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Combination of trehalose and low boron in presence of decreased glycerol improves post-thawed ram sperm parameters: A model study in boron research

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Abstract

Background: Sperm cryopreservation has been widely used in the field of reproductive biotechnology. It applies to certain males of economic and scientific values, including livestock breeds or endangered animal species. The development of a semen extender with a low cryoprotectant concentration and an appropriate amount of trehalose and boron can prevent the deterioration of sperm parameters.

Objective: The main goal of this study is to establish a suitable ram extender model, by examining different combinations of high (5%) and low (3%) glycerol concentrations (to reduce its toxic effects on sperm freezing), a fixed amount of trehalose and an increased dose of boron to prevent the deterioration of sperm parameters, and investigate the levels of gene expressions.

Materials and methods: The Merino ram ejaculates were collected. The collected ejaculates providing the defined criteria were pooled. The pooled ejaculates were divided into eight aliquots and diluted with the Tris extender including different combinations of glycerol (5% and 3%) and boron (0.25, 0.5, and 1 mM) concentrations and a fixed amount of trehalose, then frozen. After freeze–thawing process, sperm motility, mitochondrial membrane activity, plasma membrane integrity, acrosomal membrane integrity, DNA damage (single cell gel electrophoresis (COMET) and TUNEL assays)

as well as NAD(P)H quinone oxyreductase (*NQO1*), glutamate-cycteine ligase (*GCLC*), and glutathione S-transferase (*GSTP1*) for molecular mechanisms of sperm cell response to oxidative stress were assessed for different extender groups following freeze–thawing process: 5% glycerol + 0 mM boron (G5B0.00), 5% glycerol + 0.25 mM boron (G5B0.25), 5% glycerol + 0.5 mM boron (G5B0.50), 5% glycerol + 1 mM boron (G5B1.00), 3% glycerol + 0 mM boron (G3B.00), 3% glycerol + 0.25 mM boron (G3B0.25), 3% glycerol + 0.5 mM boron (G3B0.50), and 3% glycerol + 1 mM boron (G3B1.00).

Results: G3B0.25 presented higher percentages of subjective motility, mitochondrial activity, and viability of spermatozoa comparing with G5B0.00 and groups with boron. Supplementation of 0.25 mM boron with and without trehalose (G3B0.25 and G5B0.25) showed higher acrosome integrity, compared with G5B0.00, G5B1.00, G3B0.50, and G3B1.00. For TUNEL analysis, G3B1.00 showed the highest DNA integrity among the experimental groups which was statistically significant only with G5B0.50 ($p < 0.05$). The mRNA levels of NQO1 were significantly decreased in G5B1.00, G3B0.50, and G3B1.00, when compared to G5B0.00. In comparison with G5B0.00, supplementation of 1 mM boron with and without trehalose had significantly lower expression of GCLC. The level of *GSTP1* gene was significantly lower (approximately threefold) in G3B1.00, compared to G5B0.00 ($p < 0.05$).

Discussion and conclusion: It can be assumed that the increase of the boron concentration in the extender may have important adverse effects on sperm parameters and antioxidant gene expression after thawing. The results obtained from this study will help to understand the toxicity limits of boron and eliminate the toxicity of glycerol in studies of gametes and tissue freezing. Therefore, it can be concluded that the use of sufficient boron can decrease cryodamages of cryopreservation of mammalian spermatozoa as well tissue engineering.

KEYWORDS
boron, cryopreservation, ram semen, trehalose

1 | INTRODUCTION

The cryopreservation of spermatozoa has been commonly used in branch of reproductive biotechnology as an effective procedure for male fertility management. It is applicable in certain economically, biologically, or scientifically valuable males, consisting of livestock breeds or endangered animal species.[1–3] In spite of the extensive progress in this field, various physical and chemical stress factors during the freezing and thawing process, including ice crystal formation, oxidative stress, and chemical toxicity, could adversely affect sperm structure and physiological function, and thereby damaging the fertility of the spermatozoa.[4] The sperm plasma membrane of ruminants includes unsaturated phospholipids in high levels and cholesterol in low levels. The low content of cholesterol decreases the resistance of spermatozoon to freezing and thawing methods.[5,6] Redistribution of membrane phospholipids during freezing process causes to change the liquid form of some of them to gel state, and formation of extra and intracellular crystals of water molecules in which extracellular ice crystals exert external pressure on cell membrane and intracellular ice crystals damage different cell organelles and structures.[7,8] The mitochondria located in the midpiece of the spermatozoa are damaged by freezing water molecules inside the cell; thereby, free oxygen radicals are generated because of electron leakage from the disrupted membrane.[9] After all, lipid peroxidation chain reaction causes to disruption of the membrane structure. Furthermore, the oxidative stress induces the DNA damages as well.[10] Despite the fact, evidence has been shown that using sufficient cryoprotectants added to diluents can minimize the extreme damages induced by freezing–thawing process in spermatozoa.[11,12] That's why the effectiveness of various agents has been investigated in the numerous cryopreservation of gamete cells of different species.[3,9] Rams have been reported to generate more cryosensitive spermatozoa than humans, rabbits, cats, and dogs during freezing–thawing methods because of their high content of polyunsaturated fatty acids and insufficient cytoplasmic

