover slip. A total of 300 sperm cells were examined using a fluorescence microscope (Eclipse Ni-U, Nikon, Tokyo, Japan; excitation at 485/20 nm and emission at 580-630 nm) at 400× magnification to assess the sperm cell membrane integrity. It was accepted that the membranes of sperm cells reflecting green-red or red color were damaged, while green coloration was considered as an indication of an intact membrane.

The method developed for determining sperm membrane integrity rate using flow cytometry was modified from the staining protocol of Cámara et al. (2011). Thawed semen was diluted with Tris buffer solution to be 50×10^6 sperm/ml. The volume of 0.5 µl of CFDA (0.46 mg/ml stock), 0.5 µl of PI (0.5 mg/ml stock), and 200 µl of Tris buffer solution were added to 100 µl of diluted semen in the dark. Analysis was performed by counting at least 5000 sperm cells in the excitation range of 519 nm-630 nm in flow cytometry (Guava EasyCte™, Guava® Technologies, Hayward, CA, USA). Sperms with green fluorescent reflection (receiving CFDA) but not red fluorescent reflection (receiving PI) were considered "viable and with an intact membrane (CFDA +, PI -)".

Evaluation of sperm mitochondrial activity

Sperm mitochondrial activity was measured using 5,50,6,60-tetrachloro-1,10,3,30-tetraethyl benzimidazolyl-carbocyanine iodide (JC-1) staining protocol. Two straws were randomly selected for each group. After thawing, the semen samples were pooled in a 1.5 ml microcentrifuge tube and diluted with Tris-based buffer solution to 50×10^6 spermatozoa per milliliter. A hundred µl of the sample was diluted with 200 µl of Tris-based buffer, and 0.5 µl of JC-1 stock solution (3 mM JC-1 in DMSO) was added. Stained semen samples were incubated for 40 min in a water bath adjusted to 38°C (Gillan et al., 2005). At the end of the period, 300 µl of the sample was taken and transferred to 96-well microplates for analysis in flow cytometry (Guava EasyCte™, Guava®). Sperms with orange fluorescent reflection were considered as having "high mitochondrial membrane potential (hMMP)".

Examination of sperm viability

The viability of spermatozoa cells from cryopreserved semen was assessed using the Muse® Count and Viability Kit (EMD Millipore). Analysis was performed according to the manufacturer's described protocol. Briefly, 0.5 ml of semen was diluted 1:1 with Tris-buffered medium. Then, 380 µl of the kit solution was added to 20 µl of diluted semen. After 5 min of incubation in the dark, the viability percentage was determined by flow cytometry analysis.

Statistical analysis

The study was repeated ten times. All data obtained from the study were analyzed by "One-way Analysis of Variance" (ANOVA), followed by "Duncan's Multiple Range Test" to determine significant differences in all the spermatological parameters among all groups, with SPSS Version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). The results were represented as mean ± standard error. Differences with values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Total motility, progressive motility, velocity, and kinetic motion parameters determined before freezing are shown in Table 1. There was no statistical difference between the groups in terms of post-equilibration motility and progressive motility ($p > 0.05$). Rapid spermatozoa rates were highest in the control group with 76.30±2.98% and lowest in PH2.5 with 61.00±4.60%. The percentage of rapid spermatozoa was higher in the control group compared to the FH2.5 and PH2.5 groups ($p < 0.05$). The percentage of medium-speed spermatozoa was the lowest in the control group, with 16.90±2.50%, and the highest value was obtained in the PH2.5 with 29.00±3.40%. Slow spermatozoon rates did not differ between the groups. Immotile spermatozoa ratios were lowest in the FH1.0 and highest in the FH2.5 group. After equilibration, it was determined that the groups with the lowest VAP value were FH2.5 and PH2.5, which were the groups containing the highest percentage of honey. The highest VSL value was found in the PH1.0 group and the lowest in the PH2.5 group. The lowest VCL values were found in the groups diluted with extenders with high honey content (FH2.5 and PH2.5; $p < 0.05$).

Table 2 summarizes the rates of acrosomal and

total morphological defects in the groups. The percentage of acrosomal defects was statistically similar in all groups ($p > 0.05$). The highest value in total morphological defects was found in the control group with 24.40 ± 2.62% ($p < 0.05$).

