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Original Article

Effects of propolis supplementation during cryopreservation of ram semen

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Abstract

Background: Cryopreservation of ram semen is a very challenging process. Loss of motility during freezing does not allow artificial insemination of rams. **Aims:** This study aimed to determine whether the inclusion of liquid propolis extract in semen diluents affects the cryopreservation efficiency of ram semen. **Methods:** Six Akkaraman breed rams were considered for semen study. Semens were combined with Tris+egg yolk extender containing and without (control) propolis at different concentrations (0.25%, 0.50%, 1%, 2%, and 4%). Semen was frozen using routine ram semen freezing procedures. After thawing, motility and kinematic parameters were analyzed by computer assisted semen analysis (CASA), and viability, acrosomal damage level and mitochondrial membrane potential were analyzed by flow-cytometer in all groups. Additionally, fatty acid levels in total semen were analyzed using gas chromatography (GC), and vitamin and cholesterol levels were analyzed using high-performance liquid chromatography (HPLC). In addition, oxidative stress, HOS test, and morphological analyzes were performed after freezing and thawing. **Results:** The 0.5% propolis group showed a significant increase in total and rapid motility, LIN, membrane integrity, and antioxidant levels compared to the control, and a significant decrease in malondialdehyde (MDA) and low mitochondrial membrane potential (LMMP) levels. Compared to the control, the group containing 4% propolis damaged spermatozoa and caused a significant decrease in total, progressive and rapid motility and high mitochondrial membrane potential (HMMP), Glutathione S-transferase (GST), and catalase (CAT) levels. **Conclusion:** We showed that adding 0.5% propolis to semen extenders to increase the freezability level of ram semen increases the survival of spermatozoa after freeze-thaw and ensures the success of freezing.

Key words: CASA, Flow-cytometer, Propolis liquid extract, Ram, Semen

Introduction

Freezing and storing sperm is an indispensable step for reproductive technologies that play an important role in the livestock industry. Freezing ram semen is a widely used method for purposes such as genetic advancement, breed development, and preservation of genetic resources. This method ensures the preservation of valuable genetic material over time and facilitates the sharing of genetic resources. However, ram semen contains a structurally different lipid composition compared to other species. Due to this lipidic structure, ram semen is very unstable to freezing. Many researchers have studied various antioxidants into semen extenders to achieve successful freezing, the most effective way of ram semen storing for a long time, however, the desired freezing success has not been still achieved (Bailey *et al*., 2000; Bucak *et al*., 2008).

Propolis is a natural resinous substance, collected and produced by honey bees from some parts of plants (Ghisalberti, 1979). More than 300 compounds have been identified in various propolis extracts with beneficial pharmacological effects. The known components of propolis extract are vitamins B1, B2, B6, C, E, and elements such as silver, cesium, mercury, lanthanum, antimony, copper, manganese, iron, calcium, aluminum, and vanadium (Deblock-Bostyn, 1982; Debuyser, 1983). In addition, isoflavones, flavonoids and fatty acids, which are the main compounds of propolis, are known to provide antioxidant, antimicrobial, antiprotozoal, and antifungal activities to propolis (Selem, 2012). The antioxidant activity of propolis is attributed to its components, such as flavonoids, flavones, isoflavones, anthocyanins, and catechins,

which can scavenge free radicals and protect the propolis against lipid peroxidation (Moraes *et al*., 2014). The incorporation of propolis into semen diluent has been reported to enhance the sperm motility of *Salvelinus alpinus* fish and to protect sperm integrity by acting as a cryoprotective agent (El-Battawy and Brannas, 2015).

This study aimed to evaluate the semen after freezing and thawing using propolis liquid extract to eliminate the instability of ram semen in freezing process.

Materıals and Methods

Ethical approval

This study was approved by Fırat University Animal Experiments Local Ethics Committee (FÜHADYEK) on 14.04.2021 and in the 2021/7 session.

Animal material and semen collection

In this study, six Akkaraman breed rams, 2 years old, clinically healthy, with known fertility and a live weight of 55-60 kg, without any pathological findings as a result of genital organ examination, were considered. During the experiment, the animals were housed in the Fırat University Animal Hospital hospitalization unit and fed with concentrated and high-quality roughage. Drinking water was provided *ad libitum*. Experimental analyses were carried out in the andrology laboratory of the Reproduction and Artificial Insemination Department of Fırat University, Faculty of Veterinary Medicine, Biochemistry Laboratory of the Department of Biochemistry, and Biology Department of the Faculty of Science. This study was carried out during the breeding season. Semen was collected from the rams using an artificial vagina twice a week for three weeks. Semen from rams with total motility of 70% and density below 2 billion/ml were not included in the study.

Dilution of semen

The base diluent consisted of a Tris buffer solution (Tris (hydroxymethyl aminomethane) 3.63 g, fructose 0.50 g, citric acid 1.99 g and distilled water 100 ml) plus (500 IU penicillin/ml, 500 IU/ml streptomycin) and 15% egg yolk (Güngör *et al*., 2022). The semen was diluted 1:1 with Tris buffer and egg yolk diluent and pooled. The density of the pool was determined and the sample was re-diluted to 400 million motile spermatozoa per ml. The pooling was rested in an oven at 38°C for at least 15 min. The pool was divided equally into six falcon tubes of 2 ml each.

Experimental groups (G)

Fifty ml of propolis liquid extract (Hekimhan® Herbal, Türkiye) containing 70% deionized water and 30% propolis was provided. The extract was divided into falcon tubes in different proportions and diluted with distilled water and tris+egg yolk extender to prepare preliminay experimental groups. Semen samples were divided into six different falcon tubes (2 ml in each tube) and combined with the preliminary solutions containing different propolis concentrations to have final experimental groups (G1-G6). The groups included:

G1: 6 ml diluted semen containing 4% propolis
G2: 6 ml diluted semen containing 2% propolis
G3: 6 ml diluted semen containing 1% propolis
G4: 6 ml diluted semen containing 0.5% propolis
G5: 6 ml diluted semen containing 0.25% propolis
G6: 6 ml diluted semen containing 0% propolis (control)

Freezing process

After accomplishing the glycerolization-equilibration process, the sperm samples were collected into 0.25 ml mini straws with different colors according to their groups. The samples were then freezed using an automatic freezing device (Microdigitcool, IMV, Paris, France) adjusted to the ram semen freezing procedure (Fig. 1). Following the freezing process, the straws at -140°C were quickly transferred to containers containing -196°C liquid nitrogen and stored until analysis.

