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Effects of mitoquinone and caffeic acid on spermatological parameters and *OGG1/ROMO1* gene expression of post-thawed ram semen

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ABSTRACT

The aim of this study was to determine the freezability of ram semen with extenders containing mitoquinone or caffeic acid. Collected ram semen samples were pooled and divided into seven equal aliquots. Each aliquot was diluted with Tris-based extender according to control and antioxidant groups (Control, Mitoquinone (MitoQ) 100 nM, 150 nM, 200 nM; Caffeic Acid (CA) 50 μM, 100 μM, 150 μM). The post-thaw spermatological, antioxidant parameters and *OGG1/ROMO1* gene expression and methylation were evaluated. It was determined that the CASA post-thaw motility of MitoQ200, CA100 and CA150 antioxidant groups were statistically higher than the control group ($P < 0.05$). The post-thaw plasma membrane integrity of MitoQ groups was compared with CA groups, CA100 group, which had the lowest plasma membrane integrity among the antioxidant groups. The post-thaw acrosome membrane damage of all the antioxidant groups were statistically lower than control group, except CA50 and CA100 groups ($P < 0.05$). For the post-thaw mitochondrial membrane potential, all groups except the CA50 group were determined to be superior to the control group ($P < 0.05$). No statistical difference was observed among all groups including control group in terms of post-thaw DNA fragmentation, MDA and TAC values. MitoQ significantly altered the expression of the *OGG1* genes, an effect mediated through DNA methylation ($P < 0.05$). Furthermore, caffeic acid altered the *ROMO1* gene expression ($P < 0.05$). Based on the spermatological parameters and gene expression and methylation levels obtained in the current study, it was determined that mitoquinone 200 nM and caffeic acid 150 μM doses had a positive effect on the freezability of ram semen.

1. Introduction

For animal breeding studies to be carried out effectively, the genetic material of high-quality male animals must be widely transferred to the field. This is only possible with the insemination of many females with frozen semen taken from these breeding male animals (Leboeuf et al., 2000).

Cryopreservation causes structural, biochemical, functional and molecular disorders in the spermatozoa, resulting in significant decreases in the motility, viability and fertilization ability of the spermatozoon. Due to the lower cholesterol/phospholipid ratio of its plasma membrane structure, ram sperm is more sensitive to sudden temperature changes during freezing and thawing processes, than

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other animal species (Holt, 2000). The drastic osmotic and temperature changes (Cold shock) during cryopreservation disrupt the spermatozoon's membrane structure and cause mechanical and oxidative stress (OS). Reactive oxygen species (ROS) is the one of causes of OS, disrupting the fluidity of the sperm plasma membrane and inducing lipid peroxidation. Increased ROS levels lead to motility losses by damaging the bioenergy-related pathways in the spermatozoon's tail. Furthermore, a decrease in mitochondrial membrane potential (MMP) due to OS causes sperm DNA fragmentation, and ultimately cell apoptosis (Aitken et al., 2015; Malić Vončina et al., 2016). It has been reported that supplementing antioxidant substances to ram semen freezing extenders has positive effects on post-thaw spermatological characteristics by eliminating the negative effects of ROS (Amidi et al., 2016; Masoudi et al., 2018; Rostami et al., 2020).

Mitoquinone (MitoQ), a derivative of ubiquinone with the triphenylphosphonium (TPP+) cation attached, is one of the mitochondrial-targeted antioxidants and facilitates the passage of ubiquinone across the mitochondrial membrane (Ross et al., 2008). MitoQ-supplemented extenders have been reported for the cryopreservation of semen from several animal species, such as yellow catfish (Fang et al., 2014), human (Gonzalez et al., 2022), rooster (Alipour-Jenaghard et al., 2023), boar (Shi et al., 2022), bull (Câmara et al., 2022), buffalo (Tiwari et al., 2021), buck (Rezaei et al.2023; Hatami et al.,2024), and ram (Wu et al., 2021; Javaheri Barfourooshi et al., 2023).

Caffeic acid, one of the components found in propolis with various pharmacological properties, reduces free oxygen radicals by preventing the consumption of free radical scavenging enzymes found in semen (Ogeturk et al.,2005; Zhang et al.,2014). It has been reported that, when added to the extender during freezing, caffeic acid has a protective effect on motility, viability and plasma membrane integrity of boar semen (Namula et al., 2018). It also positively affects DNA integrity and total antioxidant activity in buffalo semen (Soleimanzadeh et al., 2020).

DNA bases are sensitive to oxidative damage caused by ROS (Neeley and Essigmann, 2006). The low redox potential of the guanine base makes it particularly vulnerable, leading to the formation of a large number of oxidized guanine products (Burrows and Muller, 1998). Many oxidative damages to DNA, including the oxidation of the guanine base, are repaired via base excision repair (BER), which is initiated by specific DNA glycosylases (David et al., 2007). 8-oxoguanine DNA glycosylase (*OGG1*), an important repair enzyme in the BER pathway, scans DNA for damaged bases and initiates the BER mechanism to remove 8-oxoGuanine, the most common base lesions (Klungland and Bjelland, 2007).

ROS modulator 1 (*ROMO1*) protein is the main gene localized in mitochondria and plays an important role in oxidative stress-induced mitochondrial ROS production (Shin et al., 2013). *ROMO1* has been linked to spare respiratory capacity and glucose homeostasis, emphasizing its crucial role in regulating mitochondrial dynamics, ROS sensing, and modulation (Setyawan et al., 2016). Studies have reported that there is a negative relationship between the expression of these genes and sperm quality (Qamar et al., 2020 a,b; Bang et al., 2021).

To the authors knowledge, no studies have yet been conducted on the effects of MitoQ and CA on post-thaw ram spermatological parameters, nor on the expression and methylation of ROS modulator genes (*OGG1* and *ROMO1*). The aim of this study was to determine the post-thaw spermatological characteristics (motility, acrosome, plasma membrane functional and DNA integrity and mitochondrial membrane potential), antioxidant parameters (Malondialdehyde-MDA, total antioxidant capacity-TAC) and expression and methylation levels of *OGG1* and *ROMO1* genes of ram sperm frozen with the supplementation of caffeic acid and mitoquinone.

2. Material and methods

A total of five Merino rams, aged between 2 and 4 years and with a confirmed fertility history, were supplied with fresh water and allowed to consume maize and alfalfa hay freely. They were housed and sustained by the Faculty of Veterinary Medicine at Uludag University in Northwestern Anatolia, Turkey. The Scientific Ethical Committee (Uludag University, Bursa, Turkey; no.2021–10/01) approved all protocols related to the experimental setup and evaluation techniques.

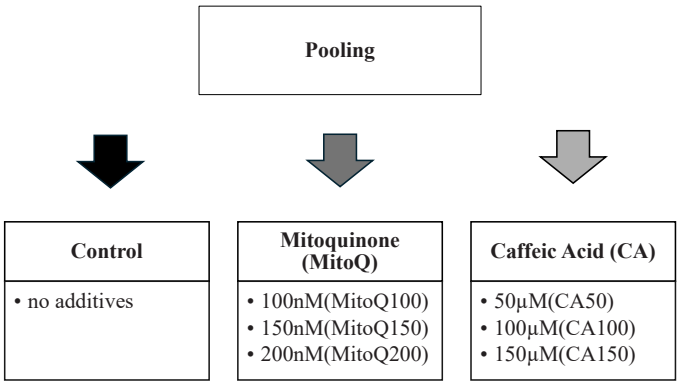


Fig. 1. The control and MitoQ or CA doses of the extender groups.