The control and study groups were similar in total and progressive motility percentages after thawing ($p > 0.05$). The rates of immotile and rapid spermatozoa were similar in all groups ($p > 0.05$). The difference between FH2.5 and PH1.0 groups was significant in the slow spermatozoa rate ($p < 0.05$). The highest percentage of spermatozoa with medium velocity was found in the FH2.5 and PH2.5 groups, while the lowest rates were obtained in the FH1.0 and control groups. In the kinetic motion parameters after thawing, the values in the FH2.5 and PH2.5 groups containing dense honey were lower than in the control and other experimental groups (Table 3).

The results of the examinations carried out with flow cytometry, fluorescence, and phase-contrast microscope after thawing are presented in Table 4. The group with the highest rate of viable spermatozoa was FH2.5 with 49.58 ± 3.57%, while the lowest rate was obtained in the control group with 35.22 ± 3.58% ($p < 0.05$). It was determined that the dilution of semen with an extender containing 2.5% flower honey increased the number of spermatozoa with high mitochondrial activity after thawing ($p < 0.05$). According to the result of flow cytometry analysis, the ratios of 1% and 2.5% of flower honey or pine honey added to the semen extender did not have a positive or negative effect on the cell membrane integrity and acro-

Table 1. Total motility (%), progressive motility (%), velocity (%) and kinetic motion parameters (μm/sec) determined by CASA after equilibration.

Groups	After equilibration								
	Motility (%)			Velocity (%)			Kinetic motion parameters (μm/sec)		
	Total	Progressive	Rapid	Medium	Slow	Static	VAP	VSL	VCL
Control	93.20 ± 1.47	42.80 ± 4.72	76.30 ± 2.98^{a}	16.90 ± 2.50^{c}	3.30 ± 0.88	3.30 ± 1.05^{ab}	134.88 ± 5.05^{a}	101.81 ± 4.75^{ab}	234.45 ± 10.46^{a}
FH1.0	94.90 ± 0.93	45.10 ± 3.27	74.90 ± 2.92^{ab}	20.00 ± 2.57^{bc}	3.00 ± 0.49	2.00 ± 0.49^{b}	128.81 ± 4.31^{a}	100.35 ± 3.89^{abc}	214.28 ± 9.31^{a}
FH2.5	90.80 ± 1.56	44.10 ± 4.00	65.30 ± 3.36^{bc}	25.60 ± 2.86^{ab}	3.80 ± 0.64	5.60 ± 1.17^{a}	111.52 ± 3.95^{b}	89.29 ± 4.52^{bc}	184.69 ± 7.09^{b}
PH1.0	92.60 ± 2.02	46.30 ± 3.67	74.50 ± 2.25^{ab}	18.10 ± 2.41^{bc}	3.00 ± 0.59	4.40 ± 1.82^{ab}	133.63 ± 5.43^{a}	105.75 ± 6.11^{a}	220.64 ± 9.85^{a}
PH2.5	90.00 ± 2.33	42.80 ± 5.65	61.00 ± 4.60^{c}	29.00 ± 3.40^{a}	4.70 ± 0.93	5.50 ± 1.54^{ab}	106.16 ± 5.81^{b}	85.25 ± 5.91^{c}	174.58 ± 7.34^{b}

Within columns, means with no common letters are different (^{abc}: $p < 0.05$)

Table 2. Percentages of acrosome and total morphological damage after equilibration.

Groups	After equilibration	
	Morphological defect (%)	
	Acrosome	Total
Control	16.20±2.50	24.40±2.62^{a}
FH1.0	10.30±1.96	18.20±1.97^{b}
FH2.5	9.30±1.79	17.70±2.12^{b}
PH1.0	9.80±2.12	15.50±2.09^{b}
PH2.5	9.50±1.56	17.00±1.53^{b}

Within columns, means with no common letters are different (^{abc}: $p < 0.05$)

Table 3. Post-thaw total motility (%), progressive motility (%), velocity (%) and kinetic motion parameters (μm/sec) determined by CASA.

