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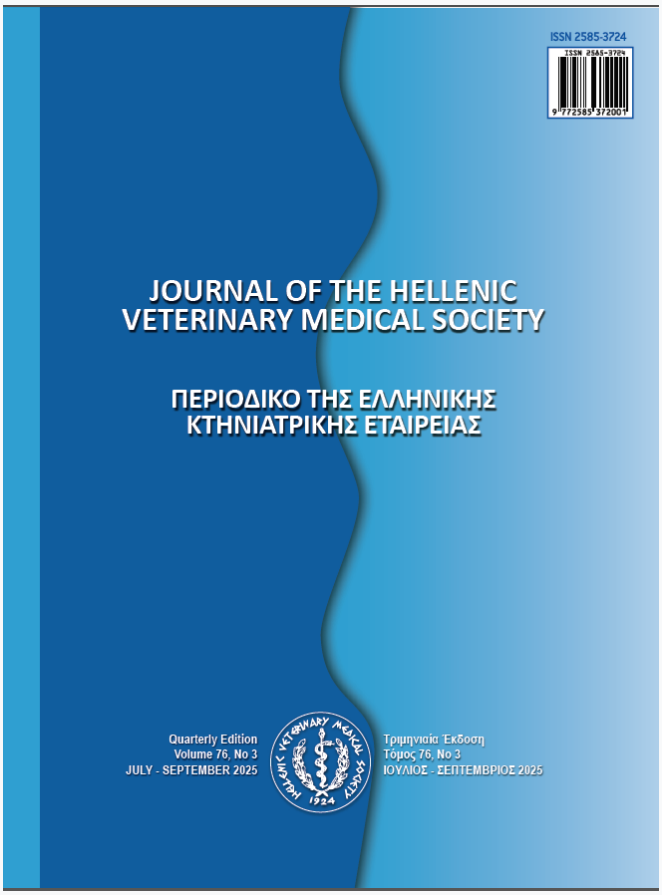

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Effects of L-Glutathione and Alpha-Tocopherol Added to Extenders on Sperm Quality in Cryopreservation of Epididymal Rat Spermatozoa

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ABSTRACT: Freezing-thawing processes are believed to be a cause of stress associated with increased reactive oxygen species (ROS) generation, causing cell damage owing to oxidative stress. Glutathione is one of the important endogenous antioxidants present in all mammalian tissues. Alpha-tocopherol is a natural non-enzymatic antioxidant that protects sperm cells from ROS damage and prevents lipid peroxidation. In this study, the effects of L-glutathione (2.5, 5, and 10 mM) and alpha-tocopherol (0.1, 0.2, and 0.5 mM) at different doses added to 8% lactose + 23% egg yolk + 10% tris solution sperm extender on the freezing of rat spermatozoa liquid nitrogen were investigated as antioxidants. Spermatological analyses of the samples were performed after the frozen straw were thawed in a 37°C water bath for 30 seconds. Compared with the control group after freezing and thawing, the highest motility was obtained in the 0.1 mM alpha-tocopherol (23.89 ± 1.82%) and 2.5 mM L-glutathione (28.00 ± 2.71%) groups ($p<0.01$). No significant difference was found between groups in terms of the rate of dead spermatozoa and abnormal acrosomes ($p>0.01$). Compared with the control group, the highest plasma membrane integrity was found in the 0.1 mM alpha-tocopherol and 2.5 mM L-glutathione groups ($p<0.01$). The highest motility values for spermatozoa endurance were determined in the 0.1 mM alpha-tocopherol and 2.5 mM L-glutathione groups compared to the control group ($p<0.01$). The rate of apoptotic and necrotic cells was lowest in the 0.1 mM alpha-tocopherol and 2.5 mM L-glutathione groups compared to the control group ($p<0.01$). As a result, it was determined that the addition of 0.1 mM alpha-tocopherol or 2.5 mM L-glutathione to the spermatozoa extender increased the sperm motility, functional plasma membrane integrity and motility endurance and decreased the evaluation apoptosis after freezing-thawing, and thus could be used successfully in the freezing extender of rat spermatozoa.

Keyword: Rat Sperm; Cryopreservation; Antioxidant; Alpha-Tocopherol; L-Glutathione.

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INTRODUCTION

Laboratory rats are considered one of the most valuable laboratory animals for biomedical and genomic research. Today, genome editing technology has facilitated the production of genetically modified mutant rats worldwide. With the production of many mutant or transgenic rat strains in the last decade (Meek et al., 2017), the demand for the conservation and production of rat strain resources has increased (Takeo et al., 2022). Rats are a preferred model for physiological manipulations in many experimental studies compared to mice due to their larger size (Abbott, 2004).

