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Rock salt and boron supplementation: Effects on oxidative stress in blood and semen of rams

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ABSTRACT: This study aimed to investigate the effects of boron and rock salt minerals, which are interesting as antioxidant minerals on malondialdehyde (MDA) and some antioxidants; glutathione level (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) enzyme activities in blood and sperm cells. For this purpose, a total of 36 rams were divided into six research groups, with six rams in each group. The research groups were designed as Control (Just 40 ml water), Salt (10 g rock salt/day), B20 (20 mg boric acid/day), B40 (40 mg boric acid/day), BT20 (20 mg boric acid + 10 g rock salt/day), BT40 (40 mg boric acid + 10 g rock salt/day). The additives were dissolved in 40 ml of water and administered orally with 50 ml syringes for 80 days. Semen and blood samples were taken from the rams at the end of the experiment. Salt added to the diet significantly increased MDA levels in blood and sperm cells. A significant decrease in plasma MDA levels were observed in the BT40 group and sperm cells in the B40 and BT40 groups. Salt and boric acid were found to be effective on GSH and antioxidant enzymes. In conclusion, it was found that boric acid added to the diet at a dose of 40 mg enhanced antioxidant metabolism and significantly reduced salt-induced lipid peroxidation.

Keyword: Antioxidants; Boron; Oxidative stress; Ram; Salt

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INTRODUCTION

Oxidative stress is defined as an imbalance between the production of free radicals and their neutralization by antioxidant defenses. A low amount of reactive oxygen species (ROS) is necessary for the organism's physiological functions; however, an excess causes oxidative damage to various molecules, negatively affects the DNA and proteins of cells and causes lipid peroxidation of cellular membranes (Corino and Rossi, 2021; Tırascı and Simsek, 2024). Oxidative stress is also associated with many pathological disorders that impair animal health, welfare and productivity parameters. Malondialdehyde (MDA), used to determine oxidative stress levels, is an end-product of polyunsaturated fatty acid peroxidation and a widely accepted biomarker for oxidative damage to cell membranes (Ponnampalam et al., 2022). Elevated MDA levels correlate with increased lipid peroxidation, indicating oxidative degradation of phospholipids in cellular membranes, which disrupts membrane integrity and function (Surai and Earle-Payne, 2022). In animal studies, MDA accumulation has been linked to heat stress, inflammatory conditions, and metabolic disorders, making it a critical indicator of redox imbalance (Salazar-Hernández et al., 2025). Antioxidants are natural or synthetic derivatives that prevent free radical reactions, inhibit the formation of these radicals, scavenge them or reduce tissue damage by promoting their decomposition (Silvestrini et al., 2023). Glutathione (GSH) is a powerful antioxidant that protects cells from free radicals' damaging effects. Glutathione peroxidase (GSH-Px) detoxifies hydroperoxides and lipid peroxides using glutathione. Superoxide dismutase (SOD) converts superoxide radicals into the less harmful hydrogen peroxide. Catalase (CAT) is an enzyme that breaks down hydrogen peroxide into water and oxygen, thus preventing cellular damage. Many intracellular antioxidants are used to prevent oxidative damage. Vitamins (C, E, etc.), enzymes (superoxide dismutase, catalase, glutathione peroxidase, etc.) and minerals (selenium, sulfur, copper, boron, etc.) have been shown to protect the cell against lipid peroxidation and DNA damage, the first step in many pathological processes (Khaliq et al., 2018; Sonawane et al., 2024).

Salt (NaCl) is essential in many plants, human, and animal diets. It consists of two elements: sodium and chloride (rock salt). Both are important for maintaining the acid-base balance of blood and osmotic pressure in tissues (AL-Enzy et al., 2020). It plays an important role in regulating the balance of body fluids and transmitting nerve impulses, maintaining rhythmic heart movements, and metabolism of carbohydrates and proteins (Nielsen, 2017). Salt has been reported to be an important mineral compound that animals may crave. In a study in poultry (AL-Enzy et al., 2020), sodium chloride supplementation to the diet was reported to have an antioxidant effect by reducing the synthesis of free radicals, increasing water and feed consumption, and compensating for the loss of electrolyte solutions.