Groups	Post-thaw								
	Motility (%)			Velocity (%)			Kinetic motion parameters (μm/sec)		
	Total	Progressive	Rapid	Medium	Slow	Static	VAP	VSL	VCL
Control	63.30 ± 6.92	28.70 ± 3.40	41.40±4.70	21.90 ± 3.19^{b}	11.30 ± 1.01^{ab}	25.50 ± 7.47	113.37 ± 4.48^{a}	93.12 ± 4.72^{a}	187.25 ± 6.40^{a}
FH1.0	65.70 ± 5.22	32.50 ± 3.05	43.50 ± 3.67	22.20 ± 2.54^{b}	12.10 ± 1.12^{ab}	22.40 ± 4.84	109.76 ± 4.40^{ab}	92.20 ± 5.06^{a}	176.63 ± 4.56^{ab}
FH2.5	77.10 ± 3.47	31.00 ± 2.14	41.30 ± 2.10	35.70 ± 2.97^{a}	10.50 ± 1.27^{b}	12.50 ± 2.32	91.08 ± 3.54^{c}	75.82 ± 2.99^{b}	148.03 ± 7.52^{c}
PH1.0	70.00 ± 2.44	29.50 ± 2.99	39.50 ± 3.40	30.50 ± 3.82^{ab}	14.30 ± 1.23^{a}	15.90 ± 1.80	100.68 ± 5.57^{bc}	84.03 ± 5.38^{ab}	164.97 ± 8.82^{bc}
PH2.5	71.40 ± 3.38	27.00 ± 1.59	37.30 ± 2.37	34.10 ± 3.32^{a}	12.20 ± 1.05^{ab}	16.40 ± 2.97	91.85 ± 2.36^{c}	76.17 ± 2.26^{b}	150.34 ± 5.51^{c}

Within columns, means with no common letters are different (^{abc}: $p < 0.05$)

Table 4. Spermatological results obtained by flow cytometry and microscopic examinations in frozen-thawed semen.

Groups	Post-thaw						
	Flow-cytometry				Microscopy		
					Florescent microscopy	Phase-contrast microscopy	
	Viability (%)	High mitochondrial activity (%)	Intact Plasma Membrane Status (%)	Percentage of spermatozoa with intact acrosomes (%)	Intact Plasma Membrane Status (%)	Acrosome defect (%)	Total morphological defect (%)
Control	35.22 ± 3.58^{b}	53.29 ± 3.71^{b}	16.52 ± 4.11	26.75 ± 2.13	34.89 ± 1.08^{b}	20.30 ± 1.92^{a}	30.20 ± 2.64
FH1.0	42.16 ± 3.18^{ab}	60.41 ± 2.49^{ab}	18.40 ± 5.69	32.25 ± 3.33	42.23 ± 1.91^{a}	13.60 ± 1.51^{b}	25.60 ± 2.01
FH2.5	49.58 ± 3.57^{a}	63.42 ± 3.12^{a}	17.53 ± 5.59	32.34 ± 2.60	41.80 ± 2.73^{a}	15.20 ± 2.06^{ab}	25.70 ± 2.21
PH1.0	45.36 ± 4.23^{ab}	61.17 ± 2.60^{ab}	14.20 ± 4.24	31.66 ± 2.91	38.15 ± 2.81^{ab}	15.90 ± 2.04^{ab}	28.30 ± 2.26
PH2.5	46.89±4.97^{ab}	61.35 ± 3.15^{ab}	15.14 ± 5.24	34.35 ± 2.84	40.10 ± 1.81^{ab}	16.10 ± 1.63^{ab}	26.50 ± 2.15

Within columns, means with no common letters are different (^{abc}: $p < 0.05$)

some integrity rate after thawing in ram semen ($p > 0.05$). On the other hand, in fluorescent microscope examination, groups containing flower honey (1.0%, 2.5%) were found to be more effective in preserving membrane integrity compared to the control group ($p < 0.05$). Morphological integrity examination with a phase-contrast microscope revealed that the rate of acrosome defect was lower in FH1.0 compared to the control group ($p < 0.05$). Total morphological defect rates did not change according to the groups ($p > 0.05$).