Sperm storage protocols vary among animal species due to the different natural sperm characteristics of each species (Barbas and Mascarenhas, 2009). Morphologically, rat sperm is extremely sensitive to suboptimal conditions compared to domestic animal sperm due to its longer tail, shape and size of the head, and membrane components (Hammerstedt et al., 1990; Nakatsukasa et al., 2001). Rat sperm is very sensitive to cold shock. The optimal liquid sperm storage temperature for most species is 4–5 °C, but for rats, storage of sperm at room temperature is an alternative method (Varisli et al., 2013). In addition, rat sperm is extremely sensitive to some environmental changes such as centrifugation, straw-feeding (Nakatsukasa et al. 2003), temperature, pH, viscosity, osmotic stress (Chularatnatol, 1982; Si et al., 2006), and freezing-thawing rate (Niwa and Chang, 1974).

Published studies in many species have shown that reactive oxygen species (ROS) are continuously produced due to cellular respiratory metabolism during cryopreservation stages and that these have important effects on the success of cryosurvival (Kobayashi et al., 1991; Gavella and Lipovac, 1992). A certain level of ROS is necessary for many fertility parameters such as spermatozoa maturation, motility, acrosome reaction, sperm capacitation, mitochondrial stability, hyperactivation and sperm-oocyte fusion (Griveau and Le Lannou, 1997; Wright et al., 2014; Bui et al., 2018). High ROS concentrations can initiate many pathological processes in the male reproductive system by damaging sperm concentration, motility, plasma membrane structure and reducing sperm fertilization, thus negatively affecting fertility (Aziz et al., 2004; Yumura et al., 2009). Therefore, the addition of antioxidants to the extender for protective purposes has been accepted as an important and successful way for sperm cryopreservation in many species.

Glutathione (GSH) is a thiol-containing tripeptide compound structurally composed of the amino acids cysteine, glycine, and glutamine (Jefferies et al., 2003). It is an important endogenous antioxidant in animals. GSH is γ-L-glutamyl-L-cysteinylglycine, which is found in all mammalian tissues at concentrations of 1–10 mM (highest in the liver) (Lu, 2013). GSH has a reducing power that protects sperm cells from oxidative stress.

Alpha-tocopherol is one of the natural antioxidants that disrupts the activity of free radicals in sperm, prevents lipid peroxide production (Capasso, 2013) and protects sperm cells against ROS damage. It is a strong (fat-soluble) antioxidant that accumulates especially in mitochondrial and microsome membranes (Aziz et al., 2004). It has an important role in spermatogenesis, spermatozoa maturation (Kurutas, 2016), maintenance and survival of spermatids (Zubair, 2017). It also plays an active role in the protection of testes against oxidative damage thanks to its ability to bind to lipid peroxyl radicals before they damage the lipid membrane of the cells. In addition, alpha-tocopherol is a non-enzymatic testicular defense system in mitochondria and spermatozoa and can inhibit peroxidation of testicular damage (Malmir et al., 2021; Wurlina et al., 2022).

However, unlike many species, limited reports have been published on cryopreservation of rat sperm, and the desired level of success and acceptable results have not yet been achieved. The first successful report on cryopreservation of rat sperm was published in 2001 (Nakatsukasa et al., 2003; Yamashiro et al., 2008; Kim et al., 2012; Nakata et al., 2012; Fujiwara et al., 2017). Viable pups have been successfully obtained using cryopreserved epididymal rat sperm in intrauterine insemination, albeit at a lower rate than fresh spermatozoa (Nakatsukasa et al., 2001). Recently, Nakagata et al. (2020) reported a limited success rat sperm cryopreservation protocol that achieved high fertilization rates via in vitro fertilization (IVF) using frozen-thawed sperm. Since a complete protocol has not been developed, studies in this area are still ongoing. To date, no studies have been identified on the use of GSH and alpha-tocopherol in the cryopreservation of rat sperm. Therefore, the aim of this study was to test the effects of adding different concentrations of GSH and alpha-tocopherol to the extender during the cryopreservation of rat sperm on sperm quality after freezing and thawing.

MATERIALS AND METHODS

Animals

In the study, 56 healthy male *Wistar-Albino* rat 12-16 weeks of age and with strong sexual desire were used. Rat were held at room temperature (24±3°C), 60% relative humidity, 12 hours of light and 12 hours of darkness. Standard care and feeding of laboratory animals were performed. Feed and water were given ad libitum. Rats were divided into 7 groups, one control and six study groups (n = 8).

