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F Yılmaz Dayanç, C Yıldız

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Effects of Melatonin and Coenzyme Q_{10} Added to Extenders on Spermatological Properties in Cryopreservation of Epididymal Rat Sperm

F. Yılmaz-Dayanc,[1]* C. Yıldız[2]

[1]*Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Hatay, TURKEY*

[2]*Yalova University, Faculty of Medicine, Department of Medical Biology, Yalova, TURKEY*

ABSTRACT: With the use of advanced genome editing technology, the production of genetically modified mutant rats worldwide, and the successful freezing and storage of their gametes, have become important in biotechnology. In the present study, the effects of different doses of melatonin (0.5mM, 1mM, 2mM) and coenzyme Q_{10} (25µM, 50µM, 75µM) added to the spermatozoa freezing medium containing 8% lactose monohydrate, 23% egg yolk (EY), and 10% trisaminomethane on the freezability of rat spermatozoa were investigated. After freezing and thawing, motility, dead spermatozoa rate, plasma membrane integrity, abnormal acrosome rate, motility longevity, apoptotic and necrotic cell rates were evaluated. Compared to the control group (12.90±1.34%), the highest motility value was found in the 1mM Melatonin group (24.58±1.68%) and 25µM Coenzyme Q_{10} group (24.23±1.95%) ($p<0.01$). The lowest dead spermatozoa rate was determined in the 1mM Melatonin group (63.08±4.89%) compared to the control group (75.47±2.27%) ($p<0.01$). Plasma membrane integrity rates were higher in the 1mM Melatonin (35.50±1.82%) and 25µM Coenzyme Q_{10} (31.31±1.93%) groups compared to the control group (18.21±1.36%) ($p<0.01$). The best motility longevity was observed in the 1mM melatonin and 25µM coenzyme Q_{10} groups compared to the control group ($p<0.01$). There was no difference between the groups in terms of abnormal acrosome rates ($p>0.01$). Apoptotic and necrotic cell rates were found to be lower in the 1mM Melatonin or 25µM Coenzyme Q_{10} groups compared to the control group ($p<0.01$). However, it was found that the use of 1mM melatonin had a better protective effect than 25µM coenzyme Q_{10} ($p<0.01$). In conclusion, the addition of 1mM Melatonin or 25µM Coenzyme Q_{10} to the rat sperm diluent effectively protected spermatozoa against cryo-damage during cryopreservation process.

Keyword: Rat Sperm Analysis, Apoptosis, Cryopreservation, Antioxidant, Melatonin, Coenzyme Q_{10}.

Correspondence author:
F. Yılmaz-Dayanc,
Hatay Mustafa Kemal University, Faculty of Veterinary Medicine,
Department of Reproduction and Artificial Insemination, Hatay, TURKEY
E-mail address: fiirdevsyiilmaz@gmail.com

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INTRODUCTION

Currently, rats are among the most commonly used laboratory animals in biomedical sciences, and many mutant strains have been developed over the past century (Festing and Greenhouse, 1992; Nakatsukasa et al., 2003). Genetic modifications through cloning, transgenics, and mutations have allowed the conservation of genetic resources from endangered animal species. The number of transgenic rat strains is increasing, which highlights the need for the preservation of rat genetic resources (Charreau et al., 1996). Rat sperm cryopreservation is considered a cost-effective and efficient method for conserving genetic resources (Nakatsukasa et al., 2001). However, success in this area has not yet reached the desired level, and the formation of reactive oxygen species (ROS) during freezing and thawing, which damages sperm cells, is one of the reasons for this failure (Lobo et al., 2010).

Melatonin, a compound secreted by the pineal gland, has strong antioxidant properties (Awad et al., 2006). It reduces ROS levels, thereby decreasing oxidative damage in sperm cells and regulating various reproductive functions (Tamura et al., 2012). Coenzyme Q_{10} (CoQ_{10}) is a powerful antioxidant that resides in mitochondria to provide energy for cells (Kapoor and Kapoor 2013; Santos-Ocana et al., 2002). It prevents lipid peroxidation, inhibits protein and DNA oxidation, and stabilizes cell membranes (El-Tohamy et al., 2012). CoQ_{10} is directly related to energy production and motility in sperm cells (Mancini et al., 2005).

This study aims to investigate the effects of Melatonin and CoQ_{10} on rat spermatozoa cryopreservation. As the first comprehensive study in this field, it seeks to explore the effects of Melatonin (0.5 mM, 1 mM, 2 mM) and CoQ_{10} (25 µM, 50 µM, 75 µM) on motility, dead spermatozoa ratio, plasma membrane integrity (Hypoosmotic Swelling Test), acrosome morphology, motility endurance (longevity), and apoptotic cell ratio. This research aims to contribute to the development of new rat sperm diluents needed in the field of cryobiology.

