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Effects of Endurance Training and *Tribulus terrestris* Extract on Oxidative Stress and Apoptosis Markers in the Liver Tissues of Rats Exposed to Arsenic

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ABSTRACT

Introduction: Arsenic (As) is a toxic metal, which causes disorders in various tissues of the body, including the liver. Studies have shown that exercise and herbs such as *Tribulus terrestris* (T) have antioxidant effects on some diseases. The present study aimed to investigate the effects of endurance training (ET) and T extract on oxidative stress and apoptosis markers in the liver tissues of rats exposed to As.

Methods: This experimental study was conducted on 49 rats, which were randomly assigned into seven groups of control, As, As with 5 mg/kg of T extract (As+T5), As with 10 mg/kg of T extract (As+T10), As with ET, As with ET and 5 mg/kg of T extract (As+ET+T5), and As with ET and 10 mg/kg of T extract (As+ET+T10). For eight weeks, study groups 2-6 consumed sodium arsenite (68 mg/l per day) dissolved in drinking water, and groups 5-7 ran on a treadmill five sessions per week (30 minutes per session) at the speed of 23 meters per minute.

Results: Exposure to As significantly increased O-6-methylguanine-DNA methyltransferase (MGMT), cytochrome C, malondialdehyde (MDA), and prooxidant-antioxidant balance (PAB), while decreasing hepatic adenosine triphosphate (ATP) (P=0.001). Training and T extract consumption reduced the concentration of MGMT, cytochrome C, MDA, and PAB, while increasing the hepatic ATP concentration (P=0.001). The T extract at the dose of 10 mg/kg could increase the ATP concentration and decrease cytochrome C, MDA, and PAB more significantly in the liver tissues compared to the dose of 5 mg/kg (P=0.001). In addition, the interactive effects of training and T extract consumption were significant on the reduction of MGMT, cytochrome C, MDA, and PAB concentrations and increasing the hepatic ATP concentration in the rats poisoned with As (P=0.001).

Conclusion: According to the results, endurance training with the consumption of *Tribulus terrestris* extract could inhibit oxidative stress and apoptosis, thereby exerting protective effects on liver tissues against arsenic poisoning. Therefore, these interventions could effectively diminish the effects of arsenic induction on liver tissues.

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Introduction

Arsenic (As) is a heavy metal and metalloid found in soil and the climate, with wide applications in cosmetics, pesticides, and even the treatment of acute promyelocytic leukemia (1). However, the abnormal doses of this element have been reported to cause cancer in various tissues, such as the liver, skin, lungs, and bladder (2). Increased reactive oxygen species (ROS) and reactive nitrogen species are the main mechanisms of the damage caused by As to various tissues, which in turn leads to the release of cytochrome C, caspase cascade activation, and cell death induction (1, 2). According to the literature, As increases ROS, followed by aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase, malondialdehyde (MDA), while also decreasing antioxidant enzymes such as superoxide dismutase (SOD) and catalase in the liver (3).

Researchers have observed that prolonged exposure to As for 4-8 weeks in animals models could lead to neurological disorders (Chang, 2015), the activation of the apoptotic mechanism (Qu, 2002), increased oxidative stress, and inhibition of nuclear gene transcription (Hu, 2020). Furthermore, the increased ROS caused by As has been reported

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to increment the tendency of ROS to binding to the proteins in the membrane and within the cell. By activating the DNA methylation pathway and destruction of the chromatin structure and genome stability, ROS impair the cell function in the transcription of the proteins that are essential to cell life (4).

Due to the irreparable damages caused by As, exercises seem to be an effective approach to the prevention and treatment of liver damage in cases with heavy metal poisoning. In other words, regular and long-term sports activities could enhance the liver function through increasing antioxidant capacity and reducing oxidative stress and apoptotic factors (5). A study in this regard indicated that eight weeks of interval and continuous training reduced the levels of caspase-3, cyclin D, and cytochrome C in the liver tissues of rats poisoned with cadmium (5, 6). Another research showed that 12 weeks of swimming could improve cellular and neurotrophic brain tissue function in the rats poisoned with various doses of As (7). Although comprehensive data are scarce regarding the effects of exercise on liver tissues and As poisoning, eight weeks of endurance training (ET; five sessions per week) have been reported to exert no significant effects on the reduction of oxidative stress in the spleen and kidney tissues of lead-poisoned rats (8).