Chemicals

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human Tubal Fluid (HTF) and were prepared according to Quinn et al. (1985).

Cryoprotectant Agents and Research Groups

Sperm extender was prepared according to the formula reported by Nakatsukasa et al. (2001). Accordingly, the diluent mixture of 8% lactose (1.08386, Sigma), 23% egg yolk, 1000U/mL penicillin G and 1mg/mL streptomycin sulphate was dissolved thoroughly by completing it to 100 ml with distilled water. The prepared mixture was centrifuged at 1600 x g for 15 minutes, and the obtained supernatant was filtered through a membrane filter (Millipore, USA). After centrifugation, 0.1% adenosine 5′-triphosphate (ATP, Sigma) was added to the mixture and the solution was thoroughly homogenized with the help of a stir plate. Different concentrations of L-glutathione (2.5 mM, 5 mM and 10 mM) (G4251, Sigma) and alpha-tocopherol (0.1 mM, 0.2 mM and 0.5 mM) (T3251, Sigma) were added to the obtained supernatant and solutions for the study groups were prepared separately. Then, the pH of each study group was adjusted to 7.4 by adding 10% tris aminomethane to the mixtures and measuring with a pH meter (Thermo Scientific 1112000, USA). The group containing 8% lactose + 23% egg yolk, to which L-glutathione and alpha-tocopherol were not added, was kept as the control group.

Collection and Dilution of Spermatozoa

To collect sperm used in the study, rats were sacrificed by cervical dislocation method after sedation under anesthesia with a ketamine/xylazine mixture. The abdominal region was cleaned with 80% ethyl alcohol in a laminar flow cabinet and the urogenital region was reached with surgical methods under sterile conditions. The testicles and the cauda epididymis (left and right) were aseptically removed and cleaned of fat tissue. The cauda epididymis was taken separately into each in a 35 mm culture dish containing 1 mL of sperm extender prepared for the control and study groups and at room temperature. In order for the spermatozoa in the epididymal tissue to come out and pass into the extender, ten-twelve deep incisions were made in the caudas using toothless forceps and sharp-tipped scissors under a stereo microscope (Euromex/Nexius Zoom, Netherlands). Then, 10 microliters of the sperm sample was taken into an micro centrifuge tube and diluted 1/10 with HTF medium in the incubator for pre-freezing spermatozoa examination and and kept at 37 °C for 10 minutes. Then, 10 µL of the semen sample in the eppendorf tube was taken, placed on a Makler counting chamber at 37°C, and sperm concentration and motility were examined with the 20X objective of a phase-contrast microscope (Olympus, CX21, Japan).

Spermatozoa Freezing and Thawing

The protocol for cryopreservation was performed by modifying the protocol reported by Nakagata et al. (2020). Sperm samples, whose microscopic examination was completed, were divided into 7 different groups and diluted with cryoprotectant extender with different antioxidant concentrations in a 35 mm culture dish. The culture dish was then left to equilibrate for 10 minutes on a thin metal plate placed on crushed ice in a styrofoam box. Each sample was processed and spermatological analyses were performed separately. Samples with a concentration of 50-100x10^6/mL and sperm motility above 70% were only frozen. After equilibration was completed, sperm were loaded (150 µL) into 0.25 mL straws (IMV, France) and the straws end were sealed with a hot press (Impulse sealer FS-200). Thus, the straws were loaded into concentrations 7-10x10^6/mL.

The straws were loaded with 30 µL HTF, 10 mm air, 150 µL sperm, 10 mm air and 30 µL HTF at 4°C, respectively, thus preventing the straws from floating in liquid nitrogen and the sperm in the straw from coming into contact with the cotton plugged part. Then, the loaded straws were left to equilibration on a metal plate on crushed ice in a styrofoam box for 30 minutes. At the end of the period, the straws were placed horizontally to a platform set at 4 cm above the liquid nitrogen level in a styrofoam box and left to equilibration for 10 minutes for pre-freezing. Thus, according to the freezing procedure, the temperature was reduced from 4°C to -140°C. Then, the straws were plunged into liquid nitrogen to complete the freezing process. The frozen straws were stored

in a liquid nitrogen container until spermatological analyses were performed.

