DOI: 10.4081/aiua.2025.14101

ORIGINAL PAPER

Impact of Tryptophan and Trehalose on post-thaw sperm quality and apoptotic gene expression in Wistar Albino rats

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Summary *Background: Cryopreservation of rat sperm cells and development of new diluents are very important in biotechnology.*
Methods: In the current study, 25 mM tryptophan and 100 mM trehalose were added separately to the cryopreservation medium containing 8% lactose monohydrate, 23% egg yolk, and 10% tris aminomethane to evaluate the cryopreservation capability of rat sperms. After freeze-thawing, motility, viable spermatozoon ratio, plasma membrane integrity, abnormal acrosome ratio, and apoptotic cell ratio and levels of Bax, Bcl-2, and Caspase-3 mRNA involved in the apoptosis pathway were evaluated.
Results: The highest viability ratio was detected in the trehalose group (26.25 ± 3.10), which was significantly higher than that in the control group (19.62 ± 1.51) ($p < 0.05$). When compared to the tryptophan group (11.87 ± 3.07), plasma membrane integrity was statistically higher in the trehalose group (20.25 ± 3.65) ($p < 0.05$). The rate of apoptotic cells was lower in the trehalose group than in the control group. Then, the effect of tryptophan and trehalose, which were added to the sperm diluent, on the expression levels of genes in the apoptosis pathway was evaluated through the RT-qPCR technique. The expression of the anti-apoptotic gene Bcl-2 increased by 25 mM tryptophan and 100 mM trehalose, while the expression of the pro-apoptotic genes Bax and Caspase-3 decreased ($p < 0.01$).
Conclusions: As a result, it was determined that trehalose, a isaccharide sugar, added to the rat sperm diluent provided more effective protection against cryodamage in the cryopreservation process than tryptophan, an aromatic amino acid, according to the parameters evaluated. From a molecular biotechnology perspective, these findings lay a valuable foundation for research on the development of next generation media for sperm cryopreservation.

KEY WORDS: *Cryopreservation; Rat; Tryptophan; Trehalose; Gene expression.*

Submitted 27 June 2025; Accepted 27 July 2025

INTRODUCTION

In molecular biology and genetics, rats are used as an alternative for creating many disease models. Therefore, the use of rats as experimental animals has been expanding day by day. The increasing demand to use rats as experimental animals has created a need for the production and preservation of transgenic and wild-type rat strains. The best way to preserve these gene resources for many years is by freezing rat embryos, sperms, and somatic cells. The successful cryopreservation of rat spermatozoa and the development of new diluents are important in biotechnology. However, compared to many species, sperm cryopreservation protocols for preserving the rat strain and protocols for in vitro fertilization of oocytes with frozen sperm remain under development. Although many relevant studies have been conducted (1-4), the ultimate objective remains unachieved. Therefore, successful cryopreservation of rat sperms would provide an important resource to preserve and increase the number of genetically valuable strains for research and applications.
Factors affecting the success of sperm cryopreservation are that the head region of rat spermatozoa is shaped in a different structure than that of mammals, the tail structure is longer than that of other animal species, and the water permeability of the plasma membrane is low (3, 5, 6). Therefore, various antioxidant substances and biomolecules are supplemented to cryopreservation media for healthy storage of rat sperm. In this study, tryptophan, an aromatic amino acid, and trehalose, a disaccharide, were supplemented to the cryopreservation diluent to increase cryosurvival.
Amino acids are the building blocks of proteins and have important roles in various biological and physiological processes (7). Their antioxidant properties provide effective primary defense for the protection of sperm plasmalemma (8). Tryptophan, an essential aromatic amino acid, is the precursor of many active molecules including serotonin, melatonin, and kynurenic acid, which are antioxidants against free radicals causing oxidative stress in spermatozoa (9, 10). It is widely used in numerous research and clinical studies (9-11) and is therefore anticipated as an additive in cryopreservation protocols.
Sugars are large molecules that interact with the plasma membrane and energize the cell. They are widely used in cryopreservation solutions as they stabilize intracellular and extracellular osmotic pressure (12). Trehalose is a

disaccharide that alters membrane fluidity and is present in the phospholipid bilayer of membranes (13).
This study aimed to evaluate the effect of the addition of the amino acid tryptophan (25 mM) and disaccharide trehalose (100 mM) to cryopreservation diluent, which have not been previously studied in rats, on the long-term cryopreservation of rat sperm. Accordingly, sperm quality parameters such as motility, viability, plasma membrane integrity, acrosome morphology, and cell apoptosis ratio, and expression of certain genes involved in the apoptosis pathway (Bax, Bcl-2, and Caspase-3) were examined to develop new and effective cryoprotectant formulations needed in the field of cryobiology.