MATERIALS AND METHODS

Animals

In the study, 56 adult male Wistar Albino rats aged 10-16 weeks were used. The rats were kept at room temperature (24±3°C) with 12 hours of light and 12 hours of darkness at 60% relative humidity. Throughout the experimental procedure, the rats were fed standard rat chow and tap water ad libitum. All procedures were carried out in accordance with the approved decision of the Hatay Mustafa Kemal University (HMKÜ), Local Ethics Committee for Animal Experiments (HADYEK) (Ethical Approval No: 2022/03-17).

Chemicals

All chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and prepared according to Quinn et al. (1985) using Human Tubal Fluid.

CPA's and Research Groups

The preparation of the cryopreservation agent (CPA) was carried out by modifying the method described by Nakatsukasa et al. (2001). The CPA contained 8% lactose monohydrate (1.07656, Sigma), 10% TRIS (tris(hydroxymethyl)aminomethane) (1.08386, Sigma), 23% egg yolk, 1 mg/ml streptomycin sulfate, 1000 IU penicillin G potassium, and 0.1% ATP (Adenosine 5'-triphosphate disodium salt hydrate, Grade II; Sigma). The preparation of the CPA: 23% fresh chicken egg yolk was added to pure distilled water, followed by 8% lactose monohydrate, 1 mg/mL streptomycin sulfate, and 1000 IU penicillin G potassium. This mixture was stirred for 5 minutes at +4 °C and then centrifuged at 1600 rcf for 15 minutes. After centrifugation, the supernatant was collected and 0.1% ATP (Adenosine 5'-triphosphate) was added. The resulting dilution was divided, and melatonin (M5250, Sigma) was added in doses of 0.5 mM, 1 mM, and 2 mM to create the melatonin groups. Coenzyme Q_{10} (C9538, Sigma) was added in doses of 25 µM, 50 µM, and 75 µM to create the Coenzyme Q_{10} groups. A control group was set up without any additional supplements. Finally, the group mixtures were adjusted to a pH of 7.4 using a pH meter (Thermo Scientific 1112000, USA) and a pre-prepared 10% TRIS (tris(hydroxymethyl)aminomethane) + distilled water solution.

Collection, Freezing, and Thawing of Spermatozoa

Rats were euthanized under anesthesia using the cervical dislocation method. Under sterile conditions, both epididymides were removed. The epididymides were transferred into 1 mL of the pre-prepared CPA (containing either control or one of the antioxidants) in a 35 mm petri dish (Nunc™, Massachusetts, USA) at room temperature. The sperm dilution and freezing process was modified from the protocol reported by Nakagata et al. (2020). The epididymides were fixed on a stereo microscope (Euromex/Nexius Zoom,

Netherlands) using fine forceps, and the tip of the scissors was used to make 10-12 deep incisions in the petri dish, allowing the sperm to enter the CPA. Fresh motility and concentration assessments were performed for each group.

For spermatozoa release and equilibration; the petri dishes were placed on a metal plate positioned on top of crushed ice in a styrofoam box and incubated for 10 minutes. At the end of the incubation period, the sperm was loaded into 0.25 mL straws (IMV, France) in the following order: 30 µL Human Tubal Fluid (HTF), 10 mm air, 150 µL sperm, 10 mm air, and 30 µL HTF, and then sealed. The straws were then placed back onto the metal plate on the crushed ice and equilibrated for 30 minutes. The temperature of the equipment used during the straw loading process (syringe connectors, straws, and HTF) was adjusted to 0°C. The straws were arranged on a straw rack placed inside a styrofoam box (270x220x270 mm), with liquid nitrogen at a depth of 3.5 cm and 4 cm above the liquid nitrogen level, where they underwent a 10-minute freezing process in the vapor of liquid nitrogen and were then immersed in liquid nitrogen. The straws were thawed in a 37°C water bath (decongelator, CITO 026897, IMV, France) for 30 seconds. The thawed straws were then transferred into 1 mL of HTF in a carbon dioxide incubator (37°C and 5% CO2).

Spermatozoa Analysis

Determination of Sperm Concentration

Sperm concentration was determined using the method described by the World Health Organization (WHO, 1999) guidelines, using the Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). Sperm samples were diluted 1:10 in fixative medium (PBS containing 10 mM formaldehyde) to immobilize the sperm. 10 µL of the fixed mixture was taken, and the number of cells within 10 squares under a 20X objective lens (Olympus CX31, Japan) was counted to determine the value in million units. Each sample was analyzed in triplicate, and the average data was calculated.