antioxidant capacity.[7] When the inadequacy of the studies on the development of ram semen extenders is added, the importance of conducting studies in the field of ram sperm protectors becomes evident.[7,9,10]

In the cryobiology, permeating and non-permeating cryoprotective are used to protect gamete cells of different species from ice crystal formation, osmotic, and chemical stress. The most commonly applied permeating cryoprotectant in ruminants during sperm cryopreservation is glycerol. One of the most important effect of glycerol is to prevent water molecules becoming large volume ice crystals during freezing procedure. Furthermore, it can also bind to the membrane of cell and organelles, reducing membrane mobility[13] and forming a particulate clump into the membrane structure because of interaction between membrane proteins and glycoproteins.[14,15] Besides, unfortunately it is cytotoxic beyond particular concentration and has some side effects by altering some proteins in spermatozoa affecting negatively sperm function.[16] Aiming to solve the situation, the best alternative is to combine low glycerol rate with non-permeating cryoprotectants such as trehalose.[9,17,18]

The role of sugars in sperm cryopreservation is notable because of regulating osmotic pressure, serving an energy source and reducing ice crystal formation.[9] Trehalose, a non-permeable cryoprotectant, interacts with membrane phospholipids by binding to their polar head groups, resulting membrane stabilization and cell dehydration prevention during freezing–thawing of spermatozoa.[2] The accessible results of trehalose on post-thaw semen motility, mitochondrial activity, and acrosome integrity have been also reported in ram semen.[19] It has been tested before that reducing the glycerol content from 5% to 3% with 60 mM trehalose supplement increased the post-thawed semen quality.[20]

Boron is an element found in soil, rock, nutrients, and water with various applications in industry, agriculture, and medicine. Note that 72.1% of world boron reserves are located in Turkey.[21] In terms of fertility and developmental toxicity, boric acid is classified as a "Category 1B" compound of the globally harmonized system (GHS), associated with risk phrases R60–61 ("may impair fertility") by the European Union.[22] Two hypotheses have appeared to account for the multiple effects. One hypothesis is that boron has a role in cell membrane function, stability, or structure, such that it influences the response to transmembrane signaling, or transmembrane movement of regulatory cations or anions, or cryoprotective effect to cold shock damage.[23,24] The other hypothesis is that boron is a negative regulator that influences a number of metabolic pathways by competitively inhibiting some key enzyme reactions.[25]

The mean safe daily intake of boron for adults was limited and endorsed (1–13 mg/day) by the World Health Organization (WHO) which intimates that intakes <1.0 mg/day is insufficient for optimal boron beneficial activity in both animal and human.[26,27] Dietary boron supplementation at 40 ppm increased sperm motility, sperm output as well as immune and antioxidant defense capacity in male goats.[28] It has been shown before that 100, 200, and 400 mg boric acid supplement can improve semen quality and has a positive effect on physiological condition in rabbits.[29] In spite of its advantages in this field, the toxic effect of high doses of boron on reproduction has been reported.[30] For example, administration of high-dose boron causes testicular atrophy in male rodents.[31–33] Moreover, according to the results of various studies on different animal species the daily boron range above 25 mg/kg causes damage in spermatological parameters and testicular morphology.[31,32,34] Despite all this, occasional reports have appeared suggesting that living areas in which abundance of boron element is high, no negative effects on the reproductive system and spermatozoa.[35–37] It was reported that Tris diluents added to 0.5, 0.7, and 0.9 g boron (na-borat) have beneficial effects on the kinetic and structural properties of Angora buck spermatozoa after freeze–thawing.[38] In another study conducted with Tris extender containing 1, 2, and 4 mM boron and 5% glycerol, the positive effect of boron on energy metabolism and DNA damage during the freezing and thawing of ram semen has been described.[39]