DISCUSSION

The stages of cryopreservation, particularly the freezing and thawing process, have harmful effects on spermatozoa. This process has led them to have capacitation-like changes and affects the other spermatological parameters (Watson, 2000). This study was carried out to determine and compare the success of flower and pine honey, which has antioxidant, antibacterial, and cryoprotectant properties, in freezing ram semen. Total and progressive motility rates were similar between the groups in the pre-freezing periods (Table 1). Different densities of flower and pine honey did not affect the total and progressive motility of spermatozoa after equilibration (5°C) of semen ($p > 0.05$). It has been reported that the addition of honey to the extender has positive effects on spermatological parameters, especially motility, in the storage of cattle and buffalo bull semen at 5°C (El-Sheshtawy et al., 2014; El-Nattat et al., 2016). Maidin et al. (2018) obtained successful motility results in cold storage of goat semen when added 2% (v/v) honey alone or combined with black seed oil to the extender. Olayemi et al. (2011) investigated the effects of adding 5, 10, and 20% honey to the extender on motility and viability in goat semen stored at cold (5ºC) and reported that 5% honey preserved sperm motility and viability. However, they reported that this positive effect decreased as the density of honey increased. On the other hand, it was reported that different monosaccharides and disaccharides added to the ram semen extender did not influence motility after cooling (Pelufo et al., 2015). Contrary to this study, in a study with ram, it was reported that different percentages of honey (1.5, 2.5 and 3.5%) added to the extender increased sperm motility in the pre-freezing period (Khatun et al., 2022). Arif et al., (2023) reported that fish semen diluted with an extender containing 0.4% honey improved motility as of the 24th hour of cold incubation (5ºC). In this study, the difference in motility percentages between the groups after equilibration may not have occurred statistically due to the short equilibration period. Likewise, the differences in the studies can be attributed to the animal species, breed, diluent types, proportions of additives, dilution, and cooling techniques.

Although it was determined that different densities of flower and pine honey did not affect motility in this study, differences were found in spermatozoon velocity values. Considering the velocity rates between the groups after equilibration, it is noteworthy that the spermatozoon velocity rates were relatively lower in dense honey groups (FH2.5 and PH2.5) (Table 1). Compared to the control group, the rapid spermatozoa rates were lower in the honey-containing groups, and this decrease was more pronounced in the groups containing dense honey ($p < 0.05$). The highest values were obtained in the dense honey groups in the medium-speed spermatozoa ratios. The decrease in the rate of fast spermatozoa and the increase in the rate of me-

dium-speed spermatozoa in dense honey groups can be attributed to the osmolarity of the medium. Honey is a very dense substance due to its natural structure, which increases the osmotic pressure of the liquid it contains. Spermatozoon velocities can vary according to the media and especially the viscosity of the media (Amann, 1989; Jerez-Ebensperger et al., 2015). Spermatozoon movements may be restricted due to physical reasons in intense environments. Findings of kinetic motion parameters also support this statement; in all three (VAP, VSL, VCL) kinetic motion parameters, fewer values are observed in dense honey groups (Table 1).

After equilibration, the most severe acrosomal damage was found in the control group, with 16.20 ± 2.50%, ranging from 9.30 ± 1.79% to 10.30 ± 1.96% in the other groups. Although the rate in the control group is statistically insignificant, it is remarkable (Table 2). The findings of acrosomal damage obtained in pre-freezing period confirm the study of Khatun et al. (2022).Likewise, the highest total morphological damage rate was found in the control group, with 24.40 ± 2.72% ($p < 0.05$). This shows that the addition of honey to semen extenders preserves the morphological integrity of spermatozoa during dilution, cooling, glycerolization, and equilibration stages. It has been reported that adding trehalose, a disaccharide, to ram semen extenders has no effect on acrosome and membrane integrity after cooling but is harmful at increasing densities (Aisen et al., 2002). Pelufo et al. (2015) reported that monosaccharides and disaccharides added to the extender did not affect acrosome integrity in ram spermatozoa after cooling, while glucose was effective in maintaining membrane integrity after cooling. The present study found that adding 1.0% and 2.5% flower and pine honey in the ram semen extender protects the morphological integrity against the harmful effects of the dilution and cooling stages (Table 2). Total motility and progressive motility values did not change between the groups after thawing, as well as before freezing (Table 3). It was determined that different ratios of flower and pine honey did not affect sperm total motility and progressive motility in the freezing of ram semen. In studies conducted on different animal species, higher motility rates were obtained after freezing with the addition of various honey or sugar into the extenders (Aisen et al., 2002; Jafaroghli et al., 2011; Jerez-Ebensperger et al., 2015; Panyaboriban et al., 2015; El-Nattat et al., 2016; El-Sheshtawy et al., 2016; Khatun et al., 2022). Although there was no statistical difference in our study, the total motility values obtained after thawing support these studies (Table 3). On the other hand, the highest rates of spermatozoa with medium velocity were found in the groups with dense honey added ($p < 0.05$). In all kinetic velocity parameters (VAP, VSL, VCL), the rates detected in dense honey groups were lower than the control group ($p < 0.05$). This difference may also be due to the limited sperm motion in dense media (Amann, 1989; Jerez-Ebensperger et al., 2015).