2.1. Chemicals and semen extender preparation

All chemicals used in the study were purchased from (Sigma St. Louis, MO, USA) and Merck (Darmstadt, Germany) unless otherwise stated.

Tris-based extenders were prepared as extender A and extender B to be used in the two-step dilution method. Extender A consisted of 223.7 mmol Tris, 55.5 mmol fructose, 66.6 mmol citric acid, 4 g/L penicillin G, 3 g/L dihydrostreptomycin, and 20 % egg yolk (v/v). Extender B was prepared by adding 100.4 mmol trehalose, 4.03 mmol EDTA, and glycerol 6 % (v/v) to the content of extender A (Aisen et al., 2000).

The different experimental groups were designed by adding various doses of either Mitoquinone (BioVision - B1309) or Caffeic acid (Sigma-Aldrich - C0625) to the prepared extender groups. The study consists of a total of seven extender groups: Control (no anti-oxidants), Mitoquinone (MitoQ100, MitoQ150 and MitoQ200 at 100, 150 and 200 nM doses, respectively) and Caffeic acid (CA50, CA100 and CA150 at 50, 100, 150 μM doses, respectively) (Fig. 1).

2.2. Semen collection, dilution, freezing, and thawing

Semen collection was carried out a total of five times every other day, using an electro-ejaculator designed for small ruminants, (REF: 11900/0000, Minitube, Germany) in the non-breeding season (Ustuner et al., 2016). The collected semen was brought to the laboratory in a warm water bath at 28–30 °C for evaluation of mass activity (0–5 scale), motility, and concentration. After assessment, semen samples with a mass activity of at least + ++ , 70 % motility, and concentration of 1×10^9 spt/mL were pooled.

The dilution process was conducted by a two-step dilution method. The dilution rate was calculated to a final concentration of 125×10^6 spermatozoa per straw. The pooled ejaculates were divided into seven equal aliquots, and each aliquot was diluted with extender A with the different treatments (Control, MitoQ100, MitoQ150, MitoQ200, CA50, CA100 and CA150). Afterward, the diluted semen samples were cooled to 5 °C within 1 hour, and then diluted with extender B (Control, MitoQ100, MitoQ150, MitoQ200, CA50, CA100 and CA150). A 2-hour equilibration process at 5 °C was done for the semen samples following the addition of the extender B.

The equilibrated semen was placed in 0.25 mL straws and frozen using a Nicool Plus PC freezing machine (Air Liquide, Marne-la-Vallée Cedex 3, France). The freezing process involved a gradual cooling of the semen from + 5 °C to −8 °C at a rate of 3 °C per minute, followed by a rapid cooling from −8 °C to −120 °C at a rate of 15 °C per minute in liquid nitrogen vapor. The frozen straws were stored in liquid nitrogen at a temperature of −196 °C for at least one month. In order to evaluate the post-thaw semen, a minimum of three straws from each group were subjected to thawing in a water bath maintained at a temperature of 37 °C for 30 seconds.

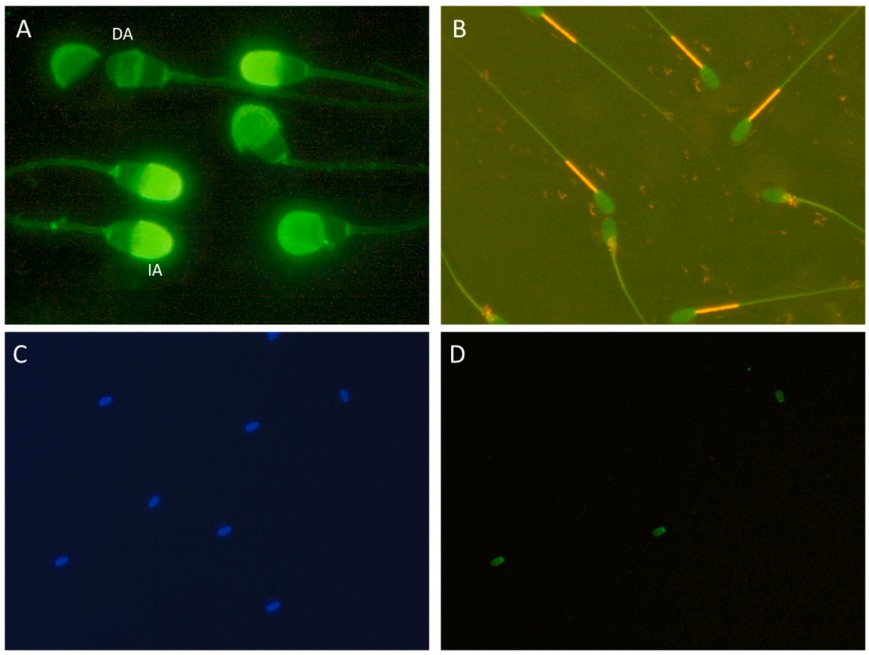


Fig. 2. Representative patterns of post-thaw ram semen as observed with FITC-PSA, JC-1 and TUNEL staining, respectively. A: FITC-PSA staining for acrosomal membrane integrity evaluation (x100). DA:Defected Acrosome, IA: Intact Acrosome. B: JC-1 staining for mitochondrial membrane potential assesment. Yellow/orange and green fluorescence associated with midpiece of spermatozoa indicated a high and a low mitochondrial membrane potential, respectively (x40). C, D: Evaluation of sperm DNA fragmentation using TUNEL assay. C: DNA in both normal and spermatozoa with fragmented DNA in DAPI (blue) and D: DNA fragmented spermatozoa stained green as TUNEL (+) cells, respectively (x40).

2.3. Semen evaluation

All the semen parameters were evaluated twice during the study: first at the native stage and second at the post-thaw stage. The semen freezing process and evaluation of semen parameters were consistently conducted by a single individual throughout the course of the study

2.3.1. Mass activity, motility and sperm kinematics

Evaluation of sperm mass activity was performed using a phase-contrast microscope (Olympus BX51) equipped with a heated mechanical stage and was assessed subjectively (Ustuner et al., 2016).

A computer-assisted sperm analyzer (CASA) (Ceros II, IMV Technologies) was used to evaluate the motility and kinematic parameters [(TM: Total Motility (%); ALH: amplitude of lateral head displacement (μm); BCF: beat cross frequency (Hz); VAP: average path velocity (μm/s); VSL: straight linear velocity (μm/s); VCL: curvilinear velocity (μm/s); LIN [VSL/VCL]: linearity (%); STR [VSL/VAP]: straightness (%); DAP: distance average path (μm); DSL: distance straight line (μm); DCL: distance curved line (μm)] of semen samples. For the CASA analysis, according to the protocol of the manufacturer, the semen sample was diluted with TRIS-based diluent, and 3 μl of the diluted sample was placed onto the specific slide heated at 37 °C (Leja®, Ref. 025107, IMV Technologies, France). The analysis continued until at least 8 areas or at least 400 spermatozoa were analyzed (Dayanikli et al.,2022).