Sperm concentration, sperm motility, dead sperm rate, abnormal acrosome rate, plasma membrane integrity, motility endurance and cellular apoptosis rates were tested in thawed straw samples. For these tests, the straws were thawed in a water bath at 37°C (decongelator (device for thawing frozen sperm straws), CITO 026897, IMV, France) for 30 seconds. Then, the thawed sperm samples were quickly transferred into 1.5 mL eppendorf tubes kept in an incubator with 5% CO_2 at 37 °C for 30 minutes and diluted (1:4) with HTF medium. Thawed sperm samples were incubated at 37 °C and 5% CO_2 for 10 minutes and then spermatological evaluations were performed.

Spermatozoa Analysis

Evaluation of Sperm Motility. The motility of sperm samples was determined using a Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel) according to the World Health Organization (WHO) (1999) guidelines. After incubation, 10 µL of the sperm samples diluted with HTF was taken and placed on a Makler counting chamber heated to 37°C in an incubator and examined with a 20X objective of a phase-contrast microscope. The numbers of motile spermatozoa with strong, smooth and linear forward movements and those that were non-motile spermatozoa were recorded in the counting area. This process was repeated 3 times and the motility value was calculated as a percentage (%).

Evaluation of Dead Sperm Rate. The eosin-nigrosin staining method was performed according to Agarwal et al. (2016). Accordingly; 10 µL of sperm sample, 20 µL of Eosin (1%) stain and 20 µL of Nigrosin (10%) stain were added on a glass slide and mixed, then sperm smears were spread and allowed to dry quickly on a hot plate (40°C). Then, 300 spermatozoa were examined with the 40X objective of a phase-contrast microscope. 300 spermatozoa in each sample were evaluated, and spermatozoa whose heads did not receive dye were considered as alive, and those whose heads received red-pink dye were considered as dead, and the ratio was determined as a percentage (%).

Evaluation of Hypo-Osmotic Swelling Test. Hypo-osmotic swelling test (HOST) was performed by modifying the formula of Jeyendran et al. (1984). Accordingly; HOST solution with low osmotic pressure of 100 mOsm was prepared by dissolving 1.1 g fructose and 0.55 g sodium citrate in 100 mL distilled water, and its osmotic pressure was measured with an osmometer (Osmomat 3000, Gonotec, USA). 100 µL HOST solution was taken and placed in eppendorf tubes labeled according to the study groups and kept in the incubator (37°C). Then, 10 µL of the incubated with HTF medium sperm samples were added in tubes and incubated for 30 minutes. After incubation, twenty µL of sperm samples were placed onto a heated (37 °C) slide and the coverslip was closed and examined with a 40X objective of a phase-contrast microscope. Spermatozoa were classified according to the tail and head shape as swollen (HOST-positive) or non-swollen (HOST-negative). HOST-positive spermatozoa (those with curled tails) were evaluated as having intact plasma membranes. For the quantification of sperm plasma membrane integrity 200 spermatozoa in each sample were evaluated and was determined as a percentage (%) of HOST-positive spermatozoa.

Evaluation of Sperm Motility Endurance. After the straws were thawed, the sperm samples reconstituted with HTF in eppendorf tubes and placed in an incubator (37°C). Motility examinations of sperm samples incubated were performed four times at thirty-minute intervals, starting from zero minutes. Ten µL of the incubated sperm samples were taken on the Makler counting chamber heated to 37°C in an incubator and counting was performed using the 20X objective of a phase-contrast microscope. Motility values were calculated as percentages (%), by dividing the ratio of forward, linear, strong moving sperms and sperms showing other forms of movement (nonmotile, circular, trembling) in the entire examined area. Thus, the motility endurance periods of sperm samples were determined.

Evaluation of Abnormal Acrosome. The evaluation of acrosome integrity was performed according to the method reported by Somfai et al. (2002). Accordingly, 500 µL of the prepared fixation solution (Hancock) was taken into an 1.5 mL eppendorf tubes, 1-2 drops of the thawed sperm sample was added to it, and the spermatozoa were fixed (Hancock, 1952). Then, fifty µL of this mixture was placed on the glass slide and the acrosome regions of the spermatozoon were examined using immersion oil with the help of the 100X objective of the phase-contrast microscope. For the quantification of sperm abnormal acrosome, a total of 300 sperms from each sample were scored and the value was expressed as a percentage (%).