The trace element boron is a dynamic nutrient of fundamental importance for animal and human biology. Inorganic borates are converted to boric acid even at low physiological pH levels and absorbed by mucosal surfaces (Khaliq et al., 2018). Boron is a multifunctional element for both humans and animals and is present in trace amounts in tissues. Animals do not experience boron deficiency as long as they receive 0.3 mg of boron per kilogram of body weight per day (mg/kg/d) in their diet. The highest tolerable level of boron is known to be 150 mg per kilogram of body weight per day (mg/kg/d) (Cengiz et al., 2020). Based on the negative effects of high doses of boron on reproductive function and development, the US Food and Nutrition Board has set the upper limit for this microelement at 20 mg/day (Estevez-Fregoso et al., 2023). Nielsen (2017) stated that boron affects the action of hormones, signal transduction in the cell membrane and the function and stability of the membrane. Boron plays an important role in various metabolic processes, such as hormone receptors, transmembrane signaling and lymphocyte proliferation. Boron deficiency is also noted for its negative effects on the immune system and metabolism (Zhao et al., 2023). The effects of boron on the antioxidant system: It has been revealed in various studies that it regulates the functions of cell membranes and has oxidative stress inhibitory properties (Khaliq et al., 2018; Ayşan et al., 2024). S-adenosylmethionine has a high affinity for boron. S-adenosylmethionine directly or indirectly affects antioxidant metabolism by taking part in methylation reactions, keeping glutathione levels in balance by regulating homocysteine metabolism, synthesis of neurotransmitters, and protection of nerve cells (Nielsen, 2017; Kan and Kucukkurt, 2023).

Climate change creates complex and multidisciplinary shifts in global and regional climate patterns that pose significant human and natural systems. It is one of the most complex multifactorial global challenges that also threatens livestock production and productivity performance. Livestock farming is vulnerable to climate change and affects many factors, such as adaptation, disease resistance, body indices, and reproductive traits (Naim et al., 2023). Livestock production based on the extensive breeding system is vulnerable to climate change. Especially, sheep breeding in the extensive system is sensitive to climate threats. Turkey's natural structure and environmental conditions offer unique advantages that make sheep breeding stand out (Kandemir and Taşkın, 2022). Research is needed to maintain the productivity and sustainability of this production under extensive conditions. Given the proven roles of salt (NaCl) and boron in antioxidant activity, metabolic regulation, and stress reduction, it was hypothesized that dietary supplementation of these minerals would improve both blood and sperm cell redox homeostasis by increasing oxidative stress resistance in rams. By examining the synergistic effects of salt and boron on oxidative stress markers in blood and sperm cells, this study aims to provide potential strategies for mitigating climate-induced stress, especially in extensive sheep farming. It also evaluates the effects on reproductive health and metabolic resistance in rams. Considering the potential benefits of mineral supplements on reproductive health and general metabolic status, this study aims to evaluate the effects of dietary salt and boron on oxidative stress levels in blood and sperm cells in rams.