2.3.2. The hypoosmotic swelling test (HOST)

To assess the functional integrity of the sperm membrane, a sample of 10 μL of semen was mixed with 100 μL of a 100 mOsM hypoosmotic solution and incubated at 37 °C for 60 minutes. After incubation, 200 spermatozoa were examined under phase-contrast microscopy to detect any coiled tails (Ustuner et al., 2016).

2.3.3. Fluorescein lectin staining assay (FITC conjugated Pisum sativum agglutinin (FITC-PSA)

The method described by Nur et al. (2010) was used to assess the integrity of the acrosome. Stained slides were prepared and examined under a fluorescent microscope, and at least 200 spermatozoa were counted for evaluation (Fig. 2).

2.3.4. TUNEL (terminal-deoxynucleotidyl-transferase-mediated-dUTP nick-end labelling) assay

To assess the integrity of DNA in the sperm cells, the slight-modified TUNEL technique recommended by the manufacturer was used. After the labeling process, slides were examined under fluorescence microscopy, and at least 100 spermatozoa were evaluated to determine the percentage of TUNEL (+) spermatozoa, which indicates sperm cells with damaged DNA (Ustuner et al.,2016) (Fig. 2).

2.3.5. Mitochondrial membrane potential (5.5′, 6,6′-tetrachloro-1, 1′, 3,3′-tetraethyl- benzimidazolo carbocyanine iodide)

Sperm mitochondrial membrane potential integrity was evaluated utilizing the JC-1 (5.5′, 6,6′-tetrachloro-1, 1′, 3,3′-tetraethylbenzimidazole carbocyanine iodide) (Invitrogen, T3168) staining method previously described by Inanc et al. (2019). The sample were analysed by microscopic examination by applying a 2.5 μL droplet onto a microscope slide, covering it with a coverslip, and evaluating at least 100 spermatozoa at 400 magnification under a fluorescence microscope (Olympus BX 51-TF). JC-1 reversibly changes from green to orange when the mitochondrial membrane potential is high. Cells with a fluorescent yellow-orange dye in the middle of the spermatozoa cell will be considered as cells with high mitochondrial membrane potential (Fig. 2).

2.3.6. Antioxidant properties

2.3.6.1. MDA (Malondialdehyde). Sperm malondialdehyde concentration, as indices of LPO in sperm was measured by microplate reader (Biotek Epoch, USA) using ELISA kit (BT LAB Sheep Malondialdehyde ELISA KIT, Cat.No E0039Sh, China). First, sperm samples were prepared according to the manufacturer's protocol and placed in microplate wells coated with MDA antibody. The biotinylated MDA antibody was added to the sample, resulting in binding to the MDA present within the sample. It then bound to the biotinylated MDA antibody by adding Streptavidin-HRP to the wells. The microplate was incubated for 1 hour at 37 °C. After the incubation, unbound Streptavidin-HRP was removed during the wash step. Then, substrate solutions were added, and color change was observed in proportion to the amount of MDA. The reaction was halted by the addition of the acidic stop solution, followed by measurement of the optical density (OD value) at 450 nm to determine the absorbance. The standard curve was constructed by plotting the average OD. The results were calculated using the standard curve in nmol/mL.

2.3.6.2. TAC (Total antioxidant capacity). The total antioxidant capacity of spermatozoa was determined using a colorimetric commercial test kit (Elabscience Biotechnology Inc., Houston, Texas, USA) following the instructions provided by the manufacturer. OD values spectrophotometrically at 520 nm, and the TAC activity was calculated according to the kit's formula in U/mL.

2.3.7. Genetic analysis

2.3.7.1. Nucleic acid isolation. Both RNA and DNA were isolated from sperm samples. The RNA samples were analyzed to assess gene expression relevant to the study, while the purified DNA samples were utilized to examine methylation status. Total RNA was isolated from frozen tissue samples using TRIpure Isolation Reagent (Roche Life Science, Penzberg, Germany), following the provided

instructions with some modifications. DNA was extracted using the ROCHE High Pure PCR Template Preparation kit (Roche Diagnostics GmbH, Mannheim, Germany) based on the manufacturer's instructions.

2.3.8. Assessment of the alterations in the ovine OGG1 and ROMO1 gene expression

After RNA isolation, cDNA synthesis was performed using the RevertAid RT Reverse Transcription Kit based on the suggestions provided by the manufacturer (Thermo Fisher Scientific, Inc.). The RNA sample was used at a concentration of 1000 ng for cDNA conversion. Real-time PCR was conducted using the LightCycler 480 II Real-Time PCR System (Roche Diagnostics, Indianapolis, USA) with SYBR Green I Master chemistry (Roche). Primers targeting the *OGG1* and *ROMO1* gene regions, along with the housekeeping genes *ACTB* (beta-actin) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), were specifically designed for the *Ovis aries* species using information from the NCBI and ENSEMBL gene databases. Primer sequences are shown in Table 1a. The specificity of these primers was verified using the BLAST program. Quantitative analysis was conducted using the LightCycler 480 Relative Quantification Software (Roche). The expression of the corresponding genes was normalized to *ACTB* and *GAPDH* expressions as internal controls, and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). In this study, initially, *GAPDH* and *ACTB* were evaluated as reference genes. Following mathematical analysis, *ACTB* was found to be more stable and was therefore selected for calculating ultimate fold change values.

2.3.8.1. Evaluation of DNA methylation status. To assess the methylation status of DNA samples, bisulfite conversion is necessary prior to Real-Time PCR analysis. The bisulfite treatment of the isolated DNA samples was conducted using the Zymo Research - EZ DNA Methylation-Gold kit (Zymo Research, Orange, CA, USA). Primers intended to evaluate the methylation status of the *OGG1* and *ROMO1* gene regions were specifically designed using the MethPrimer software. This software utilizes information from major genomic databases such as NCBI and ENSEMBL to aid in the selection and design of primers. These primers were strategically crafted to target CpG islands within the *OGG1* and *ROMO1* genes. The sequences for both methylated and unmethylated state-specific primers used in this study are listed in the 5′ to 3′ direction in Table 1b. To ascertain the methylation profile of bisulfite-converted DNA samples, primers specifically designed for the target gene regions were utilized in analyses conducted on the Roche LightCycler 480 II system. These analyses employed the LightCycler 480 SYBR Green I Master mix. The data from the Real-Time PCR were analyzed using the Tm Calling Mode of the LightCycler 480 software. The methylation status of each sample was determined by identifying a Tm shift between the methylated and unmethylated primer sets. Verification of methylation for each sample was conducted using a positive methylated control DNA.

2.4. Statistical analysis

Data obtained from the study was analysed using SPSS (SPSS 26 for Windows; SPSS, Chicago, IL) for spermatological parameters and antioxidant properties. The normality of the data was evaluated using the Shapiro-Wilk test and Levene's test assessed homogeneity of variances. For data that met assumptions of normality and homogeneity, one-way ANOVA was performed, followed by Tukey's post hoc test for pairwise comparisons of group means. Nonparametric sperm parameters were analyzed using the Kruskal-Wallis and Mann-Whitney U tests for subsequent pairwise comparisons.