Recently, there has been increasing focus on the study of the biological functions of mRNAs in spermatozoa. It is suggested that analysis of RNAs derived from spermatozoa could provide possible links between the sperm proteome and sperm freezing.[40–46] Within this scope, the genes of glutathione S-transferase pi 1 (*GSTP1*), NAD(P)H quinone dehydrogenase 1 (*NQO1*), and glutamate-cysteine ligase catalytic subunit (*GCLC*) are powerful antioxidant response genes, which were reported to involve in spermatozoa and protect the cell from reactive oxygen species generated during oxidative stress.[47–51]

Limited considerable evidence exists to determine the influence of boron on spermatological effects following sperm cryopreservation. The main goal of the present study is setting an appropriate system for demonstrating boron toxification effect via developing a ram semen extender supplemented with a low cryoprotectant concentration (to reduce its toxic effect) and a fixed amount of trehalose and increasing doses of boron to prevent the deterioration of sperm parameters (motility, mitochondrial activity, viability, acrosome integrity, DNA damage, and DNA fragmentation) as well as to investigate expression levels of *NQO1*, *GCLC*, and *GSTP1* genes following freeze–thaw process.

2 | MATERIALS AND METHODS

The experimental research was carried out at Bahri Dagdas International Agricultural Research Institute in Konya, Turkey. Artificial vagina was used to collect semen from fertile Merino rams ($n = 6$, 2–3 years of age). The Merino ram ejaculates (at least 12 ejaculates for each ram) were collected three times a week for 4 weeks during the ram breeding season. Only ejaculates with spermatozoa motility of >75%, concentration of spermatozoa $>2.5 \times 10^9$/ml were pooled and used in the study. The number of 40 ejaculates met the criteria. The experimental design included eight repetitions.

As a cryopreservation diluent,[52] the Tris-based freezing extender was used. Among the ejaculates taken every week, those who met the criteria were pooled without waiting/storing, and immediately subjected to freezing processes with different freezing extenders. To do this, the pooled ejaculates were divided into eight aliquots and diluted

with the extender including 5% glycerol and different concentrations of boron as follows: 5% glycerol + 0 mM boron (G5B0.00), 5% glycerol + 0.25 mM boron (G5B0.25), 5% glycerol + 0.5 mM boron (G5B0.50), and 5% glycerol + 1 mM boron (G5B1.00) for the first four groups. The supplementation of 60 mM trehalose to semen extender was added in order to reduce the content of glycerol (from 5% to 3%) with different boron doses as follows: 3% glycerol + 0 mM boron (G3B.00), 3% glycerol + 0.25 mM boron (G3B0.25), 3% glycerol + 0.5 mM boron (G3B0.50), and 3% glycerol + 1 mM boron (G3B1.00) for the second four groups. Each aliquot was adjusted to final concentrations of 400 × 10^6 spermatozoa/ml, loaded into 0.25 ml French straws and equilibrated for 2 h at 4°C. Afterwards, the straws were frozen in liquid nitrogen vapour for 15 min and then stored at -196°C (liquid nitrogen) for at least 1 month.

2.1 | Post-thaw microscopic sperm parameters

Frozen spermatozoa was thawed at 37°C for 30 s for the assays. The thawed samples (5 μl) were analyzed under phase contrast microscope (200×) for sperm motility.

The LIVE/DEAD Viability Kit (L 7011 Thermo Fisher, SYBR-14/PI) was used to analyze sperm viability at a fluorescence microscope (at 400× magnification). In this assay, membrane permeant SYBR-14 nucleic acid stain performs as green fluorescence to label viable spermatozoa and the other fluorescent dye; membrane impermeant propidium iodide (PI) labels the dead cells or damaged spermatozoa with red fluorescence.[53]

The mitochondrial membrane function of spermatozoa was fulfilled using the JC-1/PI staining. The stained orange or yellow fluorescence of sperm midpiece was considered to be high mitochondrial membrane potential activity, whereas the stained green of sperm midpiece indicated to be low membrane potential activity.[54]

Acrosome integrity was assessed by FITC-PNA (L7381, Sigma-Aldrich Co., USA) and PI staining. Spermatozoa emitting green fluorescence were regarded to be damaged and to have a non-intact acrosome, whereas cells displaying red fluorescence in the acrosome cap were considered to be undamaged intact acrosome.[55]