Materials and methods

Chemicals

All chemicals were purchased from *Sigma Chemical Company* (*St. Louis, MO*). *Humon Tubal Fluid* (HTF) was prepared according to the method of *Quinn et al.* (14). *Cryopreservation agent* (CPA) was prepared by modifying the method of *Nakatsukasa et al.* (6). 23% fresh chicken egg yolk, 8% lactose monohydrate, 1 mg/mL streptomycin sulfate, and 1000 iu penicillin G potassium were added into 23% fresh chicken egg yolk in pure distilled water and mixed for 5 min at +4°C. The mixture was then centrifuged at 1600 x g for 15 min. After centrifugation, the supernatant was collected, and 0.1% ATP (*Adenosine 5'-triphosphate*) was added. The resulting diluent was partitioned, and 25 mM tryptophan (*Sigma-Aldrich*) and 100 mM trehalose (*Sigma-Aldrich*) were added to form a *tryptophan* (Tryp) study group and a *trehalose* (Treh) study group. A CPA group, in which no addition was made, was used as the *control* (C) group. Lastly, the pH of the prepared study group solutions was adjusted to 7.4 with 10% tris aminomethane + distilled water solution using a pH meter (*Thermo Scientific, USA*).

Animals

In the study, 24 adult, male Wistar Albino rats aged 10-16 weeks were used. Rats were kept at room temperature (24 ± 3°C) and a relative humidity of 60% under 12 h light:12 h dark cycle. The rats were fed rat chow (pellet feed) and tap water ad libitum. All procedures were carried out with the approval (*Ethical Approval Numbered 2024/09-01*) of the *Animal Experiments Local Ethics Committee* (HADYEK) of *Hatay Mustafa Kemal University*.

Collection and freezing/thawing of spermatozoa

Rats were sacrificed by cervical dislocation under anesthesia. Under sterile conditions, both epididymides were removed, and both cauda epididymides were transferred into 1 mL CPA placed in 35 mm Petri dishes (*Nunc™, Massachusetts, USA*) at room temperature. Rat spermatozoa were reconstituted and frozen using a modified version of the protocol described by *Nakagata et al.* (3). The cauda epididymides were immobilized using flat-tipped forceps under a stereo microscope (*Euromex/Nexius Zoom, Netherlands*), and 10-12 deep incisions were made in the Petri dish using sharp-tipped scissors, and the spermatozoa were transferred into CPA. For the release of spermatozoa from the cauda and equilibration, the Petri dishes were placed on a metal plate on ice in a styrofoam box and incubated for 10 min. After incubation, 0.25 mL straws (*IMV, France*) were loaded with 30 µL HTF, 10 mm air, 150 µL sperm suspension, 10 mm air, and 30 µL HTF, respectively, and the straws were pressed. The straws were placed on a metal plate on ice and equilibrated for 30 min. The equipment used during the procedure (syringe connector, straws, and HTF) was set at a temperature of 0°C. For freezing, the straws were placed in a styrofoam box (270x220x270 mm) 4 cm above the 3.5-cm liquid nitrogen level on a straw rack and kept in liquid nitrogen vapor for 10 min. The straws were then dipped in liquid nitrogen. Thawing was performed by dissolving the straws in a 37°C water bath (*deconjelator, CITO 026897, IMV, France*) for 30 sec. The content of the thawed straws was transferred to 1 mL HTF in a carbon dioxide incubator (37°C, 5% CO_2).

Assessment of sperm concentration

Sperm density was determined using a Makler counting chamber (*Sefi-Medical Instruments, Haifa, Israel*) following the guidelines of the World Health Organization (15). Sperm samples were diluted 1:10 with *phosphate buffered saline* (PBS) fixation medium containing 10 mM formaldehyde and immobilized. From the fixed mixture, 10 µL was taken and the cells in 10 frames were counted under a 20X objective in Olympus CX31 (*Japan*) microscope and the density was calculated in millions/mL. Each sample was measured three times, and the results were averaged.