Despite the ambiguity regarding the effectiveness of ET in the treatment As poisoning, the use of medicinal plants such as *Tribulus terrestris* (T) has attracted the attention of researchers owing to the fewer side-effects compared to synthetic drugs (O'Mahony Carey, 2014). T grows widely in tropical regions and contains antioxidant compounds, polyphenols, steroid-like substances, estradiol saponins, flavonoids, and alkaloids and improves the liver, kidney, brain, and heart function (9). The dose-dependent consumption of the T extract at the doses of 100, 200, and 300 mg/kg has been reported to reduce the indicators of kidney tissue damage caused by mercury, with more favorable effects observed at the higher doses (10). In addition, the consumption of 250 mg/kg of T methanolic extract for 25 days has been shown to enhance fasting glucose, increase the antioxidant capacity, improve the lipid profile, and lead to the optimal function of the liver tissues of healthy rabbits (11).

According to a study in this regard, 750 mg/kg of T extract increased antioxidants, regulated the mitogen-activated protein kinase P38 pathway, inhibited apoptosis from the internal pathway by exerting anti-inflammatory effects, and improved the mitochondrial function in the kidney and liver tissues of rats (12, 13). Studies have also demonstrated that the consumption of T and its products along with exercise could positively influence athletes (13, 14).

Despite the high prevalence of liver damage due to exposure to heavy metals, limited studies have been focused on the simultaneous effects of exercise and T consumption on oxidative stress and apoptosis in liver tissue. Such studies could provide researchers with more information on the prevention and treatment of As poisoning. The present study aimed to investigate the interactive effects of ET and T extract on oxidative stress markers and apoptosis in the liver tissues of rats poisoned with As.

Materials and Methods

This experimental study was conducted on 49 adult male Wistar rats, which were obtained from the Center for the Breeding and Reproduction of Laboratory Animals at Islamic Azad University of Tehran Central Branch in Tehran, Iran. The mean weight of the animals was 200 grams, and they were aged 6-8 weeks. Animals food was purchased from Pars Animal Food Company (Tehran, Iran), including crude protein (23%), crude fat (3.5-4.5%), crude fiber (4-4.5%), maximum ash (10%), calcium (0.95-1%), phosphorus (0.65-0.75%), salt (5-5.5%), maximum humidity (10%), lysine (1.15%), methionine (0.33%), methionine and cysteine (0.63%), threonine (0.72%), and tryptophan (0.25%). The foods were preserved in the laboratory of the Islamic Azad University of Tehran Central Branch for one week to become adjusted to the new environment. The treatment of the laboratory animals in the present study was performed under the supervision of the Ethics Committee of the Islamic Azad University of Marvdasht Branch (ethics code: IR.IAU.M.REC.1399.030).

At the next stage, the rats were randomly assigned to seven groups, including control, arsenic (As), As with 5 mg/kg of T extract (As+T5), As with 10 mg/kg of T extract (As+T10), As with ET (As+ET), As with ET and 5 mg/kg of T extract (As+ET+T5), and As with ET and 10 mg/kg of T extract (As+ET+T10). For eight weeks, the rats consumed 68 mg/l of

sodium (meta) arsenite daily dissolved in drinking water (15). Groups 5-7 ran on a treadmill five sessions per week (30 minutes per session), and groups three, four, six, and seven were administered with specific doses of T extract daily (16). Forty eight hours after the last training session and T extract consumption, the rats were anesthetized in the fasting state using ketamine (50 mg/kg) and xylazine (10 mg/kg). At the next stage, they were sacrificed, and their liver tissues were extracted by laboratory specialists.

To separate the liver tissues, the abdominal cavity was opened, and the liver tissues were meticulously extracted. After weighing and washing, they were transferred to a special cryotype for tissue preservation. After freezing, the tissues were preserved at the temperature of -20°C with liquid nitrogen and transferred to a molecular cell laboratory immediately. ELISA kits were used for the measurement of ATP (Abnova Company, USA; catalog No. KA1661), MDA (Cusabio Company, Spain; catalog No. CSB-E08558r), cytochrome C (Cusabio Company, Spain; catalog No. CSB-E14281r), MGMT (Develop Company, USA; catalog No. DL-MGMT-Ra), and PAB.