Sperm DNA damage analysis was measured by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) and COMET assays. For TUNEL assay, the In situ Cell Death Detection Kit (POD; by ROCHE Applied Science, Germany) was used for this assessment. The counterstaining the slides with methyl green was evaluated the randomly selected 100 spermatozoa from 10 different regions under a light microscope and counted to assess the percentage of TUNEL-positive cells with light brown color in their nuclear material representing DNA fragmentation.[56] For COMET, single cell gel electrophoresis assay was applied. The 200 randomly selected nuclei were observed using a light microscope (at 400× magnification). DNA damage of the spermatozoa was investigated by a tail of fragmented DNA that migrated from the sperm head, emerging like a "comet" pattern, while the lack of a comet was considered to be undamaged DNA.[57]

2.2 | Gene expression analyses

Firstly, spermatozoa were isolated from somatic cells. To do this, the solutions of BoviPure (Nidacon International AB, Göthenborg, Sweden) were used according to manufacturer's instructions. Shortly, 0.5 ml volumes of BoviPure solution at 40% and 80% were added to centrifuge tubes. Subsequently, 0.25 ml of frozen–thawed ram semen sample was layered at the top of the tube and centrifuged at 600× *g* at 4°C for 20 min. The supernatant of the sample was discarded, and the sperm pellet was washed twice with 0.5 ml of Phosphate Buffered Saline (PBS). Finally, 0.1 ml PBS was added to sperm pellet, and it was placed at stereo microscope (40× magnification) for checking absence of somatic cells. After separation of somatic cells, total ram sperm RNA was isolated according to TRIzol method. Expression levels of *GCLC*, *GSTP1*, and *NQO1* genes were pointed out via Quantitative Polymerase Chain Reaction (qPCR).[58–60] Gene amplification was actualized by SYBR Green I dye-containing kit (Diagen, Turkey). The qPCR protocol conditions were as follows: 10 min at 95°C, followed by 10 s at 95°C, 30 s at 60°C, 5 s at 72°C, and 40 cycles in qPCR (ABI 7500 Fast, USA), while each cDNA sample was performed as duplicate, protamine 1 (*PRM1*) gene was selected as a reference gene.

2.3 | Statistical analysis

The whole data were defined as mean ± SE. The means of microscopic sperm parameters were analyzed with Duncan's post hoc test and ANOVA to adjust considerable differences. The gene expressions calculation was applied by the $2^{-\Delta\Delta Ct}$ method, and the results were explained as fold-change. Statistical analyses were verified through SPSS 13.0 (SPSS Inc., USA), and statistical relevance was adjusted at $p < 0.05$.

3 | RESULTS

The results of post-thawed sperm motility, viability, mitochondrial activity, and acrosome integrity are presented in Figure 1. G3B0.25 presented higher percentages of subjective motility, mitochondrial activity, and viability of spermatozoa (57 ± 2.00%, 55.94 ± 2.09%, and 64.47 ± 2.30%, respectively) comparing with G5B0.00 and groups with boron ($p < 0.05$, Figure 1A–C). Moreover, supplementation of 0.25 mM boron with and without trehalose (G3B0.25 and G5B0.25) showed higher acrosome integrity, compared with G5B0.00, G5B1.00, G3B0.50, and G3B1.00 ($p < 0.05$, Figure 1D). The second-best results for sperm motility, mitochondrial activity, viability, and acrosome integrity belonged to G3B0.00 (50 ± 1.58%, 49.70 ± 1.68%, 58.54 ± 1.50%, and 57.20 ± 2.09%, respectively), in comparison to groups of G5B1.00, G3B0.50, and G3B1.00 ($p < 0.05$).

The results of TUNEL and COMET assays were provided in Table 1. According to TUNEL analysis, G3B1.00 (2 ± 0.65%) showed the highest DNA integrity among the experimental groups which was statistically