Concerning the genetic analysis, comparisons through gene expression and methylation patterns were conducted using GraphPad Prism 9 software (GraphPad Software, La Jolla, USA). The Anderson–Darling test was utilized to assess the normality of the data set, ensuring the appropriateness of subsequent statistical tests. To examine the differences among experimental groups, the Kruskal–Wallis test was employed, followed by Dunn's posthoc tests for multiple comparisons. A *P*-value lower than 0.05 was considered to indicate statistical significance.

3. Results

The average (± SEM) values of the spermatological [total motility (TM), plasma membrane functional integrity (HOST), acrosome damage (FITC-PSA), low mitochondrial membrane function (JC-1) and DNA damage (TUNEL)], antioxidant (MDA (Malondialdehyde

Table 1
a) Primer sequences (5′-3′) used in this study.

Gene	Forward Primer	Reverse Primer
OGG1	CAGACTGAGGAGCATCTCTACT	TACTGTTGCACGGCCTTTAG
ROMO1	TTCCTGTCTCAGGATTGGAATG	GGCCATGAATGTGCCAAAG
*ACTB**	TCTTCCAGCCTTCCTTCCT	TAGAGGTCCTTGCGGATGT
*GAPDH**	TGAGATCAAGAAGGTGGTGAAG	GCATCGAAGGTAGAAGAGTGAG
*Housekeeping gene.		

b) Primer sequences (5′-3′) utilized in the study for both methylated and unmethylated states.

Primer type	***OGG1***	***ROMO1***
Left methylated primer	CGGGTAGATAGGTTTTGAGGTATC	TTAGGGTTTTGAGGGTTTGC
Right methylated primer	GATTAACGCGAACTAAACTCG	CGAAACTTTCTCCTAAACTAAACGA
Left unmethylated primer	GTGGGTAGATAGGTTTTGAGGTATT	ATTTTAGGGTTTTGAGGGTTTGT
Right unmethylated primer	ACAATTAACACAAACTAAACTCACT	AAAACTTTCTCCTAAACTAAACAAC

concentration), TAC (Total Antioxidant Capacity) parameters of fresh ram semen were 80.33 %; 93.67 %; 14.33 %; 6.33 %; 4.33 %; 0.71 nmol/mL and 13.40 U/mL, for TM, HOST, FITC-PSA, JC-1, TUNEL, MDA and TAC,. All fresh sperm parameters were negatively affected by the freeze-thaw process ($P < 0.01$), except TAC ($P > 0.05$).

The effects of MitoQ and CA at different concentrations on the spermatological parameters and antioxidative properties of post-thaw ram semen are shown in Fig. 3 and Fig. 4.

3.1. Sperm motility and kinematic parameters

The post-thaw motility of all groups was statistically similar to the control group, except for the MitoQ200, CA100 and CA150 groups ($P < 0.05$). The post-thaw motility of MitoQ groups was evaluated within doses, no statistical differences being observed ($P > 0.05$). Likewise, the CA groups were statistically similar, with the lowest post-thaw motility being observed in the CA50 group. When mitoquinone and caffeic acid doses were compared to each other in terms of post-thaw motility, also no statistical differences were detected ($P > 0.05$) (Table 2). The post-thaw kinematic parameters of BCF, DAP, DCL, DSL, LIN, VAP, VCL and VSL values were similar among all groups including control group ($P > 0.05$). The CA100 group, however, had the lowest ALH rate and was statistically different from the control and MitoQ100 groups ($P < 0.05$). The CA100 group, which also had the highest STR value of 91.18 %, was determined to be statistically different only from the MitoQ100 (83.03 %) group ($P < 0.05$) (Table 3).

3.2. Plasma membrane integrity

Regarding post-thaw plasma membrane integrity, only the CA50 group had no significant differences to the control ($P > 0.05$). For the post-thaw plasma membrane integrity of antioxidant groups evaluated within doses, no significant difference was observed in the MitoQ dose groups ($P > 0.05$), however, the CA150 group had a significantly higher plasma membrane integrity when compared to the CA100 group ($P < 0.05$). The post-thaw plasma membrane integrity of MitoQ groups was compared with CA groups, CA100 group, which had the lowest plasma membrane integrity among the antioxidant groups, was observed to be statistically different only from the MitoQ200 and CA150 groups ($P < 0.05$) (Table 2).

3.3. Acrosomal membrane integrity

In the evaluation of post-thaw acrosome membrane damage, all the antioxidant groups, except CA50 and CA100 groups showed statistically lower values than the control group ($P < 0.05$). No difference in terms of post-thaw acrosome membrane damage was detected between CA and MitoQ (Table 2).

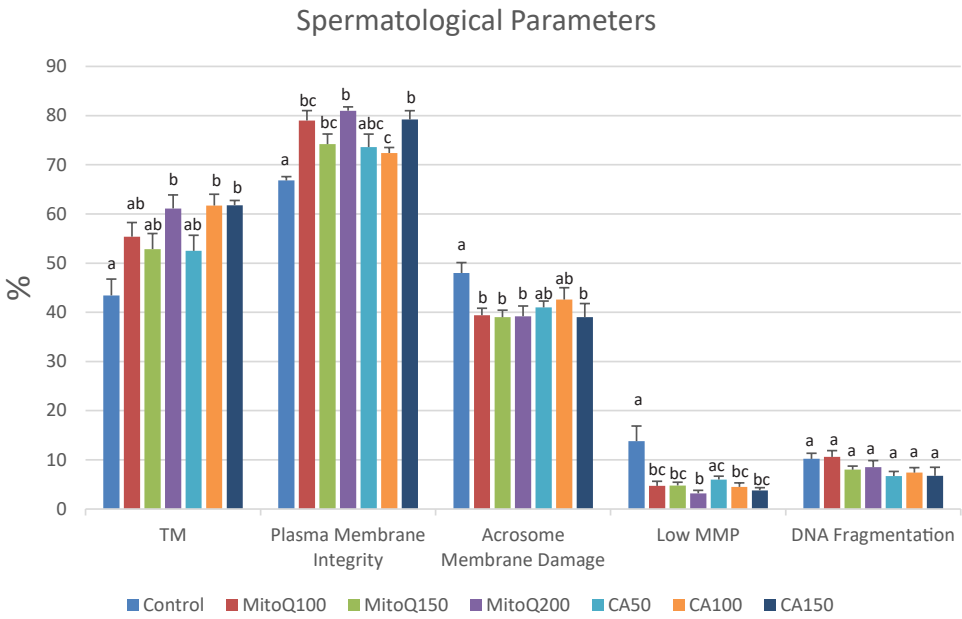


Fig. 3. The effect of different doses of Mitoquinone and Caffeic acid on post-thaw spermatological parameters [total motility (TM), plasma membrane functional integrity (hypoosmotic swelling test), acrosome membrane defect (Pisum sativum agglutinin), low mitochondrial membrane potential (MMP/JC-1), DNA fragmentation (terminal-deoxynucleotidyl-transferase-mediated-dutp nick-end labeling)], and of ram semen. Different letters indicate differences among the trial groups ($P < 0.05$).