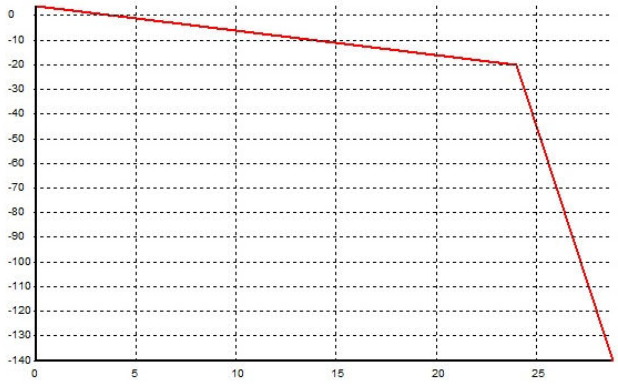


Fig. 1: Special freezing procedure set for freezing ram semen

Analysis of motility and kinematic parameters after freeze-thaw

These analyzes were performed using Computer Aided Sperm Analyzer (CASA, ISASv1, Proiser, Spain). A total of 485 µL of a previously prepared and heated Tris buffer solution was transferred to an Eppendorf tube, followed by adding 15 µL of thawed semen. Three µL of the resulting mixture was taken, dropped onto a special slide (Spermtrack 20, Proiser, Spain) and analyzed. As a result of the analysis, total, progressive, rapid, medium, slow motility (%), VCL-curvilinear velocity (µm/s), VSL-linear speed (µm/s), VAP-average road speed (µm/s), LIN-linearity of curvilinear pathways (%), STR-linearity of the average path (%), WOB-the measure of the fluctuation observed while moving on the real path (%), ALH-the distance at which the sperm head deviates laterally while advancing on the average path (µm) and BCF-frequency of the curvilinear pathway passing through the average pathway (Hertz) results were recorded. For spermatozoon speed, the speed range set by the manufacturer for rams (still <10 µm/s < slow <45 µm/s < medium <75 µm/s < fast) was used. The particle size of the device was set as 15-70 µm (Güngör *et al*., 2022).

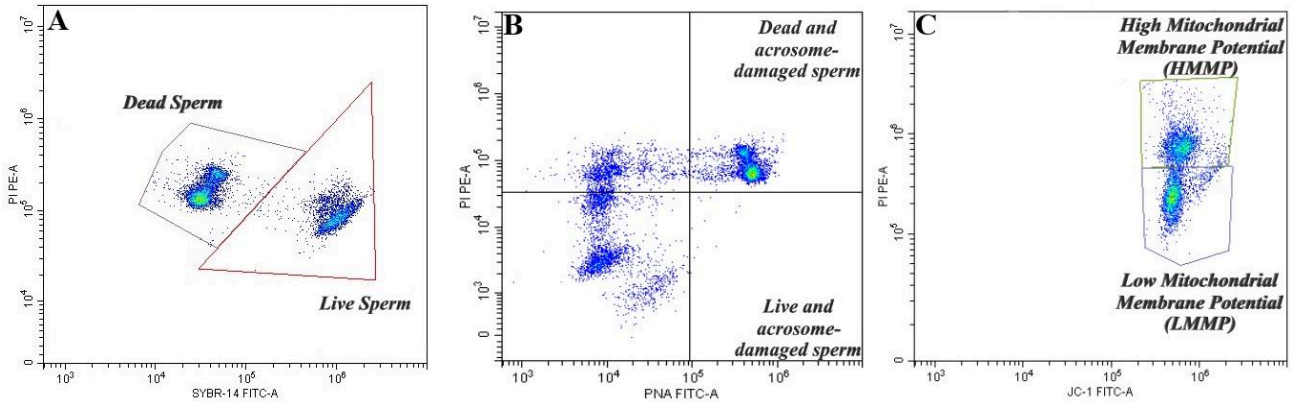


Fig. 2: Gatings taken on dot-plot cytograms as a result of flow-cytometric analyses. (**A**) Separation of dead and live spermatozoa using FITC-A/PE-A comparison cytogram, (**B**) Separation of dead and live acrosome damaged spermatozoa using FITC-A/PE-A comparison cytogram, and (**C**) Separation of high and low mitochondrial membrane potential spermatozoa using FITC-A/PE-A comparison cytogram

Morphological analysis and determination of membrane integrity after freeze-thaw

One part of the thawed semen was taken and diluted with 4 parts of Tris buffer solution. Then, 75 µL of the diluted semen was taken, dropped onto the slide, and a smear was taken. After the smears were dried in air, they were dipped into the A, B, and C solutions of the Diff-Quick sperm staining set (Gündüz Kimya, Istanbul, Türkiye), respectively. At the last stage, the smears were washed with distilled water. The dried preparations were placed on a phase-contrast microscope (Nikon, Tokyo, Japan) stand and 400 spermatozoons were counted using the ×400 magnification of the microscope. The proportion of spermatozoa with head and tail abnormalities among the counted spermatozoa was determined as the total abnormality (%). Hypoosmotic swelling test was performed to determine membrane integrity. For this purpose, 50 µL of the diluted semen (300 mOsm) was taken and transferred to a previously prepared hypotonic solution (0.49 g citric acid, 0.9 g fructose, 100 ml distilled water-150 mOsm). This mixture was incubated in an oven at 38°C for 60 min. After incubation, 50 µL of this mixture was taken, dropped onto the slide, and the coverslip was closed. 400 spermatozoons were counted using the ×400 magnification of the phase-contrast microscope. The proportion of spermatozoa with swollen heads and curled tails was expressed as a percentage (Özer Kaya *et al*., 2021).

Flow-cytometric analysis after freeze-thaw

Flow-cytometric analyses of sperm samples were performed with laser beam at 488 nm (50 mW laser output). For this purpose, 525 ± 40, 585 ± 42, and 610 ± 20 nm emission filters were used.

LIVE/DEAD Sperm Viability Kit (L7011, ThermoFisher Scientific, USA) was used to determine the viability rate. The samples were prepared with 980 µL PBS + 10 µL frozen-thawed semen + 5 µL SYBR-14 + 5 µL propidium iodide (PI) stains (Fig. 2A).

To determine the level of acrosomal damage, Lectin PNA from Arachis hypogaea (peanut agglutinin), Alexa Fluor™ 488 Conjugate (L21409, ThermoFisher Scientific, USA) was used. For preparation, 982 µL PBS and 10 µL frozen-thawed semen was added to 5 µL SYBR-14 and 3 µL PI stains (Fig. 2B).