The lowest viability detected by flow cytometry was found in the control group as 35.22 ± 3.58%, and the highest viability rate was 49.58% ± 3.57 in the 2.5% flower honey group ($p < 0.05$; Table 4). In terms of mitochondrial activity rates, more successful results were obtained in the group containing 2.5% flower honey compared to the control group ($p < 0.05$; Table 4). It can be suggested by the findings that the addition of honey to the extender partially contributes to the viability of the spermatozoon against the harmful effects of freezing, and this contribution is important in the addition of 2.5% flower honey. It has been reported that sugars (fructose, fructose-sucrose, fructose-trehalose, and sucrose-trehalose) positively affect the viability of ram spermatozoa after thawing, and even their combinations are more effective (Panyaboriban et al., 2015). El-Sheshtawy et al. (2016), in their stallion study, reported that adding honey to the extender had positive effects on viability after thawing. In ram semen frozen with extenders containing different concentrations of raffinose, trehalose, and sucrose, the viability values obtained after thawing were significantly higher than in the control group (Jafaroghli et al., 2011). It is known that there is a positive correlation between mitochondrial activity and motility (Mehdipour et al., 2017). Since energy loss that may occur because of mitochondrial damage will cause ATP deficiency, it leads to a decrease in motility parameters after the freezing-thawing processes (Medeiros et al., 2002). Maidin et al. (2018), in their study on goat semen, reported that adding honey to the extender reduces the formation of reactive oxygen species caused by cooling, freezing, and thawing processes and has curative effects on motility. The present study found that especially 2.5% flower honey was effective in maintaining sperm viability and mitochondrial activity (Table 4). When the three examination techniques related to membrane integrity are evaluated in general (florescent microscopy, flow-cytometry, phase-contrast microscopy), it can be said that flower honey is beneficial in preserving

the membrane structure of ram spermatozoa. It was stated that honey preserves membrane integrity after thawing in buffalo (El-Nattat et al., 2016) and stallion (El-Sheshtawy et al., 2016) semen. The positive effect of adding rosemary honey to ram semen extender on acrosomal integrity after thawing was reported (Jerez-Ebensperger et al., 2015). Trehalose at osmolality of 100 mOsm has a protective effect on the membrane and acrosome integrity after thawing in ram semen (Aisen et al., 2002); similarly, reduction in the membrane, acrosome, and total morphological damage rates were obtained by addition of raffinose, trehalose, and sucrose at different concentrations to ram semen extender in freezing-thawing stages (Jafaroghli et al., 2011). The results obtained in the present study support these findings.

It has been reported that the beneficial effect of honey observed in ram semen after freezing may be due to the antioxidant properties of the proteins, enzymes, amino acids, organic acids, vitamins, and phenolic compounds (Jerez-Ebensperger et al., 2015; El-Nattat et al., 2016) and sugars in its content, which are sources of energy and cryoprotectant for sperm cells during cold storage (Khatun et al., 2022).

CONCLUSION

This study observed that 1.0% and 2.5% (v/v) pine honey added to Tris egg yolk-based extender in the cryopreservation of ram semen did not have a positive effect on spermatological parameters. On the other hand, 2.5% (v/v) flower honey positively affected plasma membrane integrity, sperm viability, and mitochondrial activity after thawing and could be added to the extender for freezing ram semen.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Abdullah MH, Selman MO, Fakhrildin MBM (2015) Effect of honeybee and coq10 supplemented to cryopreservation solution on freeze-thawed human sperm parameters. World J Pharm Res 4 (12): 524-534.

Aisen EG, Alvarez HL, Venturino A, Garde JJ (2000)Effect of trehalose and EDTA on cryoprotective action of ram semen diluents. Theriogenology 53:1053-1061.

Aisen EG, Medina VH, Venturino A (2002) Cryopreservation and post-thawed fertility of ram semen frozen in different trehalose concentrations. Theriogenology 57 (7); 1801-1808.