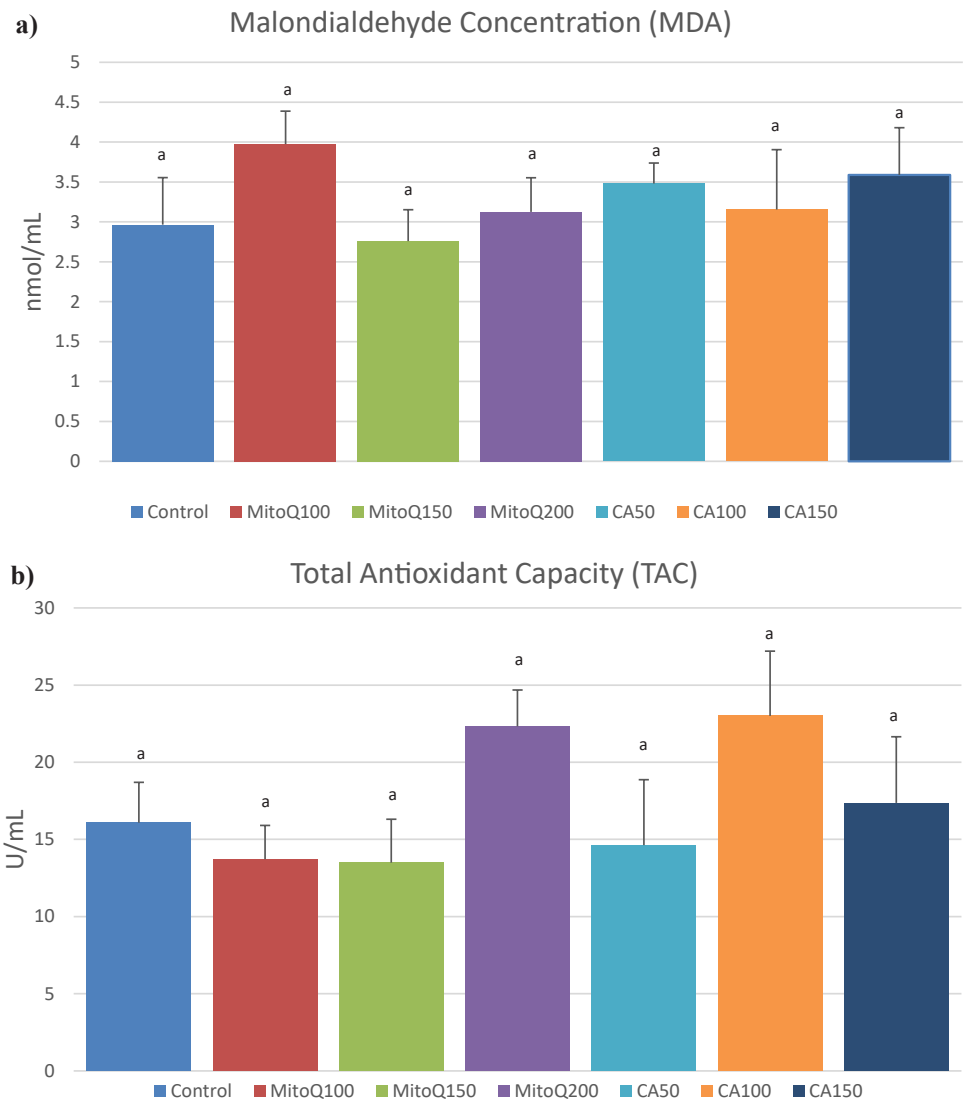


Fig. 4. The effect of different doses of Mitoquinone and Caffeic acid on post-thaw spermatological antioxidant properties of ram semen ($P > 0.05$). **(a)** Malondialdehyde concentration (MDA) and **(b)** Total antioxidant capacity (TAC).

3.4. Mitochondrial membrane potential

For the post-thaw MMP, all groups except the CA50 group were significantly higher than the control group ($P < 0.05$). No statistical difference within both antioxidant dose groups was observed ($P > 0.05$), but between the groups the highest post-thaw MMP was observed in the MitoQ200 group and was statistically different from the CA50 group ($P < 0.05$) (Table 2).

3.5. DNA fragmentation, MDA (Malondialdehyde) and TAC (Total antioxidant capacity)

No statistical difference was observed among all groups including control group in terms of post-thaw DNA fragmentation, MDA and TAC values (Table 2).

3.6. Expression and methylation levels of the ovine OGG1 and ROMO1 genes

The effect of MitoQ corresponding to changes in the gene expression and methylation status of the *OGG1* and *ROMO1* genes is shown in Fig. 5. The results indicate a dose-response effect of MitoQ for both *OGG1* gene expression and methylation levels. Increasing doses of MitoQ significantly affected changes in the expression and methylation status of the *OGG1* gene ($P < 0.05$) (Fig. 5a and b). Higher doses of MitoQ lead to decreased gene expression of *OGG1,* while increasing the methylation patterns of the same gene.

Table 2
The mean (± SEM) of spermatological parameters and antioxidant properties of post-thaw ram semen in control and different doses of MitoQ and CA groups.

Groups			Spermatological parameters				Antioxidant Properties	
	n	TM (%)	Plasma Membrane Integrity (%)	Acrosomal Membrane Damage (%)	Low Mitochondrial Membrane Potential (%)	DNA Fragmentation (%)	MDA (nmol/mL)	TAC (U/mL)
Control	15	43.44 ± 4.07[a]	66.80 ± 0.97[a]	48.00 ± 2.59[a]	13.80 ± 3.77[a]	10.20 ± 1.39	2.97 ± 0.72	16.08 ± 3.20
MitoQ100	15	55.38 ± 3.52[ab]	79.00 ± 2.47[bc]	39.40 ± 1.75[b]	4.70 ± 1.16[bc]	10.60 ± 1.57	3.97 ± 0.51	13.72 ± 2.68
MitoQ150	15	52.86 ± 3.88[ab]	74.20 ± 2.51[bc]	39.00 ± 1.76[b]	4.80 ± 0.80[bc]	8.00 ± 0.89	2.76 ± 0.48	13.49 ± 3.45
MitoQ200	15	61.12 ± 3.37[b]	81.25 ± 1.18[b]	39.50 ± 3.28[b]	3.50 ± 0.87[b]	9.37 ± 1.82	3.13 ± 0.55	22.32 ± 3.04
CA50	15	52.50 ± 3.88[ab]	73.60 ± 3.23[abc]	41.00 ± 1.58[ab]	6.00 ± 0.84[ac]	6.70 ± 1.16	3.48 ± 0.32	14.63 ± 5.20
CA100	15	61.72 ± 2.80[b]	72.40 ± 1.37[c]	42.60 ± 2.94[ab]	4.50 ± 1.00[bc]	7.40 ± 1.25	3.16 ± 0.92	23.00 ± 5.14
CA150	15	61.78 ± 1.18[b]	79.20 ± 2.20[b]	39.00 ± 3.41[b]	3.80 ± 0.66[bc]	6.76 ± 2.11	3.59 ± 0.72	17.36 ± 5.25

[a,b,c] Different letters within the same column show significant differences among the groups ($P < 0.05$).

Table 3
The mean (± SEM) of CASA kinematics of post-thaw ram semen in control and different doses of MitoQ and CA groups.