Motility Examination

The motility examination was performed using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) according to the guidelines of the World Health Organization (WHO, 1999). Briefly, 20 µL of the HTF + sperm mixture was placed on a pre-warmed (37°C) Makler chamber and examined under a phase-contrast microscope with a heating stage (Olympus CX31, Japan) at 20X magnification. The proportion of spermatozoa exhibiting progressive linear motility was counted against other movement types (vibratory, rotational, or immotile) within 100 small squares on the Makler chamber. Each sample was assessed in triplicate, and the average was calculated. Sperm motility was expressed as a percentage (%).

Determination of Dead Spermatozoa Ratio

Sperm viability was assessed using the eosin-nigrosin staining method described by Agarwal et al. (2016). A mixture of 10 µL of sperm sample and 20 µL of 1% eosin and 10% nigrosin solutions was prepared, and smears were made. The smears were air-dried within 2-3 seconds on a 60°C heated surface. Analyses were performed under a phase-contrast microscope (Olympus CX31) at 40X magnification by counting at least 300 spermatozoa. Spermatozoa with red or dark pink-stained heads were classified as dead, while those with unstained heads were classified as live. Viability was calculated as a percentage (%).

Evaluation of Plasma Membrane Integrity

The functional integrity of spermatozoa plasma membranes was evaluated using the Hypo-Osmotic Swelling Test (HOST), as described by Jeyendran et al. (1984). HOST solution with low osmotic pressure of 100 mOsm was prepared (1.1 g fructose + 0.55 g sodium citrate + 100 mL distilled water), and its osmotic pressure was measured using an osmometer (Osmomat 3000, Gonotec, USA). A mixture of 100 µL HOST solution and 10 µL sperm sample was incubated in a 1.5 mL Eppendorf tube at 37°C for 45 minutes. After incubation, the samples were examined on a pre-warmed (37°C) slide under a phase-contrast microscope (Olympus CX31) at 40X magnification. Spermatozoa with swollen or coiled tails were considered HOST positive (+), while those with no changes were considered HOST negative (-). A total of 200 spermatozoa were counted per sample, and the percentage (%) of HOST positive spermatozoa was recorded.

Motility Endurance Test (Longevity)

All groups in the study were incubated at 37°C in HTF medium after thawing. Immediately after thawing, 10 µL was taken for motility evaluation at 0 hours using a Makler counting chamber. After the initial evaluation, samples were left for incubation, and the evaluation was repeated every 30 minutes up to the 90th minute. Motility assessments were

conducted using a Makler counting chamber preheated to 37°C and examined under a phase-contrast microscope (Olympus CX31, Japan) with a 20X objective. In the Makler chamber, the percentage (%) of spermatozoa exhibiting forward, linear, and vigorous motility was calculated by comparing them to spermatozoa showing other movement patterns (vibratory, rotational, immotile) within 100 small squares.

Abnormal Acrosome Examination

Abnormal acrosome examination was conducted as described by Somfai et al. (2002). Hancock solution was used for the assessment (Hancock, 1952). A volume of 500 µL Hancock solution was added to an Eppendorf tube, followed by the addition of 50 µL sperm. From this mixture, 50 µL was placed on a slide, covered with a coverslip, and examined morphologically using an immersion oil lens at 100X magnification (Günay et al., 2003). A total of 300 spermatozoa were counted, and those with acrosomal damage were recorded as a percentage (%).

Apoptosis Examination in Spermatozoa

Apoptosis examinations were conducted on the control group and the groups that provided the best results (1 mM melatonin and 25 µM coenzyme Q_{10}). The Annexin-V FITC/PI apoptosis kit (Elabscience, E-CK-A211) was used following the manufacturer's instructions. Annexin V-FITC staining was used to identify apoptotic cells, while Propidium Iodide (PI) staining was employed to identify necrotic cells with membrane integrity loss. Preparations were analyzed using a fluorescence microscope (Eclipse Ni, Nikon) with FITC/PI filters (excitation 490 nm & emission 520 nm) under 20X and 40X objectives. Cells were classified as Annexin V-FITC (AV+) and Propidium Iodide (PI-) (green, apoptotic cells) or Annexin V-FITC (AV-) and Propidium Iodide (PI+) (red, necrotic cells). A total of 200 cells were counted, and the results were recorded as percentages (%). (Fig. 1).

Statistical Analysis

The statistical analysis of the values obtained from the semen samples was performed. Analyses were conducted using the SPSS 22.0 software package, where one-way analysis of variance (ANOVA) was applied to calculate the mean and standard error values. Duncan's multiple comparison test was used to determine the significance of differences between groups.

RESULTS

Spermatological Parameters Determined Before and After Freezing-Thawing

The motility, dead spermatozoa, abnormal acrosome, and plasma membrane integrity rates in the study groups after fresh and post-thawing processes are presented in Table 1.