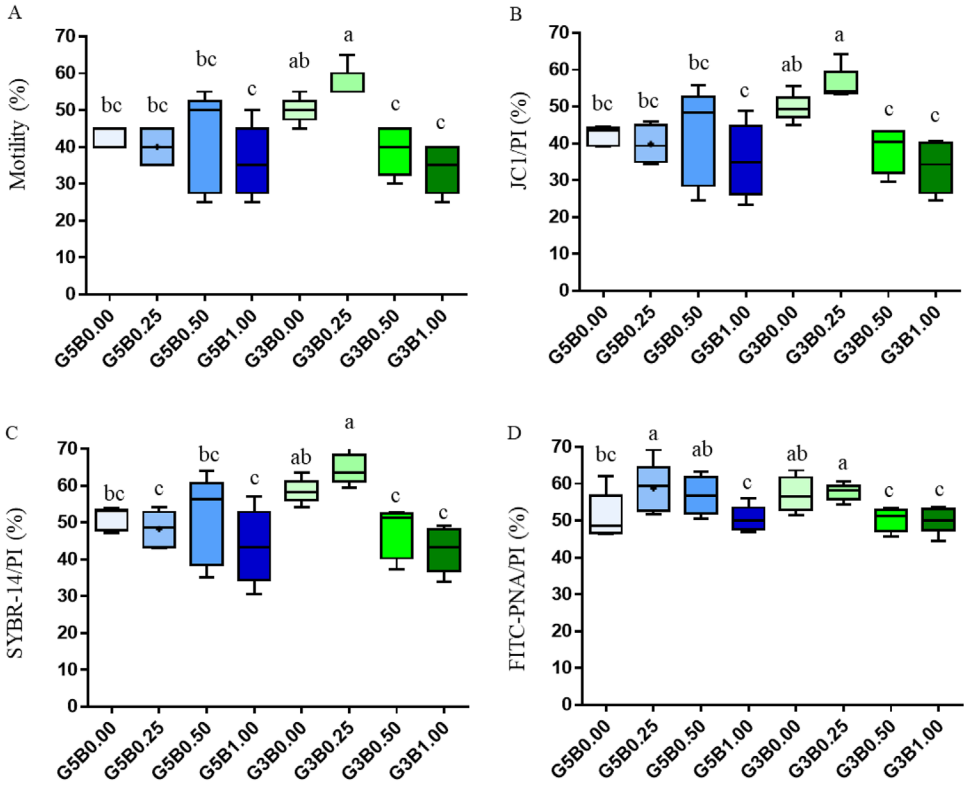

FIGURE 1 Graphs of motility (A), mitochondrial membrane integrity (JC1/PI, B), plasma membrane integrity (SYBR-14/PI, C), and acrosomal membrane integrity (FITC-PNA/PI, D) rates (mean ± SEM) in Merino ram semen supplemented with different concentrations of glycerol, boron, and trehalose after freeze–thaw process. Means with different letters (a, b) in the column of each graph demonstrate significant differences ($p < 0.05$). Abbreviations: G5B0.00, 5% glycerol; G5B0.25, 5% glycerol + 0.25 mM boron; G5B0.50, 5% glycerol + 0.5 mM boron; G5B1.00, 5% glycerol + 1 mM boron; G3B0.00, 3% 60 mM trehalose; G3B0.25, 3% glycerol + 0.25 mM boron + 60 mM trehalose; G3B0.50, 3% glycerol + 0.5 mM boron + 60 mM trehalose; G3B1.00, 3% glycerol + 1 mM boron + 60 mM trehalose

TABLE 1 Descriptive statistic data of the treatment groups for the sperm DNA damage

Groups	DNA damage % (COMET)	DNA damage % (TUNEL)
G5B0.00	19.90 ± 4.92	4.20 ± 0.68^{ab}
G5B0.25	15.80 ± 3.46	3.00 ± 0.35^{ab}
G5B0.50	17.30 ± 2.59	4.90 ± 1.44^{b}
G5B1.00	19.80 ± 5.43	4.70 ± 0.76^{ab}
G3B0.00	18.70 ± 8.00	3.00 ± 0.75^{ab}
G3B0.25	18.90 ± 7.68	3.30 ± 0.91^{ab}
G3B0.50	16.00 ± 5.53	2.5 ± 0.67^{ab}
G3B1.00	17.10 ± 5.28	2 ± 0.65^{a}

Note: Means with different letters (a, b) in the same column are significantly different from each other ($p < 0.05$).

significant only with G5B0.50 (4.90 ± 1.44%, $p < 0.05$). Increasing boron doses with 3% glycerol tended to decrease DNA damage by TUNEL, but not statistically significant. In regard to results of COMET assay, we did not find any significant differences in DNA damage of ram spermatozoa following freeze–thawing process ($p > 0.05$).

As shown in Figure 2, the mRNA levels of NQO1 were significantly decreased in G5B1.00 (0.39 ± 0.17), G3B0.50 (0.57 ± 0.12), and G3B1.00 (0.42 ± 0.11), when compared to G5B0.00 (1.00 ± 0.12) ($p < 0.05$, Figure 2A). In comparison with G5B0.00 (1.00 ± 0.16), supplementation of 1 mM boron with and without trehalose in cryopreservation extender had significantly lower expression of GCLC ($p < 0.05$, Figure 2B). The level of *GSTP1* gene was significantly lower (approximately threefold) in G3B1.00 (0.36 ± 0.13), compared to G5B0.00 (1.00 ± 0.20) (Figure 2C).