Assessment of sperm motility

Sperm motility was analyzed using a Makler counting chamber (*Sefi-Medical Instruments, Haifa, Israel*) according to the guidelines of WHO (15). Briefly, 20 µL of HTF and sperm mixture was taken and placed on a Makler slide preheated to 37°C. Samples were examined under a 20X objective in a phase contrast microscope with a heating stage (*Olympus CX31, Japan*). Within 100 small squares on the Makler slide, the ratio of spermatozoa showing linear forward movement to spermatozoa with other types of movement (tremor, rotation, or immobility) was calculated. Measurement was repeated three times for each sample, and the results were averaged. Sperm motility was expressed as a percentage (%).

Assessment of sperm viability

Sperm viability was analyzed using the eosin-nigrosin staining method described by *Agarwal et al.* (16). 10 µL of sperm samples were taken and mixed with 20 µL of 1% eosin and 10% nigrosin solutions. The mixture was swabbed onto slides and dried on a surface at 60°C for 2-3 sec. Analyses were performed under a phase contrast microscope (*Olympus CX31, Japan*) and a 40X objective by counting at least 300 spermatozoa. Spermatozoa with red or dark pink heads were classified as non-viable, while those with unstained heads were considered viable. Viability ratio was calculated as percentage (%).

Assessment of sperm plasma membrane integrity

Hypo-Osmotic Swelling Test (HOST) described by *Jeyendran*

et al. (17) was performed to evaluate the functional stability of the plasma membrane integrity of spermatozoa. 100 µL of HOST solution (1.1 g fructose + 0.55 g sodium citrate + 100 mL distilled water), of which osmotic pressure was measured with an osmometer (*Osmomat 3000, Gonotec, USA*), and 10 µL of the sperm sample were transferred into a 1.5 mL Eppendorf tube. The mixture was kept in an incubator at 37°C for 45 min. After incubation, the samples were examined on a slide preheated to 37°C under a phase contrast microscope (*Olympus CX31, Japan*) and a 40X objective. Spermatozoa with swelling or curling in the tail were categorized as HOST positive (+) and those without any morphological change were categorized as HOST negative (-). From each sample, a total of 200 spermatozoa were counted, and the ratio of HOST positive spermatozoa was recorded as percentage (%).

Acrosome assessment

Abnormal acrosome examination was performed based on the method described by *Somfai et al.* (18). Hancock's solution (19) was used in the examination. 500 µL of Hancock's solution was transferred into an Eppendorf tube, and 50 µL of sperm sample was added. 50 µL of the mixture was placed on a slide and covered with a coverslip. Morphological evaluation of the slides was performed under a microscope and a 100X objective using immersion oil (20). A total of 300 spermatozoa were examined, and the ratio of spermatozoa with acrosome damage was noted as percentage (%).

Assessment of apoptosis

Annexin-V FITC/PI apoptosis kit (*Elabscience, E-CK-A211*) was used for apoptosis examination. Kit and staining procedures were performed according to the instructions of the manufacturer. Apoptotic cells were detected with Annexin V-FITC stain, while DNA of necrotic cells with impaired membrane integrity was stained with *Propidium Iodide* (PI). The slides prepared for analysis were examined under a fluorescence microscope (*Eclipse Ni, Nikon*) and 20X and 40X objectives using FITC/PI filters (excitation wavelength of 490 nm, emission wavelength of 520 nm). Cells were classified as follows: *Annexin V-FITC positive* (AV+) and *Propidium Iodide negative* (PI-) cells (green) were defined as apoptotic cells. A total of 200 cells were counted, and apoptosis ratios were recorded as percentage (%).

Total RNA isolation

Sperm suspension were washed with PBS and centrifuged at 150 × g for 10 minutes at 4°C. To eliminate somatic cell contamination, the pellets were treated with a somatic cell lysis buffer (0.05% sodium dodecyl sulfate and 0.25% Triton X-100 in distilled water) and incubated on ice for 60 minutes. Total RNA was extracted using TRIzol reagent (*TRIzol® Reagent, Ambion*) following the manufacturer's protocol. Carefully dissected semen sample was submerged in 1 mL of TRIzol reagent and homogenized for 45 seconds with a homogenizer. The homogenate was incubated for 5 minutes and added 200 µL chloroform; shaken vigorously. The mixture was centrifuged at 12000 × g at 4°C for 15 minutes. Following this centrifugation step, the upper aqueous layer was transferred to another nuclease-free 1.5 mL tube and added 500 µL of isopropanol; shaken vigorously. The sample was centrifuged at 12000 × g at 4°C for 10 minutes to pellet the precipitated RNA. The RNA-containing pellet was washed twice with 500 µL of 70% ethanol and centrifuged at 7500 × g at 4°C for 5 minutes. Final RNA pellet was washed with 500 µL of 99% ethanol and centrifuged at 7500 × g at 4°C for 5 minutes. The RNA-containing pellet was air-dried and dissolved in 30 mL of nuclease-free water. The RNA samples were stored at -80ºC for downstream application later. The concentration and purity of RNA were assessed by measuring absorbance at 260/280 nm using a NanoDrop™ 1000 spectrophotometer (*Thermo Fisher Scientific, USA*).