Motility Values

The highest motility value (71.95±1.86%), was recorded in fresh semen. Compared to the control group after cryopreservation (12.90±1.34%), the highest motility values were detected in the 1 mM Melatonin group (24.58±1.68%) and the 25 µM Coenzyme Q_{10} group (24.23±1.95%) ($p<0.01$). When all groups were compared among themselves to Among all groups, the lowest motility value (12.90±1.34%) was observed in the control group ($p<0.01$). When the Coenzyme Q_{10}-supplemented groups were evaluated

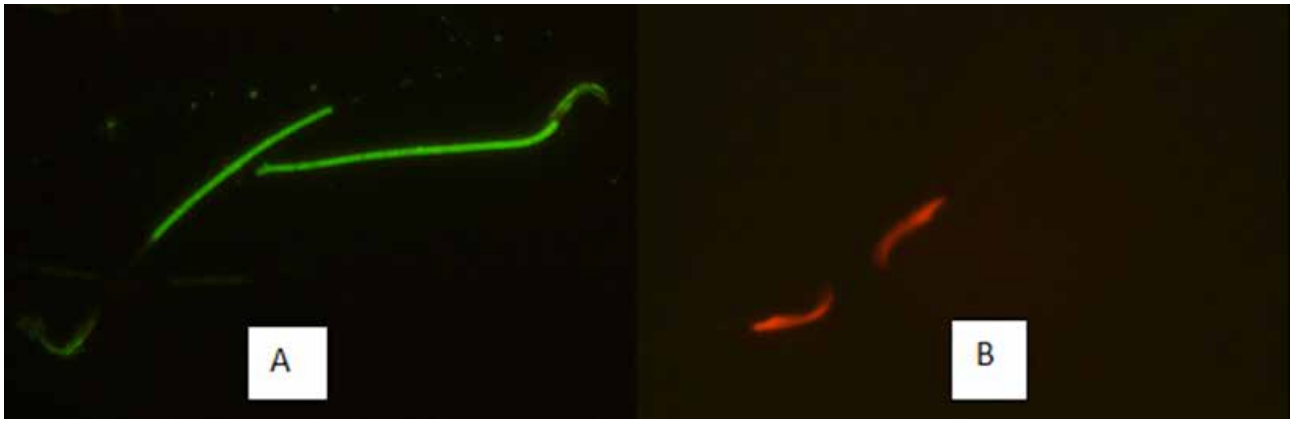

Figure 1. Apoptosis examination: apoptotic (AV+/PI-) green cell (A); necrotic (AV-/PI+) red cell (B).

Table 1. Spermatological findings determined before and after freezing and thawing in the study groups *(n = 8)*

Groups	Motility Rate (%)	Dead Spermatozoa Rate (%)	HOST Test Positive Rate (%)	Abnormal Acrosome Ratio (%)
Fresh	71.95±1.86^{a}	26.84±1.57^{e}	73.21±1.99^{a}	3.63±0.43^{a}
Control	12.90±1.34^{c}	75.47±2.27^{abc}	18.21±1.36^{c}	4.53±0.59^{a}
Coenzyme Q_{10} 25 µM	24.23±1.95^{b}	66.46±1.89^{cd}	31.31±1.93^{b}	2.69±0.33^{a}
Coenzyme Q_{10} 50 µM	14.55±1.84^{c}	82.27±1.31^{a}	19.55±2.56^{c}	3.46±0.34^{a}
Coenzyme Q_{10} 75 µM	18.00±1.86^{c}	71.80±5.50^{bcd}	23.90±2.15^{c}	3.00±0.39^{a}
Melatonin 0.5 mM	13.50±1.68^{c}	76.70±4.12^{ab}	20.30±1.84^{c}	3.50±0.43^{a}
Melatonin 1 mM	24.58±1.68^{b}	63.08±4.89^{d}	35.50±1.82^{b}	2.42±0.29^{a}
Melatonin 2 mM	15.56±1.30^{c}	69.67±2.87^{bcd}	24.33±1.97^{c}	3.44±0.82^{a}
p	p<0.01	p<0.01	p<0.01	p<0.01

ad: Different letters in the same column indicate statistical difference. (*P<0.01*)

internally, the highest motility rate was found in the 25 µM Coenzyme Q_{10} group (24.23±1.95%), while the lowest motility rates were observed in the 50 µM Coenzyme Q_{10} group (14.55±1.84%) and the 75 µM Coenzyme Q_{10} group (18.00±1.86%) (p<0.01). Similarly, when the melatonin-supplemented groups were evaluated internally, the highest motility rate was found in the 1 mM Melatonin group (24.58±1.68%), while the lowest motility rates were observed in the 0.5 mM Melatonin group (13.50±1.68%) and the 2 mM Melatonin group (15.56±1.30%) (p<0.01).