Evaluation of Apoptotic Spermatozoa. Apoptosis

examination was performed on the control group and the groups in terms of sperm parameters that provided the best values (2.5 mM L-glutathione and 0.1 mM alpha-tocopherol). Elabscience, E-CK-A211 kit was used as an Annexin V-FITC conjugated/PI staining method to determine the apoptosis rate. Staining was performed according to the manufacturer's directives and examined under an epifluorescence microscope. Spermatozoa with completely red stained heads were necrotic cells (Annexin V-FITC-/PI+), those with completely green stained were considered apoptotic cells (Annexin V-FITC+/PI-) (Fig. 1).

Statistical Analysis

All data are expressed as mean ± standard deviation. SPSS22.0 was used for statistical analysis. One-way analysis of variance (ANOVA) was used to detect differences and Duncan's multiple range test was used to indicate the significance of differences between groups. Differences were considered statistically significant at $p < 0.01$.

RESULTS

Spermatologic Values After Freezing and Thawing

The sperm motility, dead sperm rate, HOST test positive and abnormal acrosome rates after freezing and thawing in the study groups are given in Table 1.

Effects of Different Concentrations of L-Glutathione and Alpha-Tocopherol on Sperm Motility Values

The highest motility value was recorded in fresh semen (72.77±1.09%). Compared to the control group after cryopreservation (12.50±1.36%), the highest motility values were detected in the 0.1 mM alpha-tocopherol group (23.89±1.82%) and the 2.5 mM L-glutathione group (28.00±2.71%) ($p<0.01$). When all groups were compared among themselves to among all groups, the lowest motility value (12.50±1.36%) was observed in the control group ($p<0.01$). When the samples with 2.5 mM

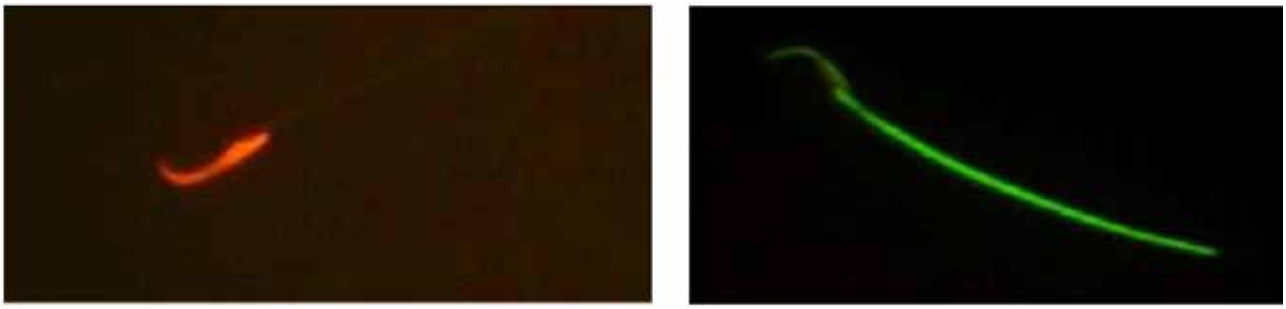

Figure 1. Spermatozoa with completely red heads were necrotic spermatozoon (A) (AV−/PI+) and spermatozoa with completely green were apoptotic spermatozoon (B) (AV+/PI−) in cellular apoptosis.

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

Groups	Sperm Motility (%)	Dead Sperm Rate (%)	HOST Test Positive Rate (%)	Abnormal Acrosome Rate (%)
Fresh	72.77±1.09^{a}	21.68±1.25^{b}	73.22±1.88^{a}	3.67±0.45^{a}
Control	12.50±1.36^{d}	72.67±2.48^{a}	22.39±2.00^{c}	4.83±0.61^{a}
Alpha-Tocopherol 0.1mM	23.89±1.82^{bc}	71.44±1.66^{a}	27.78±2.39^{b}	4.67±0.47^{a}
Alpha-Tocopherol 0.2mM	19.38±1.99^{c}	72.88±2.43^{a}	24.75±1.53^{bc}	3.38±0.42^{a}
Alpha-Tocopherol 0.5mM	18.18±1.69^{c}	73.36±1.81^{a}	24.27±1.47^{bc}	3.18±0.35^{a}
L-Glutathione 2.5mM	28.00±2.71^{b}	70.70±2.52^{a}	31.40±1.12^{b}	4.30±0.58^{a}
L-Glutathione 5mM	23.13±2.66^{bc}	69.63±2.61^{a}	29.50±1.59^{b}	4.25±0.59^{a}
L-Glutathione 10mM	23.75±2.46^{bc}	70.13±4.21^{a}	26.00±1.32^{bc}	3.63±0.32^{a}

$^{a-d}$: Within a line, means without a common superscript differed ($p<0.01$).