MATERIAL-METHODS

Establishing ethical permission and trial layout

The study received the appropriate permission from the Experimental Animals Local Ethics Board of the Firat University 21.02.2024 and 2024/04-02. The research was carried out in a community-held flock in the Elazığ Region. The animal material of the project's proposal was obtained from this herd. The male materials of the research were composed by 36 rams, who were clinically healthy, known for their fertility, and whose genital organ examination showed no pathological findings. Six rams were used for each trial group. The rams were placed in closed compartments 45 days before ramming season, with each ram being 4 m^2/ram. During the entire trial, the rams were fed quality barley and straw. The barley was given at 600 g and 1000 g was increased towards the ramming season and, 1-1.5 kg of straw per ram. Drinking water was also provided as *ad libitum*. Salt (rock salt), boric acid, and mixed supplements were individually weighed (Weightlab-WL 303) prepared each animal at the Firat University of Veterinary Zootechni AD. Since spermatogenesis in rams lasted 45-47 days (Yeni and Gündoğan, 2018), the addition of boron and salt to the ram's diet began 45 days before the ramming season. Features of salt and boric acid used in the research are presented in Table 1. Contribution to the diet continued for a total of 80 days (pre-ramming + ramming period). The recommendations of Krishnan et al. (2019) and Cortés et al. (2017) were used to determine the boron dose to be given to the animals in the applications. Rams taking 20 and 40 mg of boric acid (H3BO4) per day are estimated to take 3.5 and 6.98 mg of boron (B) per day. Rams were divided into test groups, with body conditioning scores and their age in mind. Research groups were organized as specified; Control Group (K): The group, which was given 40 ml daily tap water; the Salt Group (T) for which rams were given 10g/day rock salt; the Boric acid 20 group (B20): Group given 20 mg of boric acid/day to rams; the Boric acid 40 group (B40): 40 mg of boric acid/day to rams; the BT20 group: 20 mg of boric acid + 10 g of rock salt/day to rams; the BT40 Group: Rams were given 40 mg of boric acid + 10 g of rock salt/

Table 1. The content of boric acid and rock salt used in the research

FEATURES	Rock Salt	Boric Acid
Chemical formula	NaCl	H3BO3
Molecular mass	58,433 g/mol	61.83 g mol −1
Appearance	Colorless cubic crystals	White solid crystal
Intensity	2.1-2.6 g/cc	1.435 g/cm3
Solubility (in water)	360 g/1000 g T = 25 °C pure water	2.52 g/100 mL (0 °C) 4.72 g/100 mL (20 °C) 5.7 g/100 mL (25 °C) 19.10 g/100 mL (80 °C) 27.53 g/100 mL (100 °C)

day. During the entire research period, contributions were melted in 40 ml of water and applied virtually every day with the help of 50 ml injectors. Rams of research groups were numbered using oily paints for convenient implementation of contributions, and injectors for use in each ram group were painted the same color, minimizing confusion. Research groups were marked with different colors as Control (Blue), Salt (Black), B20 (Green), B40 (Red), BT20 (Orange) and BT40 (Yellow). Rams were accustomed to the artificial vaginas before the ramming season began. Throughout the ramming season, sheep were taken indoors to be 20 ewes/ram; free ramming was applied. At the end of the ramming season (on the 80th day of the experiment), semen and blood samples were taken. Twenty-four hours before the semen was taken, all the rams were separated from the sheep and taken into separate compartments, using artificial vagina samples. Blood samples were taken from the *V. Jugularis* to tubes containing EDTA, about 10 ml. The blood-taking area was erased antiseptically with alcohol and each animal was given a vacutainer in sterile conditions using a separate needle (Washington and Van Hoosier, 2012).

Biochemical analysis

The blood samples were taken in full blood for GSH and GSH-Px, and the remaining blood was used for transfusion of blood plasma MDA, with 10 min of acute over 3.000 cycles. The remaining erythrocyte packet was used to transfer CAT and SOD after being washed 3 times with NaCl of 0.9%. The semen was centrifuged at 4000 rpm for 15 minutes, and removed the sperm cells were removed from the pellet. The collected sperm cells were diluted at 1:10 (weight/volume) with KCl of 1.15%, homogenized in the homogenizer, and the homogenates obtained supernatant at 3500 rpm with a centrifuge of 15 minutes. These supernatants have been designated for MDA, GSH-Px, CAT, SOD, GSH, and protein. MDA level was determined according to the method of Placer et al. (1966), and the GSH level was determined according to the method of Chavan et al. (2005). Matkovics et al. (1988) method was used to measure GSH-Px activity, Góth (1991) method was used to measure CAT activity and Sun et al. (1988) method was used for SOD activity. The cyanmethemoglobin method was used for hemoglobin determination, and the modified method was used for protein measurement (Lowry et al., 1951).