Dead Spermatozoa Rate (Viability)

The lowest dead spermatozoa rate, was observed in fresh semen samples (26.84±1.57%). Among the experimental groups, the lowest dead spermatozoa rate was found in the 1 mM Melatonin group (63.08±4.89%), while the highest rate was recorded in the 50 µM Coenzyme Q_{10} group (82.27±1.31%) (p<0.01). When all experimental groups were compared after freezing-thawing, the lowest dead spermatozoa rate was observed in the 1 mM Melatonin group (63.08±4.89%), compared to the control group, which had a rate of 75.47±2.27% (p<0.01). When the Coenzyme Q_{10} groups were evaluated for dead spermatozoa rates, the 25 µM Coenzyme Q_{10} group showed the lowest rate (66.46±1.89%), while the 50 µM Coenzyme Q_{10} group had the highest rate (82.27±1.31%) (p<0.01). Among the Melatonin groups, the lowest dead spermatozoa rate was found in the 1 mM Melatonin group (63.08±4.89%), while the highest rate was observed in the 0.5 mM Melatonin group (76.70±4.12%) (p<0.01).

Plasma Membrane Integrity Rate (HOST Positive)

The highest plasma membrane integrity positive spermatozoa rate (73.21±1.99%), was observed in fresh spermatozoa (p<0.01). Among the experimental groups, after freezing and thawing, the highest HOST-positive rates were found in the 1 mM Melatonin group (35.50±1.82%) and the 25 µM Coenzyme Q_{10} group (31.31±1.93%). No statistical difference was found between these two groups (p>0.01). When the experimental groups were compared with the control group (18.21±1.36%), the 1 mM Melatonin group (35.50±1.82%) and the 25 µM Coenzyme Q_{10} group (31.31±1.93%) showed significantly higher HOST positive rates (p<0.01). However, the 50 µM Coenzyme Q_{10} (19.55±2.56%), 75 µM Coenzyme Q_{10} (23.90±2.15%), 0.5 mM Melatonin (20.30±1.84%), and 2 mM Melatonin (24.33±1.97%) groups showed no statistical differences among themselves (p>0.01).

Motility Endurance (Longevity)

Time-Dependent Motility Longevity Values After Freezing and Thawing

The motility longevity results, evaluated at 30-minute intervals after freezing and thawing, are presented in Table 2. In the first 30 minutes following the freezing-thawing process, the highest motility longevity value (18.33±1.42%) was observed in the 1 mM Melatonin group. At the end of the first 30 minutes, when all groups were compared, the lowest motility longevity value was found in the control group (7.37±1.23%), which was similar to the 0.5

Table 2. Time-dependent motility endurance values after freezing and thawing in the study groups (*n* = 8)

	0. minute	30. minute	60. minute	90. minute
Control	12.90±1.34^{b}	7.37±1.23^{c}	3.68±0.65^{c}	0.79±0.43^{c}
Coenzyme Q_{10} 25 µM	24.23±1.95^{a}	15.00±1.79^{ab}	8.85±1.62^{ab}	4.23±0.77^{ab}
Coenzyme Q_{10} 50 µM	14.55±1.84^{b}	8.09±2.36^{c}	5.64±1.36^{bc}	0.91±0.61^{c}
Coenzyme Q_{10} 75 µM	18.00±1.86^{b}	10.50±1.89^{bc}	5.50±1.57^{bc}	2.00±0.82^{abc}
Melatonin 0.5 mM	13.50±1.68^{b}	9.00±1.45^{c}	6.00±1.25^{bc}	2.50±0.83^{abc}
Melatonin 1 mM	24.58±1.68^{a}	18.33±1.42^{a}	10.00±1.51^{a}	4.58±1.44^{a}
Melatonin 2 mM	15.56±1.30^{b}	12.22±1.21^{bc}	7.22±0.88^{abc}	1.67±1.18^{bc}
p	p<0.01	p<0.01	p<0.01	p<0.01

$^{a-c}$**:** Different letters in the same column indicate statistical difference. (*p<0.01*).

mM Melatonin group (9.00±1.45%) and the 50 µM Coenzyme Q_{10} group (8.09±2.36%) (p>0.01).

At the 60th minute, the highest motility longevity value was observed in the 1 mM Melatonin group (10.00±1.51%), while the lowest motility longevity value was recorded in the control group (3.68±0.65%) (p<0.01).

At the 90th minute, the highest motility longevity value was again observed in the 1 mM Melatonin group (4.58±1.44%), while the lowest motility longevity values were recorded in the control group (0.79±0.43%) and the 50 µM Coenzyme Q_{10} group (0.91±0.61%) (p>0.01).