L-glutathione and 0.1 mM alpha-tocopherol were compared with the control group in terms of motility, the difference between them was found to be statistically significant ($p<0.01$).

Effects of Different Concentrations of L-Glutathione and Alpha-Tocopherol on Dead Sperm Rate

After freezing and thawing, no statistically significant difference was found between the control, alpha-tocopherol and L-glutathione added groups as seen in Table 1 ($p>0.01$).

Effects of Different Concentrations of L-Glutathione and Alpha-Tocopherol on HOST Test Positive Rate (Sperm Plasma Membrane Integrity)

Analysis of the data in Table 1 shows that the plasma membrane integrity of sperm in samples added with 2.5 mM L-glutathione (31.40±1.12%) and 0.1 mM alpha-tocopherol (27.78±2.39%) after freezing and thawing was significantly higher than the control group ($p<0.01$).

Effects of Different Concentrations of L-Glutathione and Alpha-Tocopherol on Abnormal Acrosome Ratio

As seen in Table 1, the abnormal acrosome ratio of sperm in samples added with alpha-tocopherol and L-glutathione after freezing and thawing was not significantly different from those in the control group ($p>0.01$).

Effects of Different Concentrations of L-Glutathione and Alpha-Tocopherol on Sperm Motility Endurance Values

Sperm motility endurance values after freezing and thawing are given in Table 2. As seen in Table 2, at the zero minute, the motility endurance values of sperm in the samples added with 0.1 mM alpha-tocopherol (23.89±1.82%) and 2.5 mM L-glutathione (28.00±2.71%) were found to be significantly higher than those in the control group (12.50±1.36%) ($p<0.01$).

Analysis of the data in Table 2 shows that at the ninetieth minute, the motility endurance parameters of sperm in the samples added with 0.1 mM alpha-tocopherol (4.44±1.00%) and 2.5 mM L-glutathione (3.50±0.76%) were found to be significantly higher than those in the control group (0.56±0.38%) ($p<0.01$).

Effects of Different Concentrations of L-Glutathione and Alpha-Tocopherol on Cell Apoptosis Examination

Cell apoptosis rates obtained from the study groups after freezing and thawing are given in Table 3. As

Table 2. Time-dependent motility endurance values in the study groups (n = 8)

	Sperm Motility Endurance Values (%)			
Groups	**0 minute**	**30 minute**	**60 minute**	**90 minute**
Control	12.50±1.36^{c}	6.67±1.14	3.33±0.57	0.56±0.38^{c}
Alpha-Tocopherol 0.1mM	23.89±1.82^{ab}	11.11±1.39	7.78±1.47	4.44±1.00^{a}
Alpha-Tocopherol 0.2mM	19.38±1.99^{b}	10.00±1.34	6.88±0.92	3.13±0.92^{abc}
Alpha-Tocopherol 0.5mM	18.18±1.69^{bc}	9.55±1.42	5.46±1.06	1.82±0.76^{abc}
L-Glutathione 2.5mM	28.00±2.71^{a}	11.00±1.45	5.50±1.38	3.50±0.76^{ab}
L-Glutathione 5mM	23.13±2.66^{ab}	10.63±1.75	6.25±2.06	1.50±1.57^{ab}
L-Glutathione 10mM	23.75±2.46^{ab}	11.88±1.88	6.25±1.25	1.88±0.92^{abc}

$^{a-c}$: Within a line, means without a common superscript differed ($p<0.01$).

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

Groups	Necrosis (AV−/PI+)	Apoptosis (AV+/PI−)
Control	35.49±3.22^{a}	37.82±3.79^{a}
Alpha-Tocopherol 0.1mM	13.77±2.99^{b}	25.03±3.86^{b}
L-Glutathione 2.5mM	11.98±4.28^{b}	23.98±2.03^{b}

$^{a-b}$: Within a line, means without a common superscript differed ($p<0.01$).

seen in Table 3, the highest rate of red-stained necrotic cells was found in the control group (35.49±3.22%). In terms of necrotic cells, the results of the control group were found to be statistically significantly higher than the results in the sperm samples added with 0.1 mM alpha-tocopherol (13.77±2.99%) and 2.5 mM L-glutathione (11.98±4.28%) ($p<0.01$).

As seen in Table 3, the highest rate of green-stained apoptotic cells after freezing-thawing was found in the control group (37.82±3.79%). In terms of apoptotic cells, the results of the control group were found to be statistically significantly higher than the results of the sperm samples added with 0.1 mM alpha-tocopherol (25.03±3.86%) and 2.5 mM L-glutathione (23.98±2.03%) ($p<0.01$).