To determine the mitochondrial membrane potential, 1,1´,3,3´-Tetraethyl-5,5´,6,6´-tetrachloroimidacarbocyanine iodide (JC-1 Dye, Mitochondrial Membrane Potential Probe, T3168, ThermoFisher Scientific, USA) was used. For preparation, 987.5 µL PBS and 10 µL frozen-thawed semen were added to 2.5 µL JC-1 (Fig. 2C).

These mixtures obtained from all analyses were vortexed and incubated at 38°C for 30 min. After incubation, they were placed in the reading section of the flow-cytometry device and the device was allowed to count 10,000 cells. After the cell count was completed, spermatozoa were separated by FSC (Forward Scatter) - SSC (Side Scatter) comparative cytogram. Then, singlet cells were determined by comparing FSC-A (Forward Scatter Area) - FSC-H (Forward Scatter Height). As a result of the PE (Phycoerythrin) - FITCH (Fluorescein Isothiocyonate) comparison among these singlet cells, live cells, cells with damaged acrosomes, and cells with high and low mitochondrial membrane potential were determined in separate compartments and expressed as a numerical value as a percentage (Güngör, 2023).

Oxidative stress analysis

The MDA level was tested according to the method described by Placer *et al*. (1966). This method was based on the reaction of thiobarbituric acid with MDA, one of the aldehyde products of lipid peroxidation. The glutathione (GSH) level was determined by the method of Ellman *et al*. (1961). This method was spectrophotometric and based on the formation of a highly stable yellow colour in sulphhydryl groups when 5,5´-dithiobis-2-nitrobenzoic acid (DTNB) was added. The CAT activity was revealed by using Aebi's method (Aebi, 1984). It was determined by measuring the resolution of hydrogen peroxide (H_2O_2) at 240 nm. Measuring of GSH-Px activity was by the Beutler method (Beutler, 1975). GSH-Px catalyses the oxidation of GSH to oxide glutathione (GSSG) using H_2O_2. The

rate of formation of GSSG was measured by the glutathione reductase reaction. The method described by Habig *et al*. (1974) was utilised to test GST activity. The enzyme activity was determined by measuring the amount of enzyme catalysing 1 µmol of 1-(S-glutathionyl)-2,4 dinitrobenzene per minute at 340 nm at 37°C using GSH and 1-chloro-2,4-dinitrobenzene. Quantifying superoxide anion (O_2.-) generation provided data for SOD activity, where generation was by xanthine and xanthine oxidases reacting with nitroblue tetrazolium (Sun *et al*., 1988). The determination of protein concentration was performed using the method described by Lowry *et al*. (1951).

Measurement of fatty acids, vitamins, and cholesterol levels in spermatozoons after freeze-thaw

After lipid exraction, 1 ml semen sample was taken and homogenized with 10 ml n-hexane-isopropanol at 3:2 (v/v) for 30 s. The mixture was then centrifuged at 4500 g for 10 min. The supernatant was then analyzed for fatty acid and vitamin (Hara and Radin, 1978).

When analyzing fatty acids in tissues and body fluids, fatty acids are analyzed by converting them into methyl esters in methanol containing 2% sulfuric acid. To do this, first the fatty acids in lipid extracts are converted into fatty acid methyl esters and then the fatty acid methyl esters are analyzed (Christie, 1990). For methyl esters, the semen lipid extract in the hexane-isopropanol phase was placed in 25 ml leak-proof test tubes. Five ml of 2% methanolic sulfuric acid was added and mixed with vortex. This mixture was incubated in an oven at 55°C for 15 h. After cooling at room temperature, 5 ml of 5% sodium chloride was added and mixed. The fatty acid methyl esters formed in the tubes were extracted with 5 ml of hexane, then 5 ml of 2% $KHCO_3$ was added and kept for 4 h to separate the phases. After the mixture containing methyl esters was evaporated at 45°C and under liquid nitrogen flow, it was dissolved in 1 ml hexane and placed in 2 ml capped autosampler vials for analysis by gas chromatography (GC) (Christie, 1990). Fatty acids in the semen lipid extract were analyzed by SHIMADZU GC 2010 Plus gas chromatography after being converted into methyl esters. SP™-2380 capillary GC column (L × I.D. 30 m × 0.25 mm, df 0.20 µm) was used for this analysis. During the analysis, the column temperature was kept at 120-220°C, the injection temperature at 240°C, and the detector temperature at 280°C. The column temperature program was set from 120°C to 220°C, and the temperature increase was programmed as 5°C/min from 200°C to 4°C/min from 200°C to 220°C. Nitrogen gas was used as the carrier gas. The mixtures of standard fatty acid methyl esters were injected and the retention times of each fatty acid were determined. After this process, the necessary programming was made and the fatty acid methyl esters of the samples were analyzed. After the analysis of fatty acid methyl esters, the levels of saturated fatty acids (SFA, %), monounsaturated fatty acids (MUFA, %), and polyunsaturated fatty acids (PUFA, %) were measured in the semen samples.

A high-performance liquid chromatography (HPLC) device was used to analyze vitamins A, D, and E and cholesterol in semen lipid extracts (Katsanidis and Addis, 1999; Sanchez-Machado *et al*., 2004; Karpinska *et al*., 2006; Lopez-Cervantes *et al*., 2006). The supernatant obtained from the extraction of lipids was divided into two equal volumes and 5% KOH solution was added to one portion. After mixing thoroughly, it was kept at 85°C for 15 min. The tubes were then cooled at room temperature. The content was vortexed thoroughly, to which 5 ml of pure water was added. Following phase separation, the upper phase was collected and its solvent was evaporated with nitrogen flow. Vitamins A, D, E, and K, cholesterol, and sterol residues remaining in the tubes were dissolved in acetonitrile/methanol mixture (50% + 50%, v/v) at a 1/1, v/v ratio and taken into autosampler vials for analysis. Acetonitrile/methanol (60% + 40%, v/v) mixture was used for the mobile phase. The mobile phase flow rate was determined as 1 ml/min. PDAUV detector and Süpelcosil™ LC 18 (15 × 4.6 cm, 5 µm) column were used for analysis. Vitamin A levels were detected at 326 nm detection wavelength, vitamins E, D, and K and cholesterol levels were detected at 202 nm.

Statistical analysis

All statistical analyzes were performed using SPSS (ver. 22.0, IBM Corp., Armonk, NY, USA). Firstly, normality analysis was performed with all parameters. It was found that the values did not show a normal distribution. For this reason, non-parametric analyzes were used. Non-parametric Kruskal-Wallis analysis of variance was used to determine the differences between the experimental groups containing propolis and the control group after the freeze-thaw process, and the non-parametric Mann-Whitney-U test was preferred for pairwise comparisons. P-value of less than 0.05 is considered statistically significant. Values are given as means±standard error means (SEM).