Ak K, Cirit Ü, Nur Z, Bacınoğlu S, Pabuccuoğlu S, Özdaş Ö et al. (2010) Effects of extender osmolarity, cooling rate, dilution rate and glycerol addition time on post-thaw ram semen characteristics and fertilization. J Fac Vet Med Istanbul Univ 36 (2): 33-46.

Akbulut M, Özcan MM, Çoklar H (2009) Evaluation of antioxidant activity, phenolic, mineral contents and some physicochemical properties of several pine honeys collected from Western Anatolia. Int J Food Sci Nutr 60 (7): 577-589.

Amann RP, Waberski D (2014) Computer-assisted sperm analysis (CASA): capabilities and potential developments. Theriogenology 81 (1): 5-17.

Amann RP (1989) Can the fertility potential of a seminal sample be predicted accurately? J Androl 10 (2): 89-98.

Arif, M, Airin, CM, Datrianto, DS, & Sejati, DAR (2023) Influence of honey on sperm traits in KUB rooster concerning cold storage. Adv Anim Vet Sci 11 (2): 246-251.

Bacinoğlu S, Cirit Ü, Nur Z, Ak K (2007) Eritilmiş koç spermasında farklı gliserol katma tekniklerinin ve soğutma hızının spermatolojik özelliklere etkisi. J Fac Vet Med Istanbul Univ 33 (1): 11-21.

Bacinoglu S, Taş M, Cirit Ü, Özdaş, ÖB, Ak K (2008) The potential fertility estimation capacity of the hypoosmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. Anim Reprod Sci 104 (1): 38-46.

Bogdanov S, Jurendic T, Sieber R, Gallmann P (2008) Honey for nutrition and health: a review. J Am Coll Nutr 27 (6): 677-689.

Balogun, KB, Nicholls, G, Sokunbi, OA, & Stewart, KR (2023) Cryoprotectant effects of natural honey on spermatozoa quality of pre-freezing and frozen-thawed boar semen. Journal of Animal Science 101.

Cámara DR, Silva SV, Almeida, FC, Nunes, JF, Guerra, MMP (2011) Effects of antioxidants and duration of pre-freezing equilibration on frozen-thawed ram semen. Theriogenology 76 (2): 342-350.

Cirit Ü, Bağış H, Demir K, Agca C, Pabuccuoğlu S, Varışlı Ö et al. (2013) Comparison of cryoprotective effects of iodixanol, trehalose and cysteamine on ram semen. Anim Reprod Sci 139: 38-44.

Demir K, Öztürk Bakırer G, Cirit Ü, Bozkurt HH, Aktaş A, Birler S et al. (2015) Effects of cooling rate on membrane integrity and motility parameters of cryopreserved ram spermatozoa. Kafkas Univ Vet Fak Derg 21: 61-67.

El-Nattat WS, El-Sheshtawy RI, El-Batawy KA, Shahba MI, El-Seadawy IE (2016) Preservability of buffalo bull semen in tris-citrate extender enriched with bee's honey. J Innov Pharm Biol Sci 3 (1): 180-185.

El-Sheshtawy RI, El-Badry DA, Gamal A, El-Nattat WS, Almaaty AMA (2016) Natural honey as a cryoprotectant to improve Arab stallion post-thawing sperm parameters. Asian Pac J Reprod 5 (4): 331-334.

El-Sheshtawy RI, El-Nattat WS, Sabra HA, Ali AH (2014) Effect of honey solution on semen preservability of local breeds of cattle bulls. Wld Appl Sci J 32 (10): 2076-2078.

Fakhrildin MBM, Alsaadi RA (2014) Honey Supplementation to semen-freezing mediumimproveshuman sperm parameters post-thawing. J Family Reprod Health 8 (1): 27.

Fuller BJ (2004) Cryoprotectants: the essential antifreezes to protect life in the frozen state. Cryo Letters 25 (6): 375-388.

Gillan L, Evans G, Maxwell, WMC (2005) Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63 (2): 445-457.

Jafaroghli M, Khalili B, Farshad A, Zamiri MJ (2011) The effect of supplementation of cryopreservation diluents with sugars on the post-thawing fertility of ram semen. Small Rumin Res 96 (1): 58-63.