DISCUSSION

Cryoprotectants and antioxidants are used as preservatives in freezing solutions to reduce cell damage and death, since they cause free oxygen radicals as a result of physical, chemical and oxidative stress during the cooling, cryopreservation or thawing of sperm cells, cold shock, intracellular crystal formation, cellular osmotic change, and lipid peroxidation in membranes. Additionally, antioxidants have been reported to have protective effects against DNA and gene damage during cryopreservation, thus preventing early embryonic deaths (Ball et al., 2001; Sieme et al., 2016; Silvestre et al., 2021). Cryoprotectants show their protective effects by increasing the unfrozen fraction in the medium, providing osmotic balance, and reducing the amount of ions (Polge et al. 1949).

Sperm motility, cellular viability, acrosomal integrity and mitochondrial function assessment are the criteria that allow a more accurate determination of the fertility capacity of spermatozoa (Graham, 2001). Therefore, all sperm parameters should be taken into account in order to evaluate the fertility capacity of sperm properly. Post-thawing sperm motility is one of the important criteria of cryopreservation and fertilization and is closely related to reproductive efficiency. As in every species, since rat sperm has its own cryobiological properties, it is important to develop freezing protocols specific to this species. In this study, the effects of L-glutathione and alpha-tocopherol were tested for the first time in the reconstitution of rat sperm. For this purpose, the protective effects of adding different amounts of L-glutathione and alpha-tocopherol to the cryopreservation extender on sperm cells were investigated. L-glutathione and alpha-tocopherol are two important antioxidants with protective properties in the cellular defense system in the field of sperm cryopreservation. Additionally, antioxidants such as coenzyme Q_{10} (CoQ_{10}), can be added to the freezing medium to protect sperm cells (Eraslan-Sakar et al., 2025). In this study where rat sperm was frozen and stored, L-glutathione (2.5 mM) and alpha-tocopherol (0.1 mM) added to the cryopreservation extender provided better results than the control group in terms of motility, plasma membrane integrity and motility endurance, which are important vitality criteria of sperm after freezing and thawing ($p<0.01$).

Cryopreservation of rat sperm is quite difficult compared to other species. A limited number of studies have been reported on this subject. Yamashiro et al. (2008), found the motility as 39.3% after cryopreservation of *Wistar* rat sperm in Krebs-Ringer bicarbonate (mKRB) containing 0.1 M raffinose, 0.75% Equex STM and 20% egg yolk. In our study, the highest motility values after cryopreservation were found in the 0.1 mM alpha-tocopherol (23.89%) and 2.5 mM L-glutathione (28.00%) groups. It was found that cryopreservation with the tested sperm extender medium significantly preserved sperm motility for *Wistar-Albino* strains compared to the control group ($p<0.01$). The sperm motility and plasma membrane integrity results obtained in the study were found to be better than the motility (4.6±0.1%) and plasma membrane integrity (9.2±3.9%) rates obtained after cryopreservation in *Sprague Dawley* male rats by Nakatsukasa et al. (2001), who also worked on rat sperm. In another study by Nakatsukasa et al. (2003); motility rates ranging from 2.0% to 12.3% were obtained by cryopreservation of epididymal sperms of different rat strains. In the present study, the motility (28.00±2.71%) and intact membrane integrity (31.40±1.12%) of sperms were found to be better than the results found by Nakatsukasa et al. (2001, 2003). These differences in sperm parameters may be due to the differences in the antioxidant substance used, the use of different methods in sperm analysis (we acknowledge that the subjective assessment of sperm motility constitutes is a limitation of our study), the selected rat strain, the packaging method of diluted sperms, the cooling rate used (Agca et al. 2002), the lack of ideal sperm extender components (Aisen et al., 2005) and suboptimal sperm manipulations during the cryopreservation procedure.

Glutathione used in the study is critical for the existence, continuity and reproductive system of healthy cells. Glutathione has strong antioxidant and free radical scavenging abilities in animals.