Results

Findings on motility and kinematic parameters

After freeze-thaw, the group containing 0.5% propolis provided a statistically significant ($P<0.001$) increase in total motility, rapid motility, and LIN parameters compared to the control group. It was revealed that the 4% propolis group caused a significant ($P<0.001$) decrease in total, progressive and rapid motility rates compared to the control group. Groups containing 2% and 4% propolis provided a significant ($P<0.05$) decrease in the slow motility parameter compared to the control. When the medium motility and kinematic parameter values (except LIN) were examined, no statistical difference could be determined between the control and experimental groups (Figs. 3A-E and 4A-C and Table 1).

Membrane integrity (HOST) and morphological analyses

After freeze-thaw, the groups containing 0.5% and 1% propolis showed better membrane integrity and a significant (P<0.05) increase compared to the control group (Fig. 4D). In morphological analysis, no statistical difference was found among the experimental groups and the control group (Table 1).

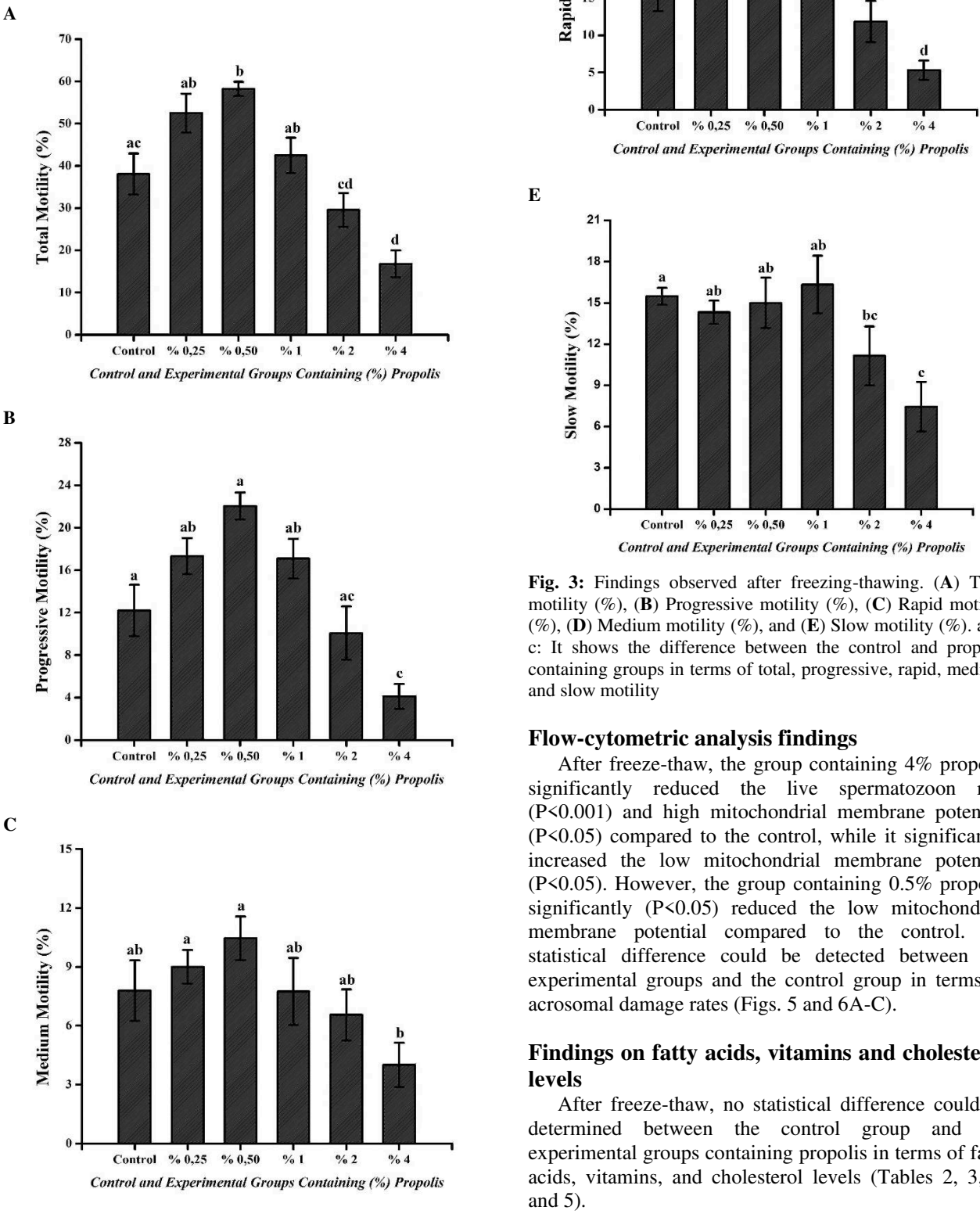


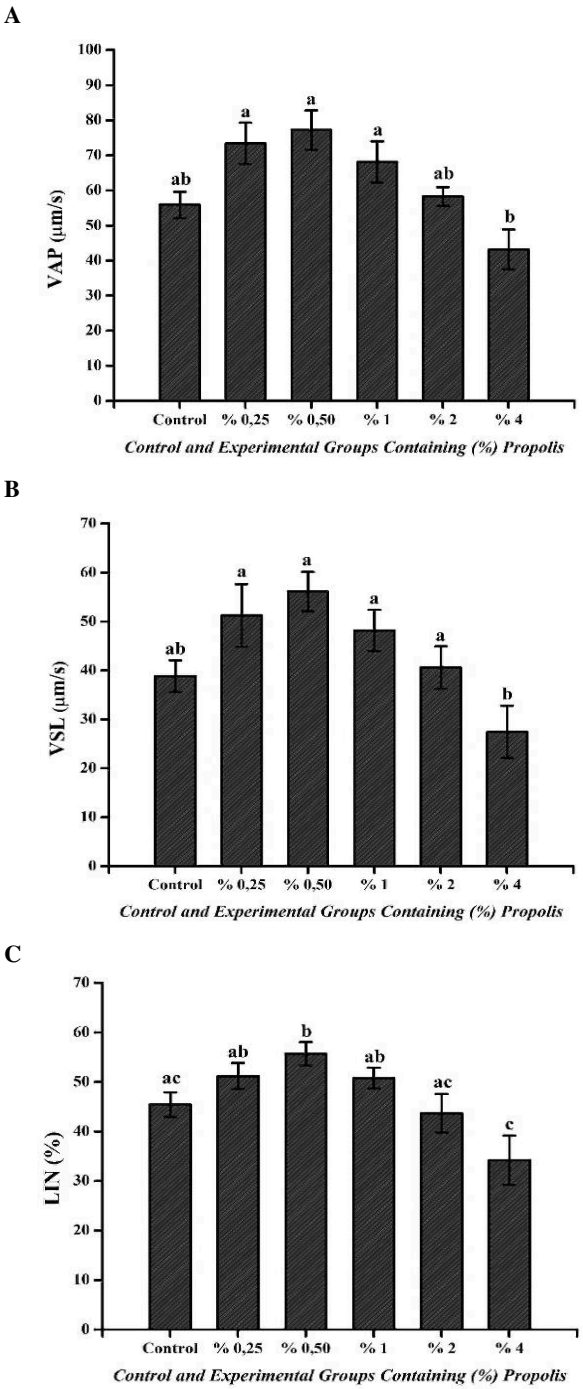
Fig. 3: Findings observed after freezing-thawing. (**A**) Total motility (%), (**B**) Progressive motility (%), (**C**) Rapid motility (%), (**D**) Medium motility (%), and (**E**) Slow motility (%). a, b, c: It shows the difference between the control and propolis containing groups in terms of total, progressive, rapid, medium and slow motility