Statistical analysis

All the data obtained by the study were analyzed for normality (Shapiro-Wilk) and found that the data were dispersed normally. Variance analysis was used to compare the biochemical parameters of the six trial groups, and further analysis took advantage of the Duncan test. All analysis is done using the SPSS 22 package program, with a level of 0.05 considered meaningful. The data is presented as average and standard error. The GraphPad Prism 8.0 program was used to deliver the data.

RESULTS

The effects of salt and boron supplements on blood tissue oxidative stress parameters in rams are given in Figure 1. Plasma MDA levels were found highest in the Salt and BT20 groups. Control was found to be similar to B20 and B40 groups and significantly lower than other groups. In the BT40 group, the MDA level is significantly reduced compared to Salt and BT20 groups ($P<0.001$). No significant differences were detected among groups at the GSH level ($P>0.05$). GSH-Px enzyme activity was found in the highest group B20, the lowest group B40 and BT40 ($P<0.01$). The Control, Salt and BT20 groups have similar values to other groups. SOD enzyme activity was found to be highest in B20=Salt>BT40=Control=B40>BT20 groups, respectively ($P=0.013$). CAT activity is highest in the B20 group and lowest in the Control group ($P<0.001$).

The effects of salt and boron supplements on oxidative stress parameters of rams in sperm cells are stated in Figure 2. The highest level of MDA in sperm cells was found in the Salt group and the lowest in the B40 and BT40 groups ($P<0.05$). The GSH level was found to be Salt>Control>B40=BT40>B20>BT20, respectively ($P<0.05$). GSH-Px activities of the sperm cells were found to be high in groups B40, BT20, and BT40 ($P<0.05$). SOD enzyme activity was found in the lowest group B40, in the highest Salt and Control groups ($P<0.001$). The highest CAT activity was detected in the Salt and BT40 groups and the lowest in the Control and B20 groups ($P=0.001$).

DISCUSSION

The increased metabolic and mechanical workload on tissues increases the oxygen consumption of tissues, and increased oxygen consumption increases ROS and thus the number of free radicals (Kalaçay and Atakişi, 2022). The structural formation of ox-