cDNA Synthesis

Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the RevertAid First Strand cDNA Synthesis Kit (*Thermo Fisher Scientific, USA*. The reaction included 1 µL of oligo(dT) (18) primer and 1 µL of random hexamer primer, which were mixed with the RNA and incubated at 65°C for 5 minutes, followed by immediate cooling on ice. Subsequently, 8 µL of cDNA synthesis mix (comprising 5X reaction buffer, RiboLock RNase Inhibitor, dNTP mix, and RevertAid M-MuLV reverse transcriptase) was added. The reaction was incubated at 42°C for 60 minutes, followed by 25°C for 5 minutes, and terminated by heating at 70°C for 5 minutes.

Quantitation of gene expression by RT-qPCR

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed using RealQ Plus 2X Master Mix Green (*Ampliqon, Denmark*). Gene-specific primers of Bax, Bcl-2, and Caspase-3, which involved in the apoptotic pathway, were used (Table 1). A total of 100 ng of cDNA was used per 25 µL reaction, which included 150 nM of each primer and 12.5 µL of RealQ Plus Master Mix. Reactions were run in a CFX96 system (*Bio-Rad*) with the following thermal profile: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 60 seconds, and 72°C for 30 seconds. Gene expression levels were normalized against housekeeping gene (GAPDH) using the 2ΔΔCt method (21). Data were reported as fold-change, with experiments conducted in two biological and technical replicates.

Table 1.
Primer sets used in the study.

Genes	Primer sequence	GenBank ID
Bax	F: 5 ′-CACGTCTGCGGGGAGTC-3 ′ R: 5 ′-TGTTGTCCAGTTCATCGCCA-3 ′	XM_063281064.1
Bcl-2	F: 5 ′-GGGCTACGAGTGGGATACTG-3 ′ R: 5 ′-GACCCCACCGAACTCAAAGA-3 ′	NM_016993.2
Caspase-3	F: 5 ′-GGAGCTTGGAACGCGAAGAA-3 ′ R: 5 ′-CCATTGCGAGCTGACATTCC-3 ′	NM_012922.3
GAPDH	F: 5 ′-CTCTCTGCTCCTCCCTGTTC-3 ′ R: 5 ′-CGACATACTCAGCACCAGCA-3 ′	NM_017008.4

Bax, Bcl-2 associated X-protein; Bcl-2, B-cell leukemia/lymphoma 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

The SPSS 22.0 software was used for statistical evaluations (*IBM Corporation, Armonk, NY*). In the evaluation of spermatological parameters, one-way analysis of variance (ANOVA) were used to compare the significance of the difference between the groups. The results were evaluated as mean ± SE and those with $p < 0.05$ were considered statistically significant. Duncan test was used to indicate the significance of the difference between the groups as a result of the analysis. For the gene expression analysis, a one-way ANOVA followed by a post hoc Tukey test was performed using GraphPad Prism 9.1.1 (*GraphPad, San Diego, CA, USA*). Statistical significance was considered at $p < 0.05$.

Results

Spermatologic Parameters Identified After Freezing-Thawing

The motility, viability rate, plasma membrane integrity rates, sperm concentration, and abnormal acrosome ratio, in the study groups after post-thawing processes are presented in Table 2.

Motility

The highest motility ratio was 9.75 ± 1.33 in the control group. This ratio was 3.12 ± 1.61 in the tryptophan group, which was significantly lower compared to the control group ($p < 0.05$). In the group to which 100 mM trehalose was added, the motility ratio was 6.87 ± 2.48, but there was no statistically significant difference between the groups ($p > 0.05$).

This indicates that tryptophan and trehalose supplemented to the cryopreservation diluent may not be effective on motility.

Viable spermatozoon ratio

There were significant differences between the groups in terms of sperm viability ratio ($p < 0.05$). The highest viability ratio was 26.25 ± 3.10 in the trehalose group, which was significantly higher than the ratio of 19.62 ± 1.51 in the control group. In the group in which 25 mM tryptophan was added, the viability ratio was 15.00 ± 3.19, which was significantly lower than the ratios in the control and trehalose groups ($p < 0.05$). These findings suggest that trehalose may improve sperm viability after freezing-thawing.