Flow-cytometric analysis findings

After freeze-thaw, the group containing 4% propolis significantly reduced the live spermatozoon rate (P<0.001) and high mitochondrial membrane potential (P<0.05) compared to the control, while it significantly increased the low mitochondrial membrane potential (P<0.05). However, the group containing 0.5% propolis significantly (P<0.05) reduced the low mitochondrial membrane potential compared to the control. No statistical difference could be detected between the experimental groups and the control group in terms of acrosomal damage rates (Figs. 5 and 6A-C).

Findings on fatty acids, vitamins and cholesterol levels

After freeze-thaw, no statistical difference could be determined between the control group and the experimental groups containing propolis in terms of fatty acids, vitamins, and cholesterol levels (Tables 2, 3, 4, and 5).



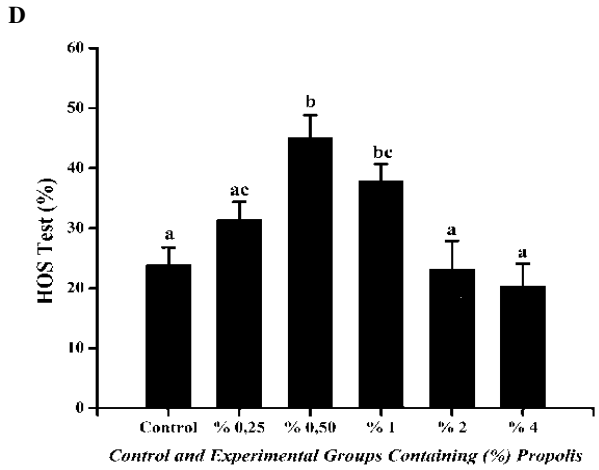


Fig. 4: Findings observed after freezing-thawing. (**A**) VAP, (**B**) VSL, (**C**) LIN, and (**D**) HOS test. a, b, c: It shows the difference between the control and propolis containing groups in terms of VAP, VSL, LIN, and HOS test

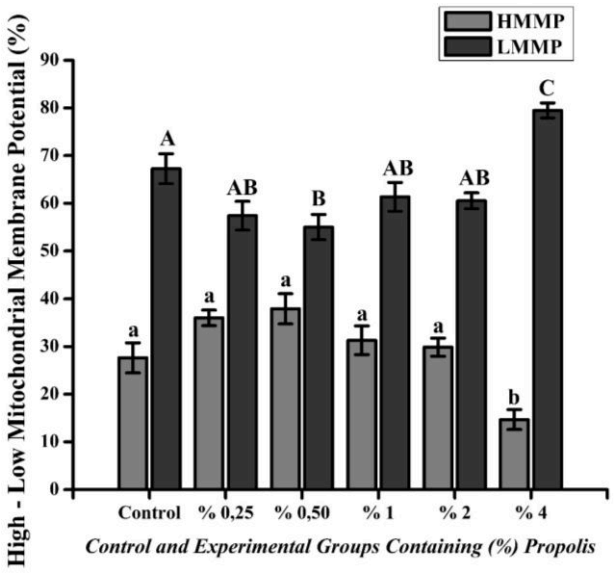


Fig. 5: High and low mitochondrial membrane potential findings observed after freeze-thawing. a, b: It shows the difference between the control and propolis containing groups in terms of HMMP, and A, B: It shows the difference between the control and propolis containing groups in terms of LMMP

Table 1: Some kinematic parameters and total abnormality values of control and frozen-thawed ram semen supplemented with propolis at different levels

Experimental groups	VCL (µm/s)	STR (%)	WOB (%)	BCF (Hertz)	ALH (µm)	Total abnormality (%)
Control	85.04 ± 3.50	69.26 ± 2.26	68.45 ± 3.73	9.50 ± 0.28	3.43 ± 0.16	4.10 ± 0.55
0.25%	100.34 ± 7.46	71.02 ± 1.85	70.96 ± 3.23	9.70 ± 0.39	3.28 ± 0.08	3.50 ± 0.47
0.50%	100.22 ± 11.42	72.88 ± 1.03	74.25 ± 3.30	8,98 ± 0.58	3.06 ± 0.17	4.90 ± 0.76
1%	94.56 ± 5.26	71.08 ± 2.84	66.48 ± 5.50	9.80 ± 0.15	3.68 ± 0.38	4.30 ± 0.46
2%	90.86 ± 2.50	69.38 ± 5.45	63.01 ± 2.38	9.10 ± 0.29	3.41 ± 0.36	3.30 ± 0.51
4%	76.95 ± 10.33	59.95 ± 8.24	57.78 ± 4.03	10.52 ± 1.28	3.05 ± 0.45	4.00 ± 0.63

No statistical difference could be detected between the control and experimental groups in terms of the parameters given above. Values are given as means±standard error means (SEM). VCL: Curvilinear velocity (µm/s), STR: Straightness (VSL/VAPx100, %), WOB: Wobble (VAP/VCLx100, %), BCF: Beat cross frequency, Hz and ALH: Amplitude of lateral head displacement (µm)