Abnormal Acrosome Rate

Regarding abnormal acrosome rates, no statistical difference was found between the fresh group (3.63±0.43%) and all other groups (4.53±0.59% in the control group, 2.69±0.33% in the 25 µM Coenzyme Q_{10} group, 3.46±0.34% in the 50 µM Coenzyme Q_{10} group, 3.00±0.39% in the 75 µM Coenzyme Q_{10} group, 3.50±0.43% in the 0.5 mM Melatonin group, 2.42±0.29% in the 1 mM Melatonin group, and 3.44±0.82% in the 2 mM Melatonin group) (p>0.01).

Cell Apoptosis Rates

Apoptotic Cell Rates Determined by Annexin V-FITC and Propidium Iodide Staining

The apoptotic cell rates determined by Annexin V-FITC and Propidium Iodide staining results obtained in the study are presented in Table 3. The highest apoptosis (AV+/PI-) cell rate was found in the control group (28.12±1.36%), while the lowest apoptosis (AV+/PI-) cell rate was found in the 1 mM Melatonin group (4.20±2.10%) (p<0.01). This result indicates that the antioxidant 1 mM Melatonin added to the diluent provided better protection against ROS (Reactive Oxygen Species) formed or potentially formed during freezing and thawing of sperm cells, compared to the 25 µM Coenzyme Q_{10} and Control groups (p<0.01). In other words, 1 mM Melatonin demonstrated better protective effects compared to 25 µM Coenzyme Q_{10}. In the statistical comparison for necrotic (AV-/PI+) cells, the lowest necrotic cell rate was found in the 25 µM Coenzyme Q_{10} group (21.59±2.00%) and the 1 mM Melatonin group

Table 3. Cell apoptosis rates after freezing and thawing in the study groups *(n = 8)*

Groups	(AV+) ve (PI-) Apoptotic Cell Rate (%)	(AV-) ve (PI+) Necrotic Cell Rate (%)
Control	28.12±1.36^{a}	33.53±2.47^{a}
Coenzyme Q_{10} 25 µM	13.21±1.45^{b}	21.59±2.00^{b}
Melatonin 1 mM	4.20±2.10^{c}	21.10±4.07^{b}
p	p<0.01	p<0.05

$^{a-c}$**:** Different letters in the same column indicate statistical difference. *(p<0.05-0.01).*

(21.10±4.07%), while the highest necrotic cell rate was observed in the control group (33.53±2.47%) ($p<0.05$).

DISCUSSION

Understanding the physiology of sperm cells is crucial to achieving maximum success during spermatozoon cryopreservation. Sperm cells, being small with a large surface area, are particularly sensitive to stress factors such as cold shock, ice crystal formation, and osmotic changes in the absence of cryoprotective agents, which can damage cellular organelles (AbdelHafez et al., 2009; Morris et al., 2012; Morris, 2006). This damage leads to the degradation of cellular structures and oxidation (O'Connell et al., 2002). During the cryopreservation process, cryoprotective and antioxidant agents are used to reduce cellular damage and the risk of cell death. These agents protect sperm cells by balancing the osmotic pressure of the medium and increasing the proportion of unfrozen fractions. Melatonin and Coenzyme Q_{10} are antioxidants with protective effects that provide protection against free radicals during sperm cryopreservation (Ball et al., 2001b; Bhattacharya, 2018; Hezavehei et al., 2018; Sieme et al., 2016; Silvestre et al., 2021; Eraslan Sakar et al. 2025). There are limited studies on the cryopreservation of Wistar Albino rat spermatozoa, and different diluents and cryopreservation techniques have yielded varying results. The structural characteristics of rat spermatozoa, especially their long tails, make them more susceptible to damage during freezing.

Yamashiro et al., in their research conducted at different times on rat sperm cryopreservation, reported the highest motility values after thawing as 35.3% ± 1.3 (2010a), 43.3% ± 2.8 (2010b), and 38.0% ± 4.1 (2007). Although these reported values are higher than the 24.58±1.68% observed in the 1 mM Melatonin group and 24.23±1.95% in the 25 µM Coenzyme Q_{10} group in our study, they are significantly higher compared to the 12.90±1.34% in the control group. Furthermore, these values were found to be better than those reported by Nakatsukasa et al. (2001) in Sprague Dawley (SD) male rats, where a diluent containing 23% egg yolk, 8% lactose monohydrate, and 0.7% Equex Stem achieved 4.6 ±0.1% motility and 9.2 ±3.9% plasma membrane integrity. Nakatsukasa et al. (2003) also used a similar diluent for cryopreservation of rat sperm and reported motility and live birth rates ranging from 2% to 12.3%, which is lower than the 24.58±1.68% motility, 35.50±1.82% plasma membrane integrity, and 63.08±4.89% dead spermatozoa rate found in the current study.