4 | DISCUSSION

Male gamete freezing is an important commonly used approach applied in various fields including assisted reproduction, protecting the several species, and saving the genetics improvement.[2] However, different cellular damages occurred during the cryopreservation process were reported. For example, ice crystal formation in the extracellular environment exerts pressure on the cell membrane and the membrane of organelles via passing through the aquaporin pores in the membrane into the cell. The mentioned osmotic pressure disrupts membranes and affects their permeability as well.[61,62] Aiming to protect the spermatozoa from cryodamage, especially ice crystal formation, cryoprotectants such as glycerol are used. Glycerol is widely used in the freezing procedure of spermatozoa of many species because of having three

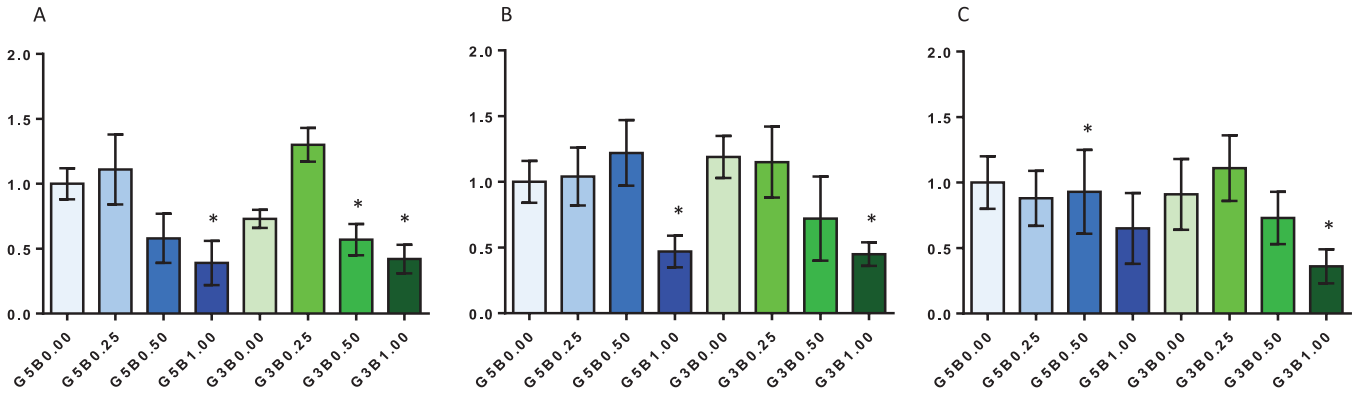

FIGURE 2 Graphs of *NQO1* (A), *GCLC* (B), and (C) *GSTP1* gene expression levels (mean ± SE) in Merino ram semen supplemented with different concentrations of glycerol, boron, and trehalose after freeze–thaw process compared to the group G5B0.00 (*$p < 0.05$)

hydroxyl groups which are able to bind three water molecules.[63] It reduces ice crystal formation in the extracellular matrix and also prevents the emerge of osmotic pressure differences by entering the intracellular matrix.[64,65] However, detrimental effect of high concentrations of glycerol during cryopreservation also has been reported in several studies.[66–68] The researchers[69] reported the highest post-thaw motility rate of swine semen in the group frozen with 3% glycerol in compared with the groups frozen with 6% and 8% of glycerol. Previous research unfolds the decrement of sperm parameters according to the reduction of glycerol concentration because of its cryoprotective effects. To deal with the problem, adding adequate doses of trehalose, which is relatively less toxic, to the semen extender were suggested to compensate insufficient effect of low glycerol concentrations. The researchers[9] reported significant improvement in post-thaw ram sperm parameters via adding 3% glycerol and 60 mM trehalose to semen extender.