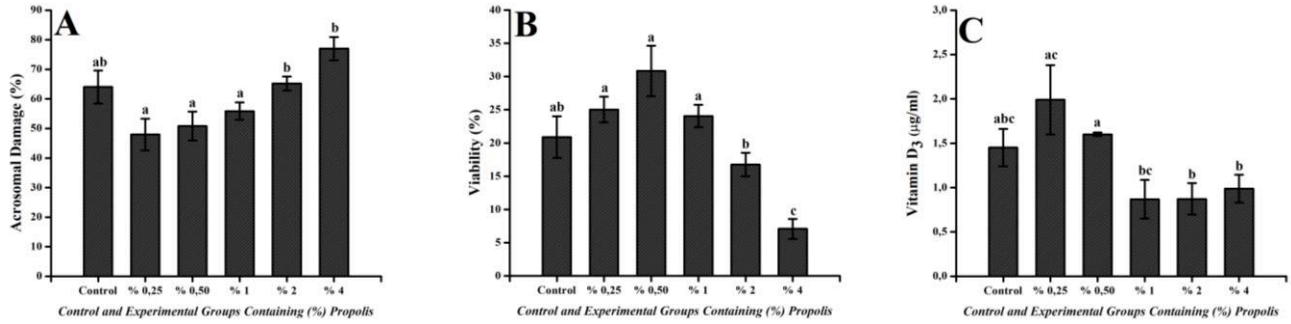


Fig. 6: Obtained after freezing-thawing. (**A**) Acrosomal damage, (**B**) Viability, and (**C**) Vitamin D_3 values. a, b, c: It shows the difference between the control and propolis containing groups in terms of acrosomal damage, viability and Vitamin D_3

Table 2: Vitamin and cholesterol levels of control and frozen-thawed ram semen supplemented with propolis at different levels

Experimental groups	Vitamin K1 (µg/ml)	Vitamin K2 (µg/ml)	α-tokoferol (µg/ml)	Ergesterol (µg/ml)	δ-tokoferol (µg/ml)	δ-sterol (µg/ml)	Kolesterol (µg/ml)
Control	0.45 ± 0.12	22.64 ± 0.37	34.62 ± 6.81	6.88 ± 1.83	2.42 ± 0.17	15.21 ± 6.41	1041.89 ± 49.41
0.25%	0.64 ± 0.18	24.84 ± 3.56	41.96 ± 7.32	6.30 ± 3.32	3.51 ± 0.47	12.64 ± 5.18	1131.69 ± 88.29
0.50%	0.43 ± 0.14	27.70 ± 3.14	37.47 ± 3.76	10.25 ± 3.35	3.29 ± 0.75	27.97 ± 8.72	1142.82 ± 65.47
1%	0.33 ± 0.10	24.71 ± 2.25	35.40 ± 2.59	5.50 ± 2.40	2.10 ± 0.43	29.32 ± 10.83	1045.29 ± 34.62
2%	0.57 ± 0.14	23.38 ± 2.36	34.59 ± 1.56	4.19 ± 1.25	2.43 ± 0.49	28.36 ± 7.14	1049.80 ± 48.74
4%	0.68 ± 0.19	16.30 ± 3.38	28.47 ± 4.85	3.58 ± 0.75	2.54 ± 0.48	17.31 ± 3.63	986.04 ± 86.17

No statistical difference could be detected between the control and experimental groups in terms of the parameters given above. Values are given as means±standard error means (SEM)

Table 3: Saturated fatty acid (SFA) levels of control and frozen-thawed ram semen supplemented with propolis at different levels

Experimental groups	Myristic acid (C14:0)	Palmitic acid (C16:0)	Stearic acid (C18:0)	∑SFA
Control	0.468 ± 0.01	24.76 ± 0.28	12.20 ± 0.15	37.34 ± 0.44
0.25%	0.467 ± 0.01	24.83 ± 0.27	10.52 ± 0.52	35.66 ± 0.47
0.50%	0.463 ± 0.01	25.02 ± 0.42	12.22 ± 0.25	37.55 ± 0.39
1%	0.454 ± 0.01	24.38 ± 0.31	11.35 ± 0.36	36.00 ± 0.62
2%	0.454 ± 0.01	24.19 ± 0.35	11.56 ± 0.26	36.14 ± 0.49
4%	0.457 ± 0.01	24.74 ± 0.29	11.57 ± 0.44	36.77 ± 0.48

No statistical difference could be detected between the control and experimental groups in terms of the parameters given above. Values are given as means±standard error means (SEM)

Table 4: Monounsaturated fatty acid (MUFA) levels of control and frozen-thawed ram semen supplemented with propolis at different levels

Experimental groups	Palmitoleic acid (C16:1 n7)	Vaccenic acid (C18:1 n7)	Oleic acid (C18:1 n9)	∑MUFA
Control	3.04 ± 0.22	1.18 ± 0.25	30.83 ± 0.61	35.05 ± 0.82
0.25%	3.30 ± 0.20	1.65 ± 0.18	35.98 ± 1.48	40.11 ± 1.55
0.50%	3.06 ± 0.24	1.22 ± 0.27	32.29 ± 0.76	36.39 ± 0.84
1%	2.99 ± 0.17	1.20 ± 0.36	32.81 ± 1.48	36.53 ± 1.59
2%	3.09 ± 0.19	1.43 ± 0.32	32.09 ± 1.27	36.14 ± 1.49
4%	3.26 ± 0.18	1.28 ± 0.29	32.81 ± 1.02	37.15 ± 1.36

No statistical difference could be detected between the control and experimental groups in terms of the parameters given above. Values are given as means±standard error means (SEM)

Table 5: Polyunsaturated fatty acid (PUFA) levels of control and frozen-thawed ram semen supplemented with propolis at different levels

Experimental groups	Linoleic acid (C18:2 n6)	α-linolenic acid (C18:3 n3)	Arachidonic acid (C20:4 n6)	Docosapentaenoic acid (C22:5 n6)	Docosahexaenoic acid (C22:6 n3)	∑PUFA
Control	14.64 ± 0.75	0.45 ± 0.31	4.93 ± 0.27	1.74 ± 0.33	1.90 ± 0.22	24.02 ± 0.82
0.25%	14.59 ± 0.61	0.46 ± 0.25	3.63 ± 0.28	1.17 ± 0.12	1.24 ± 0.29	20.81 ± 1.10
0.50%	14.61 ± 0.62	0.73 ± 0.17	4.78 ± 0.18	1.62 ± 0.20	1.70 ± 0.17	22.97 ± 0.65
1%	14.55 ± 0.81	0.71 ± 0.13	4.28 ± 0.10	1.40 ± 0.21	1.63 ± 0.33	24.09 ± 1.64
2%	14.39 ± 0.76	0.78 ± 0.06	4.51 ± 0.29	1.81 ± 0.17	1.45 ± 0.07	24.03 ± 1.63
4%	14.79 ± 0.61	0.76 ± 0.01	4.53 ± 0.35	1.52 ± 0.21	1.62 ± 0.20	22.69 ± 0.89