Groups	CASA Kinematic Parameters										
	n	ALH (μm)	BCF (Hz)	DAP (μm/s)	DCL (μm/s)	DSL (μm/s)	LIN (%)	STR (%)	VAP (μm/s)	VCL (μm/s)	VSL (μm/s)
Control	15	6.01 ± 0.61[a]	34.93 ± 1.19	36.79 ± 4.88	63.80 ± 9.71	30.26 ± 3.74	60.35 ± 4.47	85.87 ± 1.63[ab]	80.30 ± 10.00	139.38 ± 20.34	66.24 ± 7.58
MitoQ100	15	6.69 ± 0.68[a]	34.47 ± 1.53	41.45 ± 5.97	74.07 ± 10.32	32.70 ± 4.84	55.98 ± 2.70	83.03 ± 1.07[a]	93.85 ± 12.75	168.06 ± 22.84	73.43 ± 9.67
MitoQ150	15	4.53 ± 0.63[ab]	31.47 ± 1.34	26.31 ± 3.60	44.02 ± 8.77	20.96 ± 2.17	70.43 ± 3.79	89.08 ± 2.00[ab]	59.68 ± 7.12	99.12 ± 17.92	47.65 ± 4.64
MitoQ200	15	6.22 ± 1.08[ab]	32.07 ± 1.49	36.71 ± 4.24	65.59 ± 9.95	29.96 ± 3.11	59.83 ± 5.14	86.62 ± 2.33[ab]	84.01 ± 11.24	149.78 ± 25.66	68.88 ± 8.52
CA50	15	5.08 ± 0.64[ab]	32.71 ± 0.86	31.45 ± 4.18	55.81 ± 7.57	25.29 ± 3.59	63.19 ± 2.75	87.34 ± 1.39[ab]	70.66 ± 9.63	125.35 ± 17.22	57.16 ± 8.35
CA100	15	3.69 ± 0.56[b]	32.32 ± 0.62	26.11 ± 3.15	43.06 ± 6.20	22.34 ± 2.49	71.78 ± 4.04	91.18 ± 1.79[b]	59.22 ± 7.50	97.15 ± 14.43	50.58 ± 5.79
CA150	15	3.82 ± 0.77[ab]	32.16 ± 0.71	26.61 ± 4.45	43.33 ± 8.25	22.41 ± 3.47	71.96 ± 3.60	90.51 ± 1.69[ab]	66.56 ± 11.22	98.28 ± 20.13	51.28 ± 8.96

[a,b]Different letters within the same column show significant differences among the groups ($P < 0.05$).

(Fig. 5b). Although MitoQ appeared to have an effect on regulating the *ROMO1* gene, no dose-dependent effect was observed, contrary to what was observed for the *OGG1* gene. Furthermore, all MitoQ dose groups exhibited similar patterns in regulating the ovine *ROMO1* gene.

The effect of CA in the gene expression and methylation status of the OGG1 and ROMO1 genes is shown in Fig. 6. QPCR analyses indicated that increasing doses of CA induce *OGG1* gene expression. Specifically, the maximum expression of the *OGG1* gene was observed at the highest administered dose of CA (150 μM), as shown in Fig. 6a. The methylation patterns of the *OGG1* gene (Fig. 6b) across three different doses of CA (50,100 and 150 μM), however, were found to be relatively similar ($P > 0.05$). An exciting finding of our study was the observation that higher doses of CA directly increased *ROMO1* gene expression ($P < 0.05$). Notably, a significant distinction was evident between the lowest dose of CA and the highest (Fig. 6c). The highest level of *ROMO1* gene expression, along with the lowest methylation level, was observed at the highest dose group (CA150). The difference in ROMO1 gene expression between the 50 μM and 100 μM doses of CA, however, was markedly negligible (Fig. 6d). It is particularly salient that the caffeic acid 150 μM dose was correlated with the highest expression levels of both critical genes examined in this research.

4. Discussion

Reactive oxygen species play a physiological role intracellular signaling cascades mediating sperm capacitation, acrosome reaction, hyperactivation and sperm-oocyte fusion (Twigg et al., 1998). Excessive production of ROS by the spermatozoa, however, causes damage to the plasma membrane, thereby reducing sperm motility, metabolic activity, and viability (Ko et al., 2014).In order to improve the quality of frozen thawed semen, several modifications of extender have been long lasting interest of researchers. One of

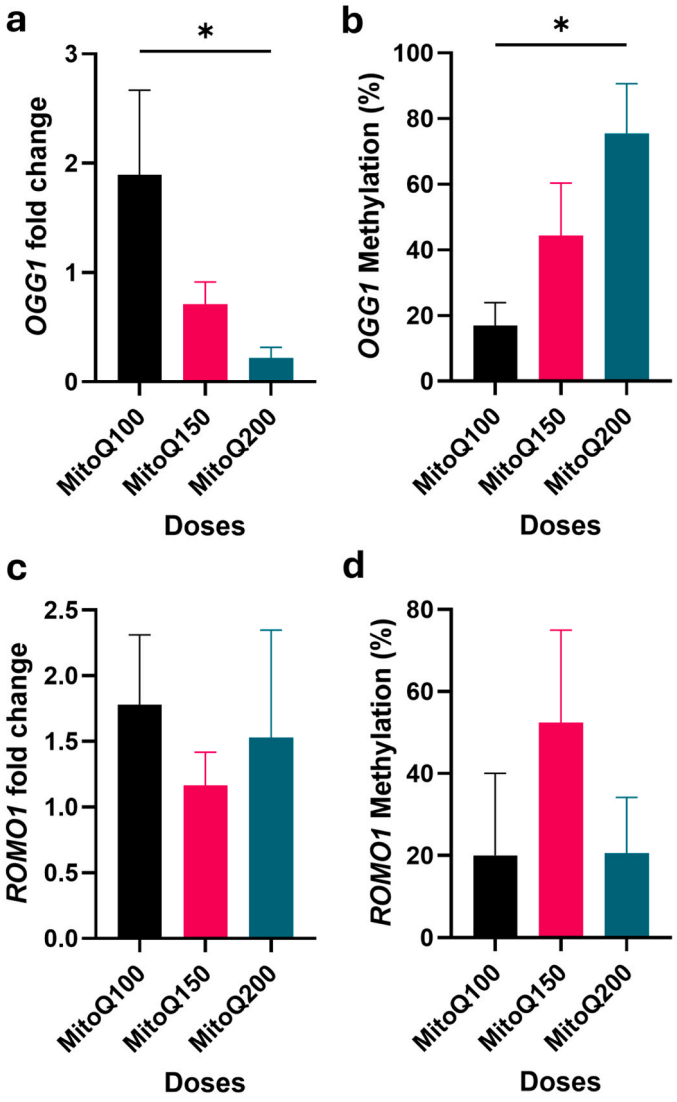


Fig. 5. Effects of three different doses of mitoquinone (100, 150, and 200 nM) on sperm *OGG1* and *ROMO1* gene regulation: **(a)** impact of mitoquinone on *OGG1* gene expression, **(b)** relationship between *OGG1* gene methylation and mitoquinone, **(c)** impact of mitoquinone on *ROMO1* gene expression, **(d)** impact of mitoquinone on *ROMO1* gene methylation. The expression levels of the target genes were normalized to ACTB as a reference gene. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method. MitoQ: mitoquinone $^{*}P < 0.05$.

these modifications is the addition of various antioxidant substances into sperm extenders (Amidi et al., 2016; Masoudi et al., 2018; Rostami et al., 2020). Here, the effects of mitoquinone and caffeic acid on the freezability of ram semen were specifically evaluated at the gene level.