The results we determined on viability parameters in this study show that the addition of glutathione to the sperm freezing medium helps improve the antioxidant capacity of sperm. Glutathione, which shows its antioxidative effect by directly neutralizing ROS or by keeping other antioxidants such as Vitamin E and C in active form (Silvestre et al., 2021), has been successfully studied in the sperm. Zou et al. (2021) reported that the concentration of glutathione (2 mmol/L) added to the extender in the cryopreservation of buck semen improved the post-freezing-thawing sperm viability (62.14%), plasma membrane integrity (37.62%) and acrosome integrity (70.87%) rates. Syafitri et al. (2022) showed that the addition of glutathione (0.1 mM) as an antioxidant agent to ram semen significantly increased motility (65.35±1.80%) and membrane integrity (68.75±1.83%) ($p<0.05$). The positive spermatological effects of glutathione added to sperm extender in different races were determined in the study conducted in the 2.5 mM glutathione group added to the extender, with similar protective effects in terms of motility, plasma membrane integrity and motility durability.

Alpha-tocopherol, an important antioxidant, was added to rat sperm cryopreservation extender and tested. Sperm motility (23.89±1.82%) and plasma membrane integrity (27.78±2.39%) were found to be the highest in the group to which 0.1 mM alpha-tocopherol was added. Alpha-tocopherol is an important antioxidant vitamin frequently used in sperm cryopreservation technique. In addition, it has been reported to increase sperm viability and fertilization rate after cryopreservation (Hu et al., 2011; Pour et al., 2013). Supplementation of alpha-tocopherol to extender during cryopreservation preserves motility endurance and viability better by reducing membrane damage caused by lipid peroxidation by neutralizing ROS accumulation (Zhu et al., 2015). Jeong et al. (2009) stated that alpha-tocopherol (200 µM) added to boar sperm extender during cryopreservation had a positive effect on the survival of thawed sperm. Ata et al. (2018) reported that 100 µM alpha-tocopherol should be added to ram sperm freezing extenders because it improves motility and membrane integrity outside the breeding season. Similarly, in the presented study, it was determined that adding appropriate amounts of alpha-tocopherol (0.1 mM) to the extender had a significant protective effect in rat sperm cryopreservation ($p<0.01$).

In the presented study, no statistical difference was found between the control and study groups in terms of acrosome integrity and dead spermatozoon rate after cryopreservation ($p>0.01$). It was determined that the use of antioxidants in the freezing of rat sperm had no effect in protecting the acrosomal region against cryo-damage. Acrosome integrity is important for thawing the zona pellucida during fertilization. Because cryopreservation procedure disrupts the lipid-lipid and lipid-protein bonds in the acrosomal membrane, this situation also affects fertility (Isachenko et al., 2008).

Apoptosis is a vital event for spermatozoa as it regulates cell number and eliminates unnecessary cells (Said et al., 2010). Determination of apoptotic cell rate in spermatozoa is closely related to ejaculate quality and fertility (Anzar et al., 2002). In this context, apoptosis examinations of sperm cells were performed in the study conducted, and while the highest necrotic cell rate after cryopreservation was found in the control group (35.49±3.22%), significant statistical differences were determined between the control group and the study groups ($p<0.01$). The highest apoptotic cell rate after thawing was found in the control group (37.82±3.79%) and in addition, statistically significant differences were determined between the study groups and the control group ($p<0.01$). The determined result showed that L-glutathione (2.5 mM) and alpha-tocopherol (0.1 mM) antioxidants added to the extender at appropriate doses protected sperm cells against ROS that may occur during cryopreservation. Briefly, in the study, that 0.1 mM alpha-tocopherol and 2.5 mM L-glutathione added to the sperm freezing medium as antioxidants protected the cells better against the negative effects of cryopreservation compared to the control group.

CONCLUSION

As a result, it was determined that the addition of 0.1 mM alpha-tocopherol or 2.5 mM L-glutathione to rat semen cryopreservation extender positively affected spermatological parameters (except for apoptosis rate) after thawing. Thus, it was concluded that these antioxidants could be used successfully in rat semen freezing extender.

Authors Contributions

R.O. and C.Y. planned the study. R.O. and C.Y. participated in sperm collection, dilution, freezing and spermatological analyses. R.O. and C.Y. performed the analyses of cell necrosis and apoptosis. R.O. and C.Y. wrote the first draft of the article and reviewed

and edited the first draft of the article. All authors had equal contribution to the interpretation of results.

Data Availability Statement

All data are available from the corresponding author.

Conflict of Interest

The authors declare no conflict of interest.

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Ethics Statement

All procedures were performed by the approved Institutional Ethical Rules of Hatay Mustafa Kemal University (Ethical Approval No: E-40595970-020-162733). The study was approved by the Local Ethics Committee on Animal Experimentation of Hatay Mustafa Kemal University (date: 30.03.2022 /decision number: 2022/03-18)

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