In this study, the differences between the findings observed in our work and those in other reports are believed to be due to several factors, primarily the effects of different antioxidant substances used, the rat strain selected, the packaging of diluted spermatozoa, and differences in the cryopreservation process. Many researchers working with rats have found that rat strain is an important factor influencing the success of spermatozoa cryopreservation (Nakatsukasa et al., 2003; Nakagata et al., 2020).

In the presented study, the use of the antioxidant melatonin in rat sperm is a first, while findings that it improves sperm parameters such as motility and viability, along with its general protective effects on sperm from other species, have been reported previously. In this context, the addition of melatonin to sperm diluents has been shown to have protective effects on motility and plasma membrane integrity after freezing and thawing in human (Karimfar et al., 2015), bull (ChaithraShree et al., 2020), ram (Putri et al., 2023), buck (Bucak et al., 2024), rooster (Mehaisen et al., 2020), dog (Fayez et al., 2023), mouse (Chen et al., 2016), fish (Yang et al., 2023), buffalo (Luo et al., 2023), and stallion (Izadpanah et al., 2015) sperm. Additionally, Chen et al. (2016) reported that supplementation of 0.125 mg/mL melatonin to mouse sperm diluents maintained motile spermatozoa and viability after freezing and thawing. In their study, Shahat et al. (2022) found that the addition of 1 mM melatonin and 1 mM L-carnitine to ram sperm diluents improved motility values and reduced total ROS levels after freezing and thawing.

Furthermore, in our study, the tested lipophilic enzyme Coenzyme Q_{10}, which is naturally found in every cell's mitochondria, is known to be a powerful antioxidant and plays a role in cellular energy production (Appiah et al., 2020). When such a lipophilic antioxidant is added to sperm, it can directly spread across the plasma membrane's polyunsaturated lipid chains, thus affecting spermatozoa structure and function by producing energy and preventing reactive oxygen species (Appiah et al., 2020). The 25 µM Coenzyme Q_{10} group in our study showed significant protective effects on spermatozoa ($p<0.01$). Coenzyme Q_{10} has been reported to protect spermatozoa in cryopreservation in species such as humans (Tas et al., 2023), bulls (Doidar et al., 2018), rams (Daghigh et al., 2017), stallions (Nogueira et al., 2022), roosters (Sharideh et al., 2019), deer (Youse-

fian et al., 2018), and mouse (Eraslan Sakar et al. 2025). Moreover, Coenzyme Q_{10} has been shown to improve sperm parameters in spermatozoa stored at low temperatures (Masoudi et al., 2019a; Nath et al., 2015; Pindaru et al., 2015; Yousefian et al., 2014). Recently, Khazravi et al. (2024) reported that the addition of 10 µM Coenzyme Q_{10} to deer sperm diluent during cryopreservation improved motility, plasma membrane integrity, and reduced ROS levels. Yi et al. (2024) reported that 150 nmol/L Coenzyme Q_{10} added to sperm diluents during the cryopreservation of buck sperm preserved motility and plasma membrane integrity. Apart from its role in cryopreservation solutions, Coenzyme Q_{10} has been reported to improve sperm quality in various animal species when administered orally (Raouf et al., 2021; Sharideh et al., 2020).

In the presented study, the highest acrosome damage rate after freezing and thawing was 4.53±0.59% in the control group, while the lowest acrosome damage rate was 2.42±0.29% in the 1 mM Melatonin group, with no significant differences found between the groups ($p>0.01$). Some researchers have reported that antioxidants added to the extender during cryopreservation interact with the phospholipids in the spermatozoon structure, protecting the cell (Rudolph et al., 1986; Aitken and Baker, 2006). In a study conducted by Varisli et al. (2009) on Sprague-Dawley and F344 rat strains, no significant difference in acrosome damage rate was found between the control and experimental groups with different extenders and cooling rates ($p>0.05$), and the acrosome damage rate was found to be higher than in the present study. In other words, in Sprague-Dawley rats, the highest acrosomal integrity was found in the control group at 96.9 ± 1.4%, while the lowest was 85.7 ± 3.3% in the 10°C cooling group. In F344 rats, the highest acrosomal integrity was 95.5 ± 1.6% in the control group, while the lowest was 84.1 ± 8.5% in the 22°C cooling group. This difference is thought to be due to the rat strain studied, the freezing protocol, the composition of the extender, and the addition of antioxidants. Additionally, in a study on rabbit sperm cryopreservation by Fadl et al. (2021), the highest acrosomal integrity was found at 77.5 ± 1.3% in the 1 mM Melatonin group. The addition of melatonin (Divar et al., 2022; Monteiro et al., 2022) or Coenzyme Q_{10} (Gardela et al., 2023; Saeed et al., 2016) to extenders in cryopreservation has been reported to preserve acrosomal integrity of spermatozoa during short-term cooling or long-term freezing.