No statistical difference could be detected between the control and experimental groups in terms of the parameters given above. Values are given as means±standard error means (SEM)

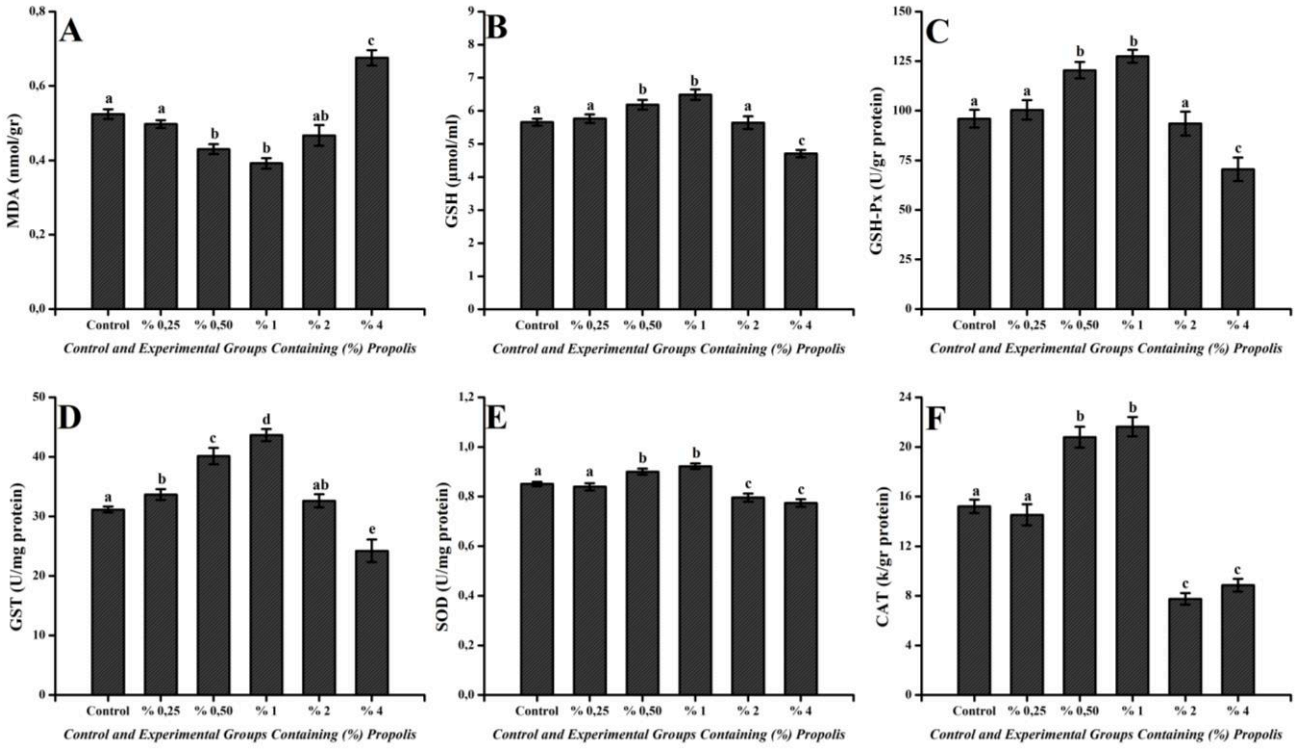


Fig. 7: Values of oxidative stress analyses after freezing-thawing. **(A)** MDA, **(B)** GSH, **(C)** GSH-Px, **(D)** GST, **(E)** SOD, and **(F)** CAT. a, b, c: It shows the difference between the control and propolis containing groups in terms of MDA, GSH, GSH-Px, GST, SOD, and CAT

Oxidative stress findings

After freeze-thaw, the experimental groups containing 0.5% and 1% propolis significantly reduced the MDA level compared to the control group (P<0.001). The experimental groups containing 0.5% and 1% propolis (P<0.001) increased GSH, GSH-Px, GST, SOD, and CAT levels compared to the control. In terms of GST levels, this positive contribution was also seen in the group containing 0.25% propolis. The group containing 4% propolis caused a significant (P<0.001) decrease in GST and CAT levels compared to the control. In terms of CAT levels, this negative effect was also seen in the group containing 2% propolis (Figs. 7A-F).

Discussion

High semen freezing success has not yet been achieved in rams, because ram spermatozoon contains high amounts of PUFA and low levels of cholesterol/phospholipid (Güngör *et al*., 2022). Reactive oxygen species (ROS), the outcome of lipid peroxidation spermatozoon, increases during freezing and causes serious damage to the spermatozoon membrane by destabilizing the stable molecules in the cell membrane (Holt *et al*., 1992; Ntemka *et al*., 2018). Spermatozoa regulate and resist this phenomenon with an antioxidant protective system in cooperation with seminal plasma, membranes, and cytoplasm, including SOD, GSH-Px, and CAT. However, this system is partially changed during freeze-thaw processes and serious damage may occur (Marti *et al*., 2008; Forouzanfar *et al*., 2013; Ntemka *et al*., 2018). Many scientific studies have been conducted on the addition of enzymatic (SOD, GSH-Px, CAT) and non-enzymatic (vitamins, minerals, fatty acids and amino acids) antioxidants to ram semen to reduce or prevent the harmful effects of oxidative stress induced by the freeze-thaw process on semen quality (Ntemka *et al*., 2018).

Propolis is a natural resinous substance containing more than 300 identified phenolic compounds belonging to three main groups (Simoes *et al*., 2004). Propolis liquid extract contains many antioxidants and substances that have a protective effect on the spermatozoon membrane, such as flavonoids (Chrysin, Tectochrysin, Acacetin, Quercetin, Kaempferide, Rhamnocitrin, Galangin etc.), benzoic acid and its derivatives (Benzoic acid, Salicylic acid, Gentisic acid, Gallic acid etc.), benzaldehyde derivatives (Vanillin, Isovanillin), cinnamic acid and its derivatives (Cinnamic acid, Caffeic acid, Ferulic acid etc.), other acids and its derivatives (Myristic acid, Sorbic acid, Stearic acid etc.), minerals (Na, K, Mg, Ca, Ba, Sr, Zn, etc.), sterols (Cholesterol, Stigmasterol etc.) and sugars (d-fructose, d-glucose, sucrose etc.) (Walker and Crane, 1987). These components posess antioxidant, antimicrobial, antiviral, and anti-inflammatory properties (Kumazawa *et al*., 2004; Paulino *et al*., 2008). In a study in which propolis was added to semen extenders for the purpose of freezing buffalo semen, researchers suggested that 2-4 µg/ml propolis increased progressive motility, viability, and membrane integrity and reduced abnormality after