Jerez-Ebensperger RA, Luno V, Olaciregui M, Gonzalez N, de Blas I, Gil L (2015) Effect of pasteurized egg yolk and rosemary honey supplementation on quality of cryopreserved ram semen. Small Rumin Res 130: 153-156.

Khatun, A, Fazili, MR, Malik, AA, Naikoo, M, Choudhury, AR, Shah, S, ... & Hussain, I (2022) Can honey improve the quality of cryopreserved cross bred ram semen added to tris egg yolk extender?. Cryoletters 43 (6): 334-340.

Kwakman, PH, Zaat SA (2012) Antibacterial components of honey. IUBMB life 64 (1):48-55.

Maidin MS, Padlan MH, Azuan SAN, Jonit R, Mohammed NH, Abdullah R (2018) Supplementation of Nigella sativa oil and honey prolong the survival rate of fresh and post-thawed goat sperms. Trop Anim Sci J 41 (2): 94-99.

Marco-Jiménez F, Puchades S, Gadea J, Vicente, JS, Viudes-de-Castro MP (2005) Effect of semen collection method on pre-and post-thaw Guirra ram spermatozoa. Theriogenology 64 (8): 1756-1765.

Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL (2002) Current status of sperm cryopreservation: why isn't it better?. Theriogenology 57 (1): 327-344.

Mehdipour M, Kia HD, Nazari M, Najafi A (2017) Effect of lecithin nanoliposome or soybean lecithin supplemented by pomegranate extract on post-thaw flow cytometric, microscopic, and oxidative parameters in ram semen. Cryobiology 78: 34-40.

Motamedi-Mojdehi R, Roostaei-Ali Mehr M, Rajabi-Toustani R (2014) Effect of different levels of glycerol and cholesterol-loaded cyclodextrin on cryosurvival of ram spermatozoa. Reprod Domest Anim 49 (1): 65-70.

Ögretmen F, İnanan BE (2014) Evaluation of cryoprotective effect of turkish pine honey on common carp (Cyprinus Carpio) Spermatozoa. Cryo Letters 35 (5): 427-437.

Olayemi FO, Adeniji DA, Oyeyemi MO (2011) Evaluation of sperm motility and viability in honey-included egg yolk based extenders. Global Vet 7 (1): 19-21.

Ouchemoukh S, Louaileche H, Schweitzer P (2007) Physicochemical characteristics, and pollen spectrum of some Algerian honeys. Food Control 18 (1): 52-58.

Özkök A, Sorkun K, Salih B (2016) The microscopic and GC-MS analysis of Turkish pine honey. J Biol & Chem 44 (4): 375-383.

Özmen MF, Cirit Ü, Arıcı R, Demir K, Kurt D, Pabuccuoğlu S et al. (2020) Evaluation of synergic effects of iodixanol and trehalose on cryosurvival of electroejaculated ram semen. Andrologia 52 (9): e13656.

Öztürkler Y, Baran A, Evecen M, Ak K, İleri İK (2001) Comparison of ovine spermatozoal morphological features after staining of fixation and assessment of morphological abnormalities in dead/live spermatozoa. Turk J Vet Anim Sci 25 (5): 675-680.

Panyaboriban S, Suwimonteerabutr J, Phutikanit N, Swangchan-Uthai T, Tharasanit T, Techakumphu M (2015)Effect of various combinations of sugar supplementation in the extender on frozen-thawed ram se-

men quality and fertility. Thai J Vet Med 45 (2): 229.
Pelufo V, Armengol ML, Malcotti V, Venturino A, Aisen EG (2015) Effects of glycerol and sugar mixing temperature on the morphologic and functional integrity of cryopreserved ram sperm. Theriogenology 83 (1): 144-151.
Salamon S, Maxwell WM (2000) Storage of ram semen. Anim Reprod Sci 62: 77-111.
Valente SS, Pereira RM, Baptista MC, Marques CC, Vasques MI, Pereira MS et al. (2010) In vitro and in vivo fertility of ram semen cryopreserved in different extenders. Anim Reprod Sci 117 (1-2): 74-77.
Watson PF (2000) The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 60: 481-492.
Yimer N, Muhammad N, Sarsaifi K, Rosnina Y, Wahid H, Khumran AM et al. (2015) Effect of honey supplementation into Tris Extender on Cryopreservation of Bull Spermatozoa. Mal J Anim Sci 18 (2): 47-54.