The effect of boron on the reproductive system has been investigated and it has been found to have a cytotoxic effect at high doses.[34,70] Also, male reproductive status in a boric acid/borate production zone at Bandırma, Turkey were investigated, and a weak correlation was observed between blood boron levels and mean DNA strand breaks in spermatozoa.[35] In another study, it was reported that boron compounds do not have a genotoxic effect even at the highest concentrations, but cause oxidative stress in increasing doses.[71] In the present study, different concentrations of boron (0.25, 0.5, and 1 mM) were added to 5% glycerol and 3% glycerol + 60 mM trehalose. Among all of the groups, the highest percentages of the most of post-thaw parameters belonged to G3B0.25 group in which boron amount was 0.25 mM. It was statistically significant for subjective motility, mitochondrial activity, and viability of spermatozoa comparing with G5B0.00 and other groups with boron. Furthermore, G3B0.00 had the second higher percentages of the same parameters and acrosome integrity as well in comparison with G5B1.00, G3B0.50, and G3B1.00. On the other hand, glycerol and boron concentrations used in G5B1.00 and G3B1.00 groups led to reduced motility, viability, mitochondrial activity, and acrosome integrity compared with G5B0.00 and G3B0.00, which were statistically significant for all of the parameters. As a result, these findings emphasize that a combination of 0.25 mM boron supplementation along with 3% glycerol and 60 mM trehalose in freezing extender serves an effective cryopreservation process particularly in terms of motility, mitochondrial activity, and viability of ram spermatozoa. A further finding demonstrated that 0.25 mM boron supplementation with 3% or 5% glycerol with or without trehalose can increase the acrosome integrity in comparison with the less or more boron concentration. It is by now generally accepted that, the increased boron concentration in the extender may cause to important adverse effects on the mentioned microscopic parameters. So, one could draw a conclusion of using adequate amount of boron to prevent side effect of cryopreservation of ram spermatozoa.

There are other studies on adding boron to diluents in freezing and preserving various cells, including stem cell and spermatozoa. Boron (20 μg/ml) has been shown to have a protective role in the viability of human tooth stem cells in short- and long-term cryopreservation.[72] Some positive effects of increasing doses of boron (sodium pentaborate) added instead of glucose components of Tris-based extender on spermatological parameters (such as total motility, progressive motility, semen kinetic parameters) have also been reported in freezing Ankara goat semen. However, in the presence of glucose, the administration of increasing doses of boron decreased motility.[73] It was shown that supplementation of the extender with boron (0.4 mg/ml) increased fertilization rates and also decreased DNA damages post-thaw in trout spermatozoa.[74] In another study,[39] it was observed that increasing doses of boron in the freezing of ram semen adversely affected sperm parameters. The toxic effect of increasing doses of boron (0.5 and 1 mM) was also demonstrated in this study which is in in accordance with our findings.

Sperm DNA damage accompanied by a decrease in fertility and pregnancy rates is a useful biomarker for the diagnosis of male infertility.[75] It is known that there is an increase in DNA single/double strand breaks, DNA condensation, and fragmentation after the freezing and storage of spermatozoa.[76] The effect of different components (antioxidants, sugars, etc.) in the cryoprotectant medium on DNA integrity has been extensively investigated.[77–81] TUNEL and COMET tests are methods that directly measure sperm DNA damage.[82] Both methods are frequently used both in assisted reproduction techniques studies of various animal species.[83–86] In this study, these two

methods, which are effective in detecting DNA integrity, were applied and as a result, no statistically significant effect on preventing DNA double strand break was been reported in COMET assay. By contrast, increasing boron concentration with 3% glycerol caused to decrease the DNA damage in which G3B1 had the highest significant DNA integrity comparing with G5B0.50. On the other hand, in another study, Ramlıç ram spermatozoa were frozen in diluents prepared with boron (1, 2, and 4 mM) added to the Tris-based extender containing 5% glycerol, and the DNA damage after the thawing was investigated by COMET assay. The DNA damage in the 1 mM boron group was significantly decreased compared to the other boron doses (0, 2, and 4 mM).[39] However, 1 mM boron added to the basic diluent containing 3% glycerol + 60 mM trehalose was found to inhibit DNA damage compared to the G5B0.50 (5% glycerol + 0.5 mM boron) group. Significant decrease in DNA fragmentation in G3B1.00 compared to G5B0.50 could be explained by lower glycerol concentration of G3B1.00 compared to G5B0.50 group. In addition, it seems that higher concentration of boron (1 mM) along with trehalose could have synergic effect to preserve sperm DNA integrity because by increasing the concentration of boron the percentage of spermatozoa with DNA damage were decreased.