Plasma membrane integrity ratio

According to the results of HOST assay performed to evaluate sperm cell membrane integrity, there were significant differences between the groups ($p < 0.05$). The highest HOST positive ratio was determined in the control group with (22.87 ± 1.10) and in the trehalose group (20.25 ± 3.65). There was no statistical difference between the groups ($p > 0.05$). The HOST positive ratio was 11.87 ± 3.07 in the tryptophan group and significantly lower compared to the control group ($p < 0.05$).

Spermatozoa density

Spermatozoa density was determined to verify the amount of cells required for analyses to be performed after freezing-thawing and to standardize experimental procedures. Sperm concentration did not differ significantly among the experimental groups ($p > 0.05$).

Abnormal acrosome ratio

There was no statistically significant difference between the groups in terms of abnormal acrosome ratio ($p > 0.05$).

Table 2.
Spermatological findings determined after freezing-thawing in the study groups (n = 8).

Groups	Progressive motility (%)	Viability (%)	Intact plasma membrane integrity (%)	Sperm concentration (×10^6 cells/mL)	Abnormal acrosome (%)
Control (C)	9.75 ± 1.33	19.62 ± 1.51	22.87 ± 1.10	221.00 ± 3.40	6.37 ± 0.99
Tryptophan (Tryp) (25 mM)	3.12 ± 1.61	15.00 ± 3.19	11.87 ± 3.07	245.75 ± 5.74	4.75 ± 0.75
Trehalose (Treh) (100 mM)	6.87 ± 2.48	26.25 ± 3.10	20.25 ± 3.65	245.5 ± 4.25	4.87 ± 0.63
C *vs* Tryp	*p < 0.05*	*p < 0.05*	*p < 0.05*	*p < 0.05*	*p < 0.05*
C *vs* Treh	*p < 0.05*	*p < 0.05*	*p < 0.05*	*p < 0.05*	*p < 0.05*
Tryp *vs* Treh	*p < 0.05*	*p < 0.05*	*p < 0.05*	*p < 0.05*	*p < 0.05*

The abnormal acrosome ratio was 6.37 ± 0.99 in the control group, 4.75 ± 0.75 in the tryptophan group, and 4.87 ± 0.63 in the trehalose group.

Apoptotic cell ratios determined with Annexin V-FITC/PI staining

The apoptotic cell ratios determined with Annexin V-FITC and PI staining are presented in Figure 1. Accordingly, the lowest ratio of apoptotic cells was determined in the group in which 100 mM trehalose was added (22.37 ± 2.94); however, this value was not significantly different from the ratio of 33.00 ± 4.55 determined in the control group ($p > 0.05$). The highest ratio was determined as 55.00 ± 3.95 in the tryptophan group, which was significantly higher than that in the control and trehalose groups ($p < 0.01$).

Effects of Tryptophan and Trehalose Supplementation on The Expression of Apoptosis-Related Genes in Frozen-Thawed Sperm Using Real-Time PCR

To investigate whether tryptophan and trehalose can alle-

Figure 1.
Apoptotic spermatozoa evaluation. Apoptosis was assessed by Annexin V-FITC/PI assay in epididymal rat sperm. Spermatozoa with completely green heads were early apoptotic (a), spermatozoa with green-red heads were late apoptotic (b-c), spermatozoa with completely red heads were necrotic (d), and quantification of the apoptosis index (%) of sperm cells in each group (e). Data were shown as the mean ± SE (n = 8 in each group). Values with different superscripts differ significantly (p < 0.001).