The current study further confirmed that cryopreservation negatively affects spermatological parameters of ram semen, as shown previously by Peris et al. (2007), Mahmuda et al. (2015), Ustuner et al. (2016) and Banday et al. (2017). That detrimental effect, however, was more pronounced in the control group when compared with the MitoQ and CA groups.

Motility is essential for a spermatozoon to progress to the fertilization area, for passing through both the cumulus oophorus complex and zona pellucida of the oocyte (Bacinoglu et al.,2008). The post-thaw motilityof MitoQ200, CA100 and CA150 antioxidant groups was significantly higher than the control group ($P < 0.05$), although there were no dose-dependent response. In the study conducted by Wu et al. (2021) using 50, 100, 150 and 200 doses of MitoQ in Hu sheep, it was observed that the post-thaw motility values were significantly lower than in the present study. Another study, by Javaheri Barfourooshi et al. (2023) used 1, 10, 100 and 1000 nM doses of MitoQ in rams, and it reported post-thaw motility values of 55 % for a 100 nM dose, a similar value to those obtained in the present study. The extender formulation, cryopreservation technique, ram breed and season of semen collection all play a role in the variability of spermatological parameters. Doğu et al. (2021) stated that increasing the antioxidant dose in the diluent reduces the oxidant level, resulting in a better preservation of motility. The better protection of motility by 100 μM caffeic acid compared to 50 μM

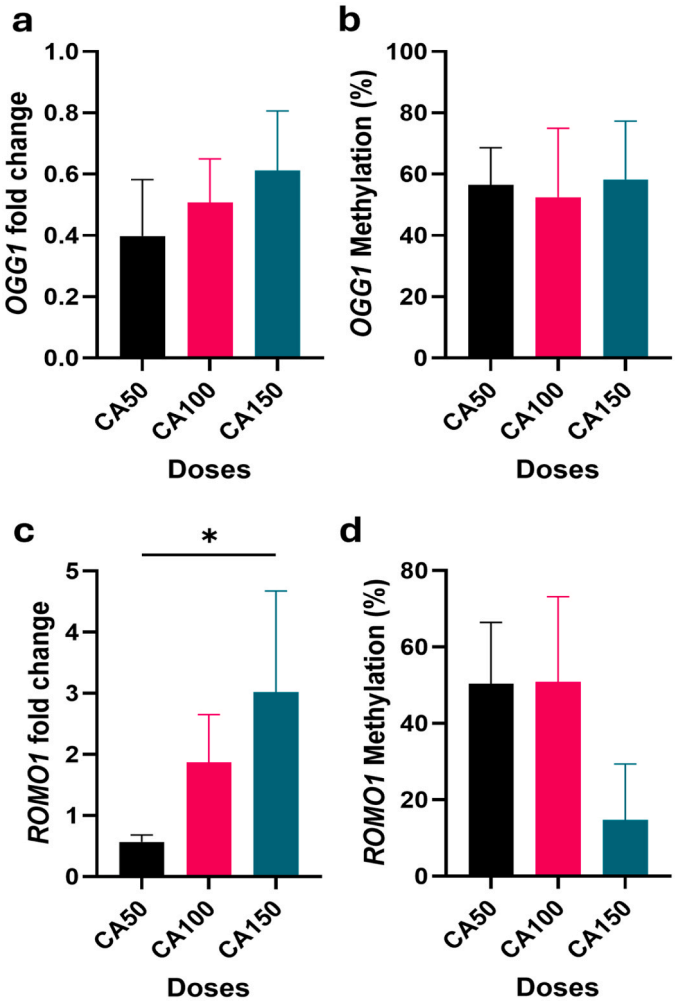


Fig. 6. Assessment the effects of three caffeic acid doses (50, 100, and 150 μM) on sperm *OGG1* and *ROMO1* gene regulation, examining: **(a)** caffeic acid impact on *OGG1* gene expression, **(b)** the relationship between *OGG1* gene methylation and caffeic acid, **(c)** caffeic acid impact on *ROMO1* gene expression, and **(d)** caffeic acid effect on *ROMO1* gene methylation. Gene expression levels were normalized using *ACTB* as an internal control, and fold changes were calculated employing the $2^{-\Delta\Delta Ct}$ method. CA: caffeic acid. $^{*}P < 0.05$.

dose we observed, is also consistent with Namula et al. (2018) and Soleimanzadeh et al. (2020). Consistently with those studies, we also conclude that lower antioxidant doses are insufficient to preserve post-thaw motility.

The CA100 group exhibited the lowest ALH value (3.69 μm), which was significantly lower than both the control (6.01 μm) and MitoQ100 (6.69 μm) groups ($P < 0.05$). Previous studies by Javaheri Barfourooshi et al. (2023), Rezaei et al. (2023), and Hatami et al. (2024) also reported that varying MitoQ doses did not affect post-thaw ALH values. Similarly, Soleimanzadeh et al. (2020) found no significant differences in ALH values among groups in their study on CA freezing of buffalo semen. While these studies observed no differences in ALH values, they did report significant variations in post-thaw motility. Santiago-Moreno et al. (2017) noted that ALH increased due to early capacitation. In our study, the CA100 group demonstrated higher post-thaw motility compared to the control ($P < 0.05$) and MitoQ100 ($P > 0.05$) groups, aligning with the ALH findings. Sönmez and Fırat (2022) stated that the STR value ranges from 0 to 100, with values closer to 100 indicating a more linear progression trajectory of the spermatozoon. The highest STR (%) value we obtained was observed in the CA100 group, although only signlificantly different from the MitoQ100 group ($P < 0.05$). Additionally, Soleimanzadeh et al. (2020) and Rezaei et al. (2023) reported no significant differences in post-thaw STR values among all groups in their studies involving CA and MitoQ, respectively. Evaluating motility and kinematic parameters, it was determined that the addition of antioxidants preserved sperm movement, with the MitoQ200, CA100, and CA150 groups being particularly effective. Lower doses of antioxidants were insufficient to protect sperm motility from the adverse effects of cryopreservation.

It is possible for spermatozoon to exchange substances with the external environment, bind sperm to oocytes during storage in the female reproductive system, and initiate the acrosome reaction by preserving the functional integrity of the plasma membrane (Kadirvel et al.,2012). Cold shock, osmotic pressure change, ice crystals and ROS formation during cryopreservation cause lipid composition changes in the cell membrane and loss of function in the plasma membrane (Medeiros et al., 2002). The plasma membrane

functional integrity values varied between 35.21 % and 71.70 %, at post-thaw and these results were similar to previous studies (Ustuner et al., 2016; Banday et al., 2017; Soleimanzadeh et al., 2020; Jia et al.2022; Rezaei et al., 2023). We observed that post-thaw plasma membrane integrity values of antioxidant groups were generally higher than this range. CA50 dose group was insufficient to protect plasma membrane integrity and was not found to be different from the control group.

Cryopreservation induces premature capacitation or acrosome reactions (Neild et al., 2003). The obtained data related to the post-thaw acrosome defects in the present study are in agreement with several studies that reported 35–69 % rate of acrosome defects resulting from the freeze thaw process (Ustuner et al., 2016; Jia et al., 2022; Hatami et al., 2024). Apartfrom the CA50 and CA100 doses, all antioxidant groups tested here were found to protect acrosome integrity better than the control group.