Freezing–thawing process differentially affects the molecular activity of spermatozoa.[87] As it is well known, the molecular regulation of the organism is under the control of complex mechanisms at the mRNA and even miRNA levels.[88] Antioxidant regulators have vigorous roles for the fate of cells.[89] *NQO1* gene, one of these regulators, is a member of NADPH dehydrogenase family and is essential for the response to oxidative stress. It encodes the enzyme which reduces quinones to hydroquinones by catalyzing two-electron-mediated reaction.[49,90] In many tissues, such as breast, colon, and lung, cellular antioxidant capacity increases with higher *NQO1* mRNA levels.[90] Upregulation of *NQO1* gene expression occurs under cellular stress responses notably to oxidative stress.[90] In this study, the upregulation of *NQO1* gene was in G5B0.25 and G3B0.25 groups. In addition to this fact, interestingly the significant downregulation of this gene was observed in G5B1.00, G3B0.50, and G3B1.00. With the results of sperm quality parameters, it seems that lower boron administration has ameroliating effects both on sperm parameters and antioxidant genes, whereas high levels of boron has adverse effects, thus 1 mM boron dose decreased sperm quality. *GCLC* gene is assumed to be upregulated via nuclear Nrf2 pathway and increasing testicular antioxidant capacity because of cellular response to oxidative stress.[50,91] In a study, decreasing in *GCLC* was demonstrated in mice with nuclear Nrf2 knockout, subsequent to decreasing in testicular antioxidant capacity which led to decreased sperm motility and sperm production, while oxidative stress was increased.[50] In another study,[46] it was stated that *GCLC* gene expression level was 1.31-fold more in frozen–thawed bull spermatozoa in comparison with fresh samples. It is accepted that spermatozoa because of the high polyunsaturated fatty acid content are susceptible to oxidative damage; so, *GCLC* upregulation in frozen–thawed spermatozoa is considered as a protective response of spermatozoa to chilling and oxidative stress. Moreover, *GCLC* gene was known as a part of glutathione metabolic pathway as well,[46] and also was reported that *GCLC* tends to increase in bull spermatozoa after freeze–thaw procedure.[46] In this study, as *GCLC*, the transcriptional level of this gene was found remarkably lower in 5% glycerol + 1 mM boron and 3% glycerol + 60 mM trehalose + 1 mM boron groups compared to the control (5% glycerol). *GSTP1* gene was suggested as another protective gene against oxidative DNA damage in spermatozoa.[92] The researchers[93] noted that, in human prostate tissue, *GSTP1* prevents the cell against oxidative damage. In the current study, *GSTP1* gene expression was significantly decreased in 3% glycerol + 60 mM trehalose + 1 mM boron group compared to the control group. It was thought that the mRNA activities of these genes might reflect the powerful antioxidant effects of combination of trehalose and boron with appropriate concentrations. According to the mRNA levels of *NQO1*, *GCLC*, and *GSTP1* genes, it is possible to argue that the higher concentrations of boron have potential suppressive effect on oxidative status of ram semen after freezing–thawing process. In overall, the higher but not significant expression of antioxidantive genes (*NQO1*, *GCLC*, and *GSTP1)* indicated that these genes were not overexpressed in the mature spermatozoa because it seem that, mature spermatozoa is relatively silent transcriptionally. Therefore, supplementation of antioxidant such as boron along with trehalose only preserved these genes against detrimental effect of cryopreservation. So, we did not expect overexpression of these kind of genes by supplementation of boron or such antioxidants.

5 | CONCLUSIONS

Boron concentration of 0.25 mM added to 3% glycerol + 60 mM trehalose resulted in higher percentages of subjective motility, mitochondrial activity, and viability of spermatozoa comparing with G5B0.00 and groups with boron. G3B0.25 and G5B0.25 showed higher acrosome integrity, compared with G5B0.00, G5B1.00, G3B0.50, and G3B1.00. For TUNEL analysis, G3B1.00 showed the highest DNA integrity among the experimental groups which was statistically significant only with G5B0.50. The expression levels of NQO1 were significantly decreased in G5B1.00, G3B0.50, and G3B1.00, when compared to G5B0.00. In comparison with G5B0.00, supplementation of 1 mM boron with and without trehalose had significantly lower expression of GCLC. The level of *GSTP1* gene was significantly lower (approximately threefold) in G3B1.00, compared to G5B0.00. It can be assumed that increased boron concentration in the extenders may cause important adverse effects on post-thaw sperm parameters and antioxidant gene expressions. These results obtained from the study will shed light in understanding boron toxicity limits and eliminating glycerol toxicity in gamete and tissue freezing studies. So, one could draw a conclusion of using adequate amount of boron to decrease cryodamages of cryopreservation of mammalian spermatozoa as well tissue engineering.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

AUTHOR CONTRIBUTIONS

Mustafa Numan Bucak and Nuri Başpınar designed the study. Mustafa Numan Bucak, Mustafa Bodu, Ali Erdem Öztürk, Bülent Bülbül, Mesut Kırbaş, and Şükrü Dursun collected, freezed sperm samples, and performed sperm quality analysis. Nazan Keskin and Pınar İli performed COMET and TUNEL analyses. Hüseyin Özkan performed gene analysis. Fateme Frootan performed statistical analysis. Mustafa Numan Bucak and Fateme Frootan designed table and figures, and wrote and edited the manuscript.

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