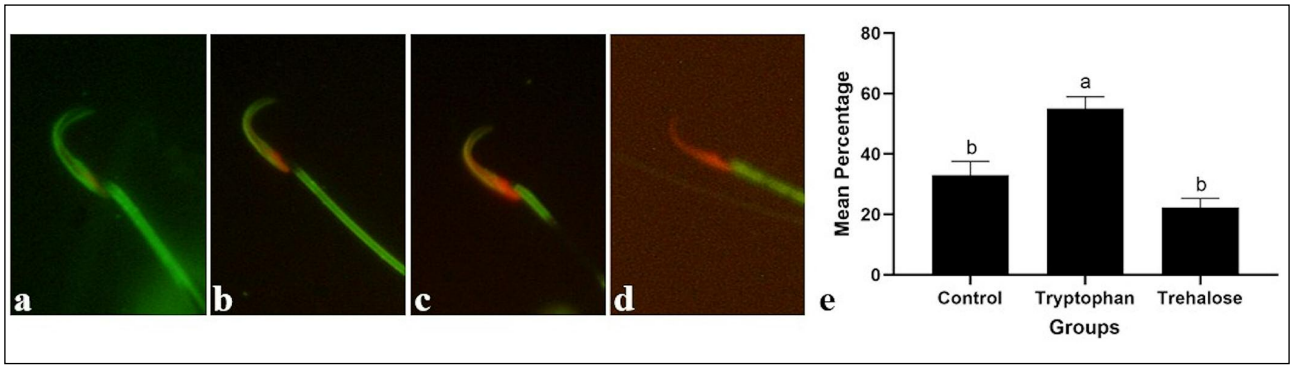
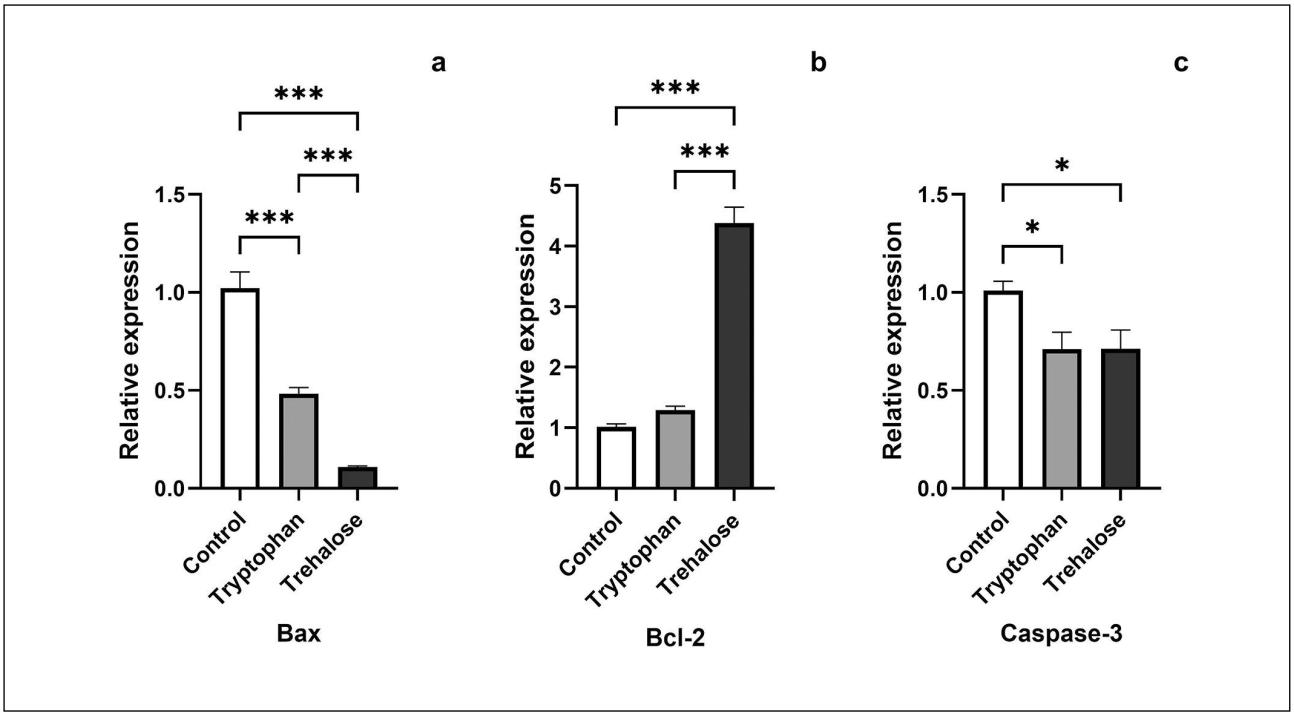


Figure 2.
Quantitative PCR analysis of the relative expression of Bax (a), Bcl-2 (b), and Caspase-3 genes (c) on frozen-thawed epididymal rat sperm. All data were presented as mean ± SEM (n = 8 in each group).
**p < 0.05, **p < 0.01, and ***p < 0.001 vs the other group.*