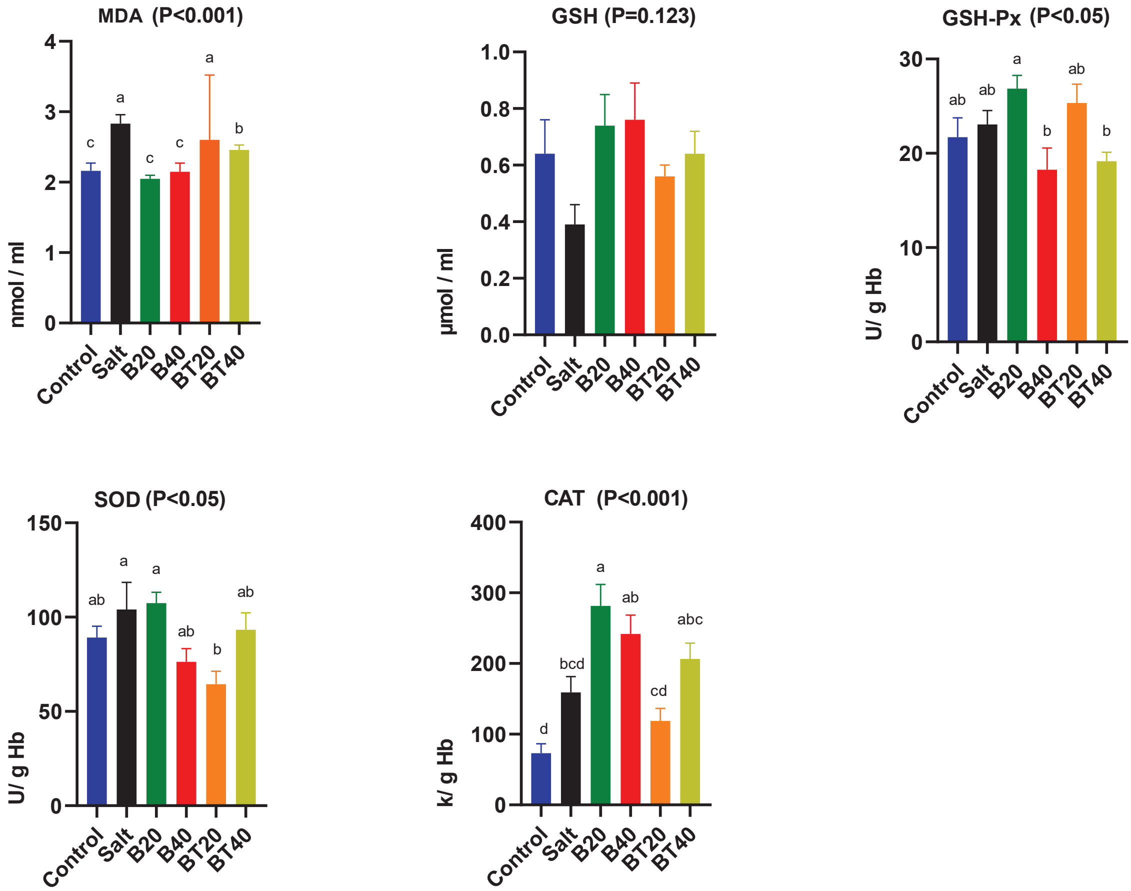

Figure 1. The effects of boric acid and salt supplements on lipid peroxidation and some antioxidants in the blood

idants is balanced by producing antioxidants at a similar rate. The oxidants and antioxidant species imbalance causes oxidative stress due to lipid peroxidation. Increased MDA concentrations reflect the extent of oxidative stress (Sogut et al., 2015; Cordiano vd., 2023). Salt is often used as a carrier to ensure trace mineral intake, and NaCl addition to the diet of sheep and goats is necessary to meet their mineral needs (Rankins and Pugh, 2011). Giving 8-11 g of salt daily to sheep. Minerals in salt participate in numerous metabolic activities, but few studies have examined the effect of salt on oxidative stress metabolism (AL-Enzy et al., 2020; Mahmood et al., 2020). Recent research suggests that high salt intake may exacerbate oxidative stress by increasing MDA levels, as observed in both blood and sperm tissues in recent studies and in the current study (Mohamed et al., 2024; El-Kazazaz et al., 2020). Although the effects of boron on cell membrane functions and enzymatic reactions are well known, its effects on oxidative stress are among the current and controversial issues. Although studies show that boron decreases MDA levels by reducing the oxidation of lipids (Kan and Kucukkurt, 2023; Cakir et al., 2018), Özdemir et al. (2016) concluded that boron supplementation in the diet for six weeks did not change blood MDA levels in Japanese quail. Gu et al. (2007) emphasized that the dose used is important for boron to be metabolically effective and may have toxic effects if used excessively. In the current study, the effects of different doses of rock salt and boron salt (boric acid) on lipid peroxidation and antioxidant enzymes were investigated in rams. It was thought that these salts alone and/or synergistically would positively affect tissue lipids and decrease lipid peroxidation due to the active components in their structures. However, when the study's findings were considered, it was determined that MDA levels were higher in both the blood and sperm tissues of the salt group compared to the control group. The elevated MDA levels in the Salt group compared to Control, indicating salt-induced oxidative stress. Plasma MDA levels were