Determination of MMP and DNA fragmentation in the evaluation of sperm quality are two important parameters used in estimating natural pregnancy rates, especially in infertile individuals (Malič Vončina et al., 2016). During cryopreservation, unsaturated fatty acids in mitochondria disrupt electron flow, increasing ROS and reducing sperm motility (Nowicka-Bauer and Nixon et al.,2020). Decrease in MMP due to oxidative stress causes sperm DNA fragmentation and ultimately cell apoptosis (Aitken et al., 2012). According to our results, mitochondrial membrane damage of the control group (13.80 %) was significantly higher than all antioxidant groups except CA50 (6.0 %). As mentioned earlier, the primary reason for using mitoquinone in this study was its mitochondria-targeted effect as an antioxidant. Despite not having difference in MMP between different mitoquinone doses, the highest protection was found in the MitoQ200 dose group. While not having a mitochondria-specific mechanism of action, CA100 and CA150 doses do not have a mitochondria-targeted mechanism of action, CA had an equivalent positive effect as MitoQ. Unlike the higher CA doses, CA50 did not have a sufficient antioxidative effect as demonstrated by the previously mentioned parameters. The positive effect of CA on sperm mitochondrial membrane potential is likely due to the double-sided binding of the quinone group conjugated to the catechol group in its chemical structure, increasing the stability of the quinone radical and the antioxidant activity of CA by causing delocalization of electrons.

The fact that the antioxidants tested had a positive effect on mitochondrial membrane potential, but meaningful effect on DNA fragmentation, suggests that the TUNEL method is not sensitive enough. Similar results were observed in other studies evaluating sperm DNA fragmentation using the TUNEL method, which supports the findings found herein (Ustuner et al.,2018; Öztürk et al., 2020; Onder et al., 2022).

For the post-thaw MDA levels examined, no significant differences were observed. The MDA values of MitoQ different dose groups were found to be lower than the values of Wu et al. (2021) and Javaheri Barfourooshi et al. (2023). Similarly, although there was no statistical difference among the groups in terms of TAC levels, the highest TAC values were obtained for MitoQ200, CA100 and CA150 groups. As a result of the literature review, no study evaluated the MDA and TAC levels of caffeic acid added to the cryopreservation medium in ram semen.

This study analyzed the expression levels of *ROMO1* and *OGG1* to validate data from MDA, TAC, mitochondrial membrane potential, and TUNEL assays. Understanding oxidative stress's role in male fertility, the benefits of antioxidants on sperm quality, and the genetic regulation of these processes are crucial in reproductive biology.

Understanding the underlying genetic mechanisms of the related pathways, such as the role of *OGG1* in DNA repair or the impact of the gene on reproductive parameters, will provide further valuable insights (Garcia-Rodriguez et al., 2018). In this study, higher doses of MitoQ were associated with reduced *OGG1* gene expression, suggesting a suppression of transcriptional activation in antioxidant response genes, particularly evident at 150 and 200 nM doses. Additionally, an increase in methylation of the *OGG1* gene was observed with increasing MitoQ doses, which likely contributes to the decreased gene expression. These findings indicate that MitoQ induces methylation changes that result in diminished gene expression of *OGG1*, corroborating the interconnected nature of gene expression and methylation responses. Increasing doses of caffeic acid progressively enhanced the expression of the *OGG1* gene. It has been reported that *OGG1* gene expression showed a significant negative correlation with increased DNA damage in spermatozoa (Garcia-Rodriguez et al., 2018). The differences observed among 50, 150 and 200 nM dose groups of MitoQ in terms of *OGG1* gene expression and methylation levels indicate that the 200 nM dose of MitoQ protects DNA integrity at the molecular level. The same effect was not detected in DNA integrity rates determined by TUNEL method. Notably, the peak expression level was attained at the highest dose administered, CA150. Contrastingly, the methylation patterns of the *OGG1* gene across three varying doses did not show significant differences. This consistency in methylation suggests that the dose-responsive increase in *OGG1* expression induced by caffeic acid is likely independent of changes in DNA methylation status. Thus, other regulatory mechanisms might be responsible for modulating the observed upregulation in gene expression in response to caffeic acid.

Romo1 protein is the main gene localized in mitochondria and plays an important role in oxidative stress-induced mitochondrial ROS production (Shin et al., 2013). We observed that a 150 nM dose of MitoQ significantly increased methylation levels, which was associated with reduced expression of the *ROMO1* gene compared to other doses. Additionally, MitoQ doses of 100 and 200 nM demonstrated similar regulatory effects on the ovine *ROMO1* gene. A notable finding from our study is the significant impact of CA on *ROMO1* gene expression. Increasing doses of caffeic acid led to higher *ROMO1* gene expression, with a significant difference observed between the 50 μM and 150 μM doses. In studies conducted on the addition of different antioxidant substances to cryopreservation extender in canine semen, low *ROMO1* gene expression levels were reported in groups with high post-thaw spermatological parameters. (Setyawan et al., 2016; Qamar et al., 2020a,b). It has been reported that the addition of antioxidants to the extender used in cryopreservation of various species semen decreased *ROMO1* gene expression at the post-thaw stage and a negative correlation was observed between high mitochondrial membrane potential and *ROMO1* expression (Bang et al., 2021; Qamar et al., 2019, 2020a). The negative correlation between *ROMO1* expression and sperm parameters observed in the mentioned studies was in direct conflict with the results of the present study. Khan et al. (2024) conducted a study in bull semen and found that while statistical significance was observed between antioxidant dose groups in terms of spermatological parameters, *ROMO1* gene expression levels did not differ

statistically between dose groups. It was clearly suggested that the expression level of the *ROMO1* gene may vary according to the animal species.

To the best of our knowledge, this study is the first to demonstrate that DNA methylation modulates the expression levels of the *OGG1* and *ROMO1* genes in response to mitoquinone and caffeic acid and the impact of this regulation on sperm quality and male fertility outcomes in rams. The observed differences in gene expression levels are, therefore, consistent with current knowledge and may offer valuable insights into sperm RNA and transcription activity. The noted expression differences in key ROS regulatory genes in our study are particularly significant. These findings highlight the potential of epigenetic mechanisms to influence gene expression and underscore their significance in assessing the effect of antioxidants used in cryopreservation.

5. Conclusion

Based on the spermatological parameters and gene expression and methylation levels measured in the current study, MitoQ 200 nM and CA 150 μM doses had a positive effect on the freezability of ram semen. This study is the first to demonstrate that MitoQ significantly alters the expression of the *OGG1* genes, an effect mediated through DNA methylation. Furthermore, CA altered the *ROMO1* gene expression. These results indicate that assessing the expression and methylation status of ROS modulator genes is crucial for a better understanding of the genetic basis of spermatological parameters in ram semen.

CRediT authorship contribution statement

Burcu Ustuner: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization. **Tugaycan Cimen:** Methodology, Formal analysis, Data curation. **Sena Ardicli:** Writing – review & editing, Visualization, Supervision, Methodology, Formal analysis, Data curation. **Mehmet Melih Yılmaz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Burcu USTUNER reports financial support was provided by Bursa Uludag University. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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