viate cryodamage in spermatozoa after frozen-thawed were analyzed the expression of apoptosis-related genes. Addition of 25 mM tryptophan and 100 mM trehalose to the cryoprotectant agent affected Bax, Bcl-2, and Caspase-3 mRNA levels in comparison to the control group (Figure 2). Bax gene expression was significantly down-regulated in both the tryptophan and trehalose-treated groups compared to the control group ($p < 0.001$). Furthermore, mRNA expression levels in the trehalose group were significantly lower than those observed in the tryptophan group ($p < 0.001$). Bcl-2 gene expression reached its highest level in the trehalose group when compared to the control and tryptophan groups ($p < 0.001$). No statistically significant difference was detected between the control and tryptophan groups in terms of Bcl-2 expression ($p > 0.05$). These findings suggest that supplementation of the cryopreservation extender with trehalose enhances the post-thaw expression of the anti-apoptotic Bcl-2 gene. Caspase-3 gene expression did not differ significantly between the tryptophan and trehalose groups ($p > 0.05$); however, when compared to the control group, both the tryptophan and trehalose groups showed a significant decrease in caspase-3 expression levels ($p < 0.05$). Collectively, these results indicate that the

freeze-thaw process induces apoptosis in rat spermatozoa, and the addition of amino acid (tryptophan) and disaccharide (trehalose) to the cryoprotectant medium reduces the expression levels of genes involved in the apoptotic pathway after thawing.

Discussion

Cryopreservation is known to be a more challenging process for rat sperm compared to other mammalian species (6, 22). Main reason for this is that rat spermatozoa are highly sensitive to all kinds of manipulations and that these procedures constitute a significant source of stress on the cell. Furthermore, the molecular structure of rat sperm is remarkably different from that of other mammalian sperm, especially due to differences in the lipid content and composition of the sperm membrane (23, 24).

Motility is considered a criterion of cell activity and viability after cryopreservation. In this study, motility was found to be higher in the control group (9.75 ± 1.33) compared to the trehalose group (6.87 ± 2.48), but the difference was not statistically significant ($p > 0.05$). Interestingly, although the freezing protocol used in this study was the same as the protocol reported by *Nakatsukasa et al.* (6), the addition of 100 mM trehalose to the diluent increased the post-thaw motility (6.87 ± 2.48), which was higher than the value reported by *Nakatsukasa et al.* (6) (4.6 ± 0.1). Moreover, in another study conducted by the same author (2), post-thaw motility ratios of rat sperm was reported to range from 2% to 12.3%. The motility observed in the present study with 100 mM trehalose falls within this range, suggesting that trehalose contributes to maintaining motility after freezing and thawing. Similar stabilizing effects of trehalose have also been reported in other species, including ram (25) and goat (26) sperm. In contrast, tryptophan at 25 mM had minimal impact on motility. Similar findings were reported by *Koçak* and *Yıldız* (27) in mouse sperm, where the same concentration had only a modest effect on motility (20.83 ± 3.96) but improved fertilization capacity. This suggests that tryptophan's effect on motility is indirect and may depend on its antioxidant activity rather than structural stabilization (28).

When viability rate was considered, the highest post-thaw viability (26.25 ± 3.10) was observed in the trehalose group and was significantly higher than in the tryptophan group ($p < 0.05$). Trehalose, as a non-permeable disaccharide, reduces osmotic stress during dehydration and stabilizes sperm cell structures, leading to better survival (29). This result is consistent with findings in pig (30), ram (31), human (32), fish (33, 34), rabbit (35), buffalo (36), and mouse (27) sperm cryopreservation, where trehalose enhanced viability. By comparison, tryptophan showed weaker effects on viability, likely because it mainly reduces oxidative stress without improving osmotic stability. Since rat sperm membranes have a high lipid content and are prone to oxidative damage (28, 37), tryptophan may have more impact on DNA or membrane integrity than on viability itself.

Plasma membrane integrity is a critical indicator for assessing sperm quality and reproductive potential after cryopreservation. The trehalose group maintained the highest membrane integrity (20.25 ± 3.65), while tryptophan had a reduced effect ($p < 0.05$). Trehalose likely provides superior protection by stabilizing the plasma membrane bilayer and minimizing osmotic imbalance during freezing (35). This is consistent with studies on ram (25, 38), goat (26), and bull (39) sperm where trehalose preserved membrane structure more effectively than amino acids.

Sperm concentration did not differ significantly among the experimental groups. This was expected, as sperm concentration is determined prior to freezing and is used to confirm the amount of cells needed for post-freeze-thaw analyses and to standardize experimental procedures ($p > 0.05$).