freeze-thaw compared to the control group. They also determined that 2 µg/ml propolis increased the antioxidant capacity (TAC, SOD, GPx, and CAT) of semen and significantly reduced ROS and MDA levels compared to the control group. In addition, they revealed that 2-4 µg/ml propolis protected the acrosome and plasma membrane integrity at a better level than the control group after freeze-thaw (Abdelnour *et al*., 2023). In a study in which propolis was added to semen extenders for freezing bull semen, researchers determined that 100 and 200 µg/ml propolis caused significant decreases in motility and kinematic parameters compared with the control group. However, they suggested that 25 and 50 µg/ml propolis reduced tail abnormalities compared to the control group. In particular, they found that 50 µg/ml propolis had a positive effect on tail length, movement and DNA, while 100 and 200 µg/ml propolis caused DNA damage (Yeni *et al*., 2022). In a study conducted to freeze fish semen, it was reported that groups containing propolis (0.2, 0.4, 0.6, 0.8, and 1 mg/ml) increased sperm motility and motility duration after freeze-thaw compared to the control group, and the use of propolis made a significant contribution to the cryopreservation process (Öğretmen *et al*., 2014). Many similar studies have also shown that propolis has a protective effect on sperm motility, viability, and membrane integrity during freeze-thaw processes (Castilho *et al*., 2009; El-Harairy *et al*., 2018; Al-Nawab *et al*., 2021).

In our study, propolis was added to semen extenders in different percentages. Following the freeze-thaw process, the group containing 0.5% propolis provided a significant increase in total motility, rapid motility, and LIN values, and a significant decrease in low mitochondrial membrane potential, compared with the control group. In terms of HOST values, the group containing 0.5% and 1% propolis significantly increased spermatozoon membrane integrity compared to the control. Considering oxidative stress parameters, the group containing 0.5% and 1% propolis significantly reduced the MDA level compared to the control, while it caused a significant increase in GSH, GSH-Px, GST, SOD, and CAT levels. The group containing 0.25% propolis created a significant increase in the GST level compared to the control. Among the experimental groups, the group containing 4% propolis caused significant decreases in total, progressive, and rapid motility, high mitochondrial membrane potential, and GST and CAT levels compared with the control group. It has been determined that the addition of propolis in semen extenders at this rate harms spermatozoa.

The essential role of propolis in protecting live sperm during preservation is thought to be related to the basic biological ability of propolis in diluting media. It has been stated that the strong antioxidant activity of propolis is due to the large amount of antioxidative compounds such as caffeic acid, ferulic acid, caffeic acid phenethyl, and kaempferol (El-Seadawy *et al*., 2017). Additionally, some researchers have concluded that propolis extract may show the activation of antioxidant enzymes (Rizk *et al*., 2014; Zaghloul *et al*., 2016). Approximately, 26% of propolis consists of sugars such as fructose, glucose, and mannitol. These sugars are actively used in semen freezing procedures. Propolis can contribute to the membrane integrity during freezing of semen (Warnecke and Pluta, 2003). Additionally, they can be used to provide energy to spermatozoa, such as glucose (Lahnsteiner *et al*., 1997), or as a non-electrolyte, such as mannitol, to make the medium isotonic to the seminal plasma (Morisawa *et al*., 1983). Propolis has also been reported to activate antioxidant enzymes such as superoxide dismutase and catalase against free radicals (Jasprica *et al*., 2007). In our study, the positive effect of 0.5% propolis on parameters related to motility and membrane integrity is thought to be due to the membrane protective effect of propolis (Walker and Crane, 1987) and its strong antioxidant effect (El-Seadawy *et al*., 2017). Antioxidant substances are responsible for keeping ROS levels within physiological limits. The amount of ROS exceeding physiological limits causes damage to the cell membrane, causing damage to the cells (Türk, 2015). It is thought that propolis prevents this damage with its strong antioxidant activity due to its antioxidative compounds (El-Seadawy *et al*., 2017). In addition, propolis can activate SOD and CAT antioxidant enzymes (Jasprica *et al*., 2007). Therefore, adding 0.5% propolis to sperm provides protection from oxidative stress and increases both motility and linear movements of spermatozoa such as LIN compared with the control. The energy metabolism of spermatozoa varies depending on the amount of fructose/glucose in the environment. The high sugar content of propolis resulted in a notable reduction in cells exhibiting low mitochondrial activity in the group treated with 0.5% propolis. It is thought that the reason for this decrease is due to the basic biological content of propolis (Warnecke and Pluta, 2003). Additionally, due to the density of ram semen, the amount of K is quite high in this species. K is a metabolic inhibitor, while the K/Na ratio balances this metabolic situation (Çevik and Tuncer, 2005). Propolis may preserve this energy balance due to having high Na. In this study, no difference was found in semen fatty acids in the propolis groups with different concentrations compared with the control after the freeze-thaw process. During the freezing process of sperm, the cell membrane are damaged alot. The membrane of the ram spermatozoon is very rich in fatty acids. Possibly, fatty acid migration from the cell to the extracellular fluid is expected due to freezing-induced membrane disruption. However, in this study, the fatty acid level in total semen, not spermatozoon, was measured. No difference was determined in the level of fatty acids in frozen-thawed semen supplemented with 0.5% propolis compared with the control group. However, a significant increase was observed in other spermatological parameters. This can be explained by the fact that 0.5% propolis additive protects the cell membrane against freezing and thus reduces the passage of fatty acids from the membrane to the extracellular fluid. After freezing and thawing, the group containing

0.5% propolis showed a numerical increase in levels of all vitamins and cholesterol, except vitamin K1, compared with the control. It is thought that this increase is due to the basic biological content of propolis (Warnecke and Pluta, 2003). Addition of propolis to semen extenders at a high rate (4%) caused a decrease in levels of motility, mitochondrial activity, GST, and CAT compared with the control. It is thought that the reason for this decrease may be due to the fact that high amounts of propolis disrupt the osmolarity of the semen diluent by increasing the amount of sugar in the diluent.

It is proposed that the addition of 0.5% propolis to semen diluents may enhance the viability of ram semen during freezing. This approach could improve the survival of spermatozoa following freezing and thawing, thereby, increasing the success of freezing procedures.

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Conflict of interest

None of the authors have any conflict of interest to declare.

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