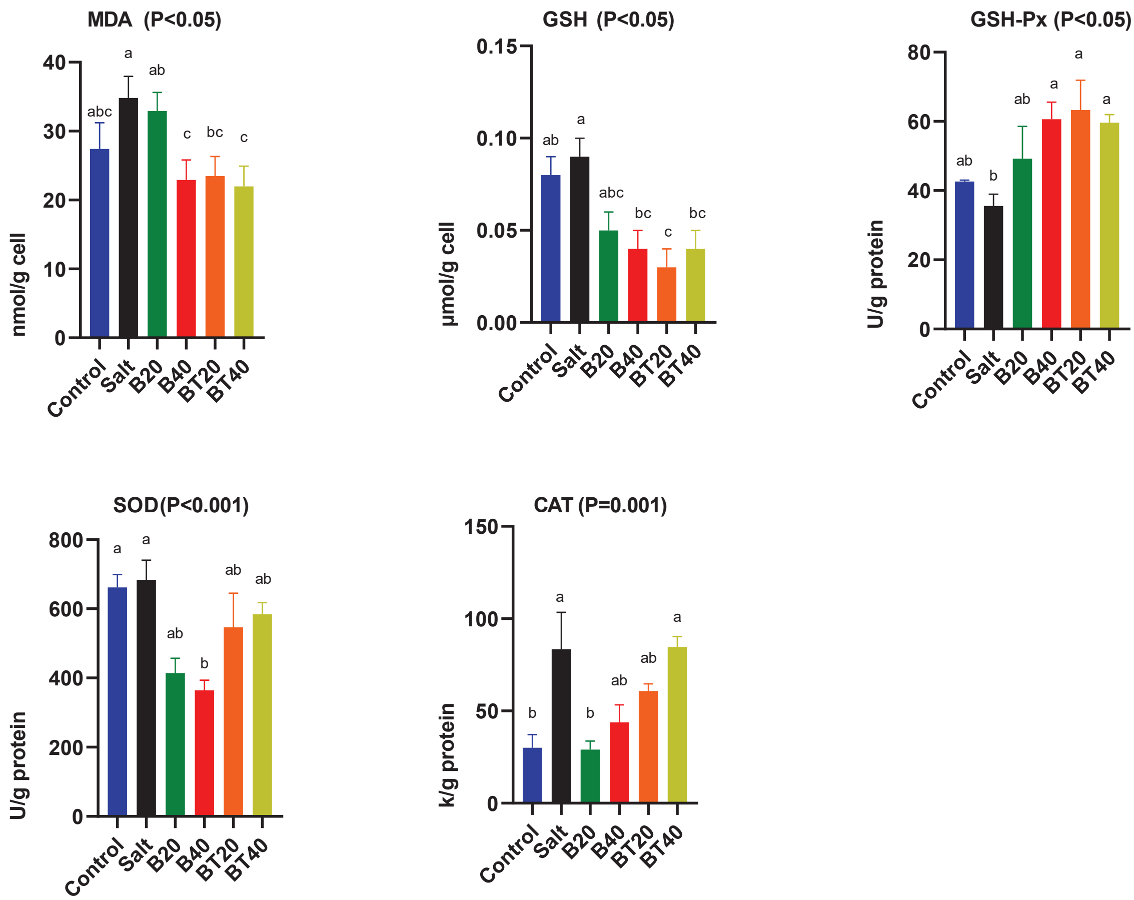

Figure 2. The effects of boric acid and salt supplement on lipid peroxidation and some antioxidants in sperm cells

significantly higher in the Salt group, while MDA levels in the B20 and B40 groups were similar to the control group. In the groups in which salt and boron were used together, the toxic effect of salt was found to be significant in the BT20 group, and the plasma MDA level decreased significantly in the BT40 group. These results are consistent with the findings of Kan and Küçükkurt (2023) who reported the antioxidative properties of boron under stress conditions. In sperm cells, the observed reduction in MDA levels in the B40 and BT40 groups suggests that boron may counteract salt-induced lipid peroxidation. This is consistent with studies demonstrating boron's protective role against oxidative damage in reproductive tissues (Krishnan et al., 2019; Avdatek et al., 2022). Furthermore, the synergistic effect of boron and salt in reducing MDA levels (particularly in the BT40 group) highlights its potential as an antioxidant modulator (Kucukkurt et al., 2015; Ince et al., 2020).