When examining the abnormal acrosome ratio, the lowest acrosome damage was found in the trehalose (4.87 ± 0.63) and tryptophan (4.75 ± 0.75) groups compared to the control (6.37 ± 0.99), although the differences were not statistically significant ($p > 0.05$). These findings indicate that trehalose and tryptophan have comparable cryoprotective effects in preserving acrosome integrity. Trehalose stabilizes the plasma membrane by reducing osmotic stress, while tryptophan limits oxidative damage through its antioxidant properties. Similar effects were reported by *Gholami et al.* (40) for trehalose in ram sperm, and by *Koçak* and *Yıldız* (27), who showed that 25 mM tryptophan reduced acrosome damage in mouse sperm (11.17 ± 1.22) compared to the control (12.83 ± 1.22). The comparable outcomes in our study suggest that both additives have potential to reduce acrosomal damage, but larger sample sizes may be needed to reveal significant differences.

Apoptosis is a mechanism of programmed cell death, being a physiological process that eliminates cells that threaten survival, and is critical for spermatozoa (16). Therefore, in this study, the Annexin V-FITC/PI test was performed to determine whether tryptophan and trehalose added to the cryopreservation diluent had any effect on the apoptotic mechanism after thawing. According to the test results, spermatozoa with the head stained completely green were considered as early apoptotic, spermatozoa with green-red staining were considered as late apoptotic, and spermatozoa with red staining were considered as necrotic. When apoptotic cell ratios were evaluated together with apoptosis-related gene expression, trehalose demonstrated a clear cryoprotective advantage. The Annexin V-FITC/PI assay demonstrated a significantly reduced apoptosis index (%) in the trehalose group (22.37 ± 2.94) compared with controls (33.00 ± 4.55, $p < 0.01$), aligning with the downregulation of pro-apoptotic Bax ($p < 0.001$) and Caspase-3 ($p < 0.05$) expression and the upregulation of anti-apoptotic Bcl-2 expression ($p < 0.001$). These findings are consistent with previous studies (41-44). In canine sperm, trehalose supplementation during cryopreservation caused slight numerical increases in Bcl-2 and decreases in Bax expression (45). Similarly, in rat ovaries, *Xie et al.* (41) reported that adding trehalose to the cryoprotective agent significantly reduced the apoptotic cell ratio and suppressed Bax gene expression. However, *Bumbat et al.* (46) reported that trehalose supplementation in the cryopreservation

medium of cancer cells paradoxically upregulated not only the anti-apoptotic gene Bcl-2 but also the pro-apoptotic genes Bax and Bad, suggesting that such variations may depend on tissue type or experimental conditions. Overall, trehalose consistently reduced both apoptotic cell counts and pro-apoptotic gene expression, whereas tryptophan showed more variable effects. Although tryptophan also lowered Bax and Caspase-3 expression, the apoptosis index in the tryptophan group (55.00 ± 3.95) was unexpectedly higher than in the control, suggesting that the 25 mM concentration may not be optimal and that its effects could be dose-dependent, as reported in several sperm cryopreservation studies (27, 34, 36).

In summary, trehalose provided more consistent cryoprotective benefits than tryptophan by improving viability, preserving plasma membrane integrity, reducing apoptosis, and maintaining acrosomal structure.

Tryptophan offered limited benefits, mainly through its antioxidant action, and its effectiveness may vary with concentration. These findings indicate that optimizing trehalose concentration is a promising approach for rat sperm cryopreservation, while further research is needed to explore the dose-response effects of tryptophan and to evaluate fertility outcomes *in vivo*.

Conclusions

In conclusion, according to the parameters evaluated, the addition of the disaccharide sugar trehalose to rat sperm diluent provided more effective protection against cryodamage during cryopreservation than the aromatic amino acid tryptophan. The use of these substances may provide a significant advance in overcoming the difficulties encountered in the cryopreservation process of rat sperm and may lay a strong foundation for future studies.

Declarations

Ethical approval: *Ethical approval for this study was obtained by the Animal Experiments Local Ethics Committee (HADYEK) of Hatay Mustafa Kemal University, Hatay, Türkiye (Ethical Approval Numbered 2024/09-01).*

Availability of data and material: *The data used in this study were generated by the author(s) and are available from the corresponding author upon reasonable request.*

Competing interests: *None of the authors have any conflicts of interest to declare that are either directly or indirectly related to the study design, execution or reporting of this work.*

Funding: *This study did not receive any financial support.*

Authors' contributions: *F.Y.D., O.K.Y. and A.E.Ş. designed the study, performed the experiments, performed the analyses, and prepared the first drafts of the manuscript based on the data obtained. Then, they made final revisions on the manuscript. All authors equally contributed to the interpretation of the results.*

Acknowledgments: *We would like to express our gratitude to all researchers for their valuable contributions.*

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