Apoptosis, a programmed cell death mechanism, is a physiological event that is vital for spermatozoa, as it regulates cell numbers and eliminates unnecessary cells that threaten survival (Said et al., 2010). The presence of apoptotic spermatozoa is related to ejaculate quality and fertility (Anzar et al., 2002). In the present study, the best results in terms of sperm parameters after freezing and thawing were obtained from the Coenzyme Q_{10} and Melatonin groups, and these groups were evaluated for apoptotic (AV+/PI-) and necrotic (AV-/PI+) cell rates, with statistical differences found between the groups ($p<0.01$; Table 4.3).

When evaluated for apoptotic (AV+/PI-) cell rates after freezing and thawing, the lowest apoptotic cell rate was found in the 1 mM Melatonin group (4.20±2.10%), while the highest apoptotic cell rate was in the control group (28.12±1.36%), and significant statistical differences were found between all groups ($p<0.01$). Regarding necrotic cell rates, the lowest necrotic (AV-/PI+) cell rates were found in the 1 mM Melatonin (21.10±4.07%) and 25 µM Coenzyme Q_{10} (21.59±2.00%) groups, while the highest necrotic cell rate was in the control group (33.53±2.47%) ($p<0.05$). These values are consistent with the routine spermatozoon examination values (motility, viability, etc.). Thus, it is thought that the addition of Melatonin (1 mM) and Coenzyme Q_{10} (25 µM) at appropriate doses to the CPA protected the sperm cell from cryo-oxidative damage. In the present study, the apoptotic and necrotic cell rates determined through the beneficial effects of Melatonin and Coenzyme Q_{10} were lower than those in the control group ($P<0.01$).

Melatonin, in addition to acting as a hormone, is a potent scavenger of metabolites and free radicals and is known to be protective against oxidative damage (Sun et al., 2020). Due to its antioxidant properties, melatonin is commonly used in extenders to protect spermatozoa of various species from ROS-induced damage during freezing and thawing (Ofosu et al., 2021). A study on fish found that the addition of 1 µM melatonin had a significant effect on the percentage of apoptotic sperm (Yang et al., 2023). Chaudhary et al. (2021) reported that the addition of 1 mM melatonin to the extender during buffalo sperm cryopreservation could increase spermatozoon motility and viability and reduce the number of apoptotic sperm in frozen-thawed spermatozoa ($p<0.01$). Perumal et al. (2018) reported that the addition of 3 mM melatonin to the extender during

bull sperm freezing protected spermatozoa with a lower percentage of apoptotic sperm compared to the control group ($P<0.05$). These results are consistent with those of the present study, indicating that melatonin is a powerful antioxidant that protects spermatozoa from ROS damage.

As a sign of apoptotic-like changes, the translocation of phosphatidylserine from the inner to the outer side of the plasma membrane increases during the cryopreservation and thawing process. The addition of Coenzyme Q_{10} at optimal concentrations to the cryopreservation medium has been reported to reduce apoptotic-like changes due to its ability to stabilize the plasma membrane (Masoudi et al., 2018). In the present study, similar findings were observed, where melatonin and coenzyme Q_{10} reduced apoptotic sperm cell rates during sperm cryopreservation. These findings have been reported by several researchers in different animal species, including fish (Ruan et al., 2024, Yang et al., 2021), deer (Khazravi et al., 2024), sheep (Asadzadeh et al., 2021, Masoudi et al., 2019b), rooster (Alipour-Jenaghard et al., 2023), and bull (Elkhawagah et al., 2024, Chaudhary et al., 2021).

CONCLUSIONS

It was found that the addition of 1 mM Melatonin and 25 μM Coenzyme Q_{10} to the extender during rat sperm cryopreservation increased motility, viability, plasma membrane integrity, and motility endurance after freezing and thawing, and protected the cells against apoptosis and necrosis. Thus, it was determined that the above-mentioned beneficial concentrations of Melatonin and Coenzyme Q_{10} can be successfully used in rat sperm cryopreservation protocols.

Author's Contribution

F.Y.D. and C.Y. designed the study, conducted the experiments, performed the analyses, and drafted the initial manuscript. They then carried out the final revisions of the draft. All authors equally contributed to the interpretation of the results.

Author Confirmation Statement

Prof. C.Y., Yalova University, Faculty of Medicine, Department of Medical Biology, Yalova, TURKEY; Dr. F.Y.D., Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, Hatay, TURKEY. This study is the PhD thesis of one of the authors, Firdevs Yilmaz-Dayanc.

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Author Disclosure Statement

All authors declare that they have no conflicts of interest.

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