Glutathione and GSH-Px are the most important antioxidants at the cellular level and are critical defense mechanisms against oxidative stress. SOD and CAT are antioxidant enzymes involved in the removal and detoxification of free oxygen radicals. Mohammed et al. (2019) reported that saline water adversely affected oxidative markers in sheep, corroborating the findings of elevated oxidative stress in the Salt group. Similarly, Mahmood et al. (2020) found that high-salt environments triggered oxidative stress in goats, which was alleviated by antioxidant supplementation. They found that vitamin E and selenium given to pregnant goats reduced lipid peroxidation, improved enzymatic and non-enzymatic antioxidant enzymes, and improved birth weights, survival and growth characteristics in kids. In previous studies (Khaliq et al., 2018; El-Saadany et al., 2016), it was found that boron derivatives increased SOD activity and decreased glutamyl transpeptidase activity by regulating NADPH production, and this mechanism was found to be effective in making cells more resistant to oxidative stress by increasing the overall amount of antioxidants in the body. There are also findings that boron reduces oxidative damage by promoting the production of glutathione and its derivatives or by increasing the activity of agents that neutralize ROS (Kucukkurt et al., 2015). Turkez et al. (2007), observed that low doses (15 mg/L) of boron compounds of blood concentration increased both SOD and CAT activities in erythrocytes compared to the control group, while high doses (500 mg/L) decreased both SOD and CAT activities, making erythrocytes sensitive to oxidative damage. While no significant difference was detected in the GSH levels of the research groups, plasma GSH-Px activity was found to be low in the B40 and BT40 groups. This was associated with the metabolic utilization of this enzyme. The high plasma SOD activity in the salt and B20 groups suggests a cellular response for detoxification of superoxide radicals. High SOD activity indicates that superoxide radicals are overproduced as a result of increased oxidative stress and that the cell activates defense mechanisms against this stress. While plasma SOD and CAT activities were effective on salt stress in B40 and BT40 groups, the dose of boric acid was not effective in overcoming the stress in the BT20 group. In the male reproductive system, testes produce high levels of ROS compared to other tissues and are more vulnerable to oxidative damage despite having stronger antioxidant capacities. Oxidative stress damages the sperm cytoplasmic membrane, which is rich in highly unsaturated fatty acids and phospholipids (Barbagallo et al., 2020). High MDA levels indicate increased cellular damage in sperm, which may lead to fertility problems. Mohamed et al. (2024) added salt levels (350 ppm, 4557 ppm and 8934 ppm) to the drinking water of rams for five months. In medium and high salt groups, blood MDA, nitric oxide and cortisol levels increased significantly while hormone levels decreased. Blood GSH level and SOD activity were found to be significantly lower in low and high salt groups. Although there were pathologic changes in sperm cells and testicular tissue in these groups, the motile sperm rate was similar in the research groups. In the present study, GSH level in sperm cells was found to be significantly higher in the Salt group, and GSH-Px activity was significantly higher in the B40, BT20 and BT40 groups. At the same time, the SOD enzyme was high in Control and Salt groups, while the CAT enzyme was low in Control and B20 groups. Anti-oxidant mechanisms and different modes of operation of enzyme systems have been considered as mechanisms that are activated to prevent lipid peroxidation in sperm cells (Simsek et al., 2024). In recent studies (Krishnan et al., 2019; Avdatek et al., 2022), it was determined that different boron salts at appropriate doses added to the diet significantly improved sperm quality.

CONCLUSION

As a result, it was determined that salt at the given dose (10 g/day) increased oxidative stress, increased MDA levels, and damaged blood and sperm cells. Boric acid functioned significantly at the cellular level in the fight against oxidative stress and reduced cellular damage by neutralizing free radicals. Boric acid was found to be more effective under high oxidative stress caused by salt. High doses of boric acid were especially effective in reducing oxidative stress by increasing antioxidant enzyme activities such as GSH-Px and CAT. In this direction, it can be emphasized that effective doses of boron derivatives have the potential to reduce oxidative stress and protect sperm health. It was determined that boron salts have an important antioxidant potential for the continuation of productivity in sheep and goat species, which are likely to be exposed to salt stress in barren soils in parallel with climate changes and global warming in the future.

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