Training Protocol

In order for the animals to adapt to the treadmill, the rats of the exercise groups ran on a treadmill for three sessions (5-10 minutes each) at the speed of 4-5 meters per minute. In the present study, moderate-intensity training (50-55% V_{O2max}) with physiologically efficient training was implemented. The exercise groups trained on a treadmill five days per week for eight weeks, with the frequency of three days of exercise and one day of rest as the training protocol. In each training session, five minutes was allocated to a warm-up and five minutes to cool-down at the rate of 4-5 m/min, which was added to the main training time. In addition, no electric shocks were used during the training program, and the animals were forced to continue training by using their hands or making an audio stimulus on the cover of the treadmill if necessary. The rats performed ET five days a week for two months at the speed of 23 meters per minute (30 minutes per day). All the animals accomplished the two-month training protocol (17).

Preparation of the Tribulus terrestris Extract

To prepare the T extract, the fruit of the plant was obtained from the botany department of the university. The fruit was ground initially, and 100 grams of the powder was added to 80 milliliters of 70% alcohol. The solution was preserved in the laboratory for three days. Afterwards, the solution was initially filtered through a filter paper; the liquid part was obtained using a vacuum purifier, and the dry extract of the plant was also obtained. After concentrating the extract with normal saline, the rats were administered with a specific dose daily (16). Notably, T extract was approved and used by the specialists of the Botany and Pharmacology Laboratory of the university.

Statistical Analysis

Data analysis was performed in SPSS version 22 using the Shapiro-Wilk test to assess the normal distribution of the data, and the effects of As on the research variables were evaluated using independent sample t-test. In addition, the main effects of training and T extract and the interactive effects of training and T extract were determined using the two-way analysis of variance (ANOVA). The differences between the doses were also evaluated using Bonferroni comparison of means ($P \leq 0.05$).

Results

Figures 1-5 depict the levels of the research variables. According to the results of independent sample t-test, the levels of MGMT (P=0.001), cytochrome C (P=0.001), MDA (P=0.001), and PAB (P=0.001) were significantly higher in the poisoned control group compared to the healthy control group. However, the concentration of ATP in the poisoned control group was significantly lower compared to the healthy control group (P=0.001).

The results of the two-way ANOVA indicated that training (P=0.001) and T extract consumption (P=0.001) could significantly reduce the concentration of hepatic MGMT in the rats poisoned with As. Furthermore, the interactive effect of training and T extract consumption was significant on the reduction of the hepatic MGMT concentration (P=0.001). The results of Bonferroni post-hoc test also showed that the T extract at the doses of 5 mg/kg (P=0.001) and 10 mg/kg (P=0.001) significantly affected the reduction of the hepatic MGMT concentration, while no significant difference

was observed between the doses of 5 and 10 mg/kg in this regard (P=0.07) (Figure 1).

According to the obtained results, training (P=0.001) and T extract consumption (P=0.001) could significantly increase the ATP concentration in the liver of the rats poisoned with As. In addition, the interactive effects of training and T extract consumption were considered significant on increasing the concentration of hepatic ATP (P=0.001). The consumption of the T extract at the concentrations of 5 mg/kg (P=0.001) and 10 mg/kg (P=0.001) significantly increased the liver ATP concentration. In addition, the ATP concentration at the dose of 10 mg/kg was higher compared to the dose of 5 mg/kg (P=0.001) (Figure 2).

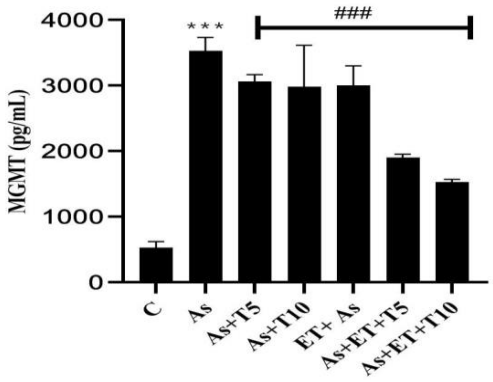

Figure 1. The results of the two-way analysis of variance (ANOVA) for MGMT levels in the research groups
C: Control, As: Arsenic, T5: 5 mg/kg of Tribulus terrestris, T10: 10 mg/kg of Tribulus terrestris, ET: Endurance Training
***Significant increase compared to the C group (P = 0.001)
###Significant decrease compared to the As group (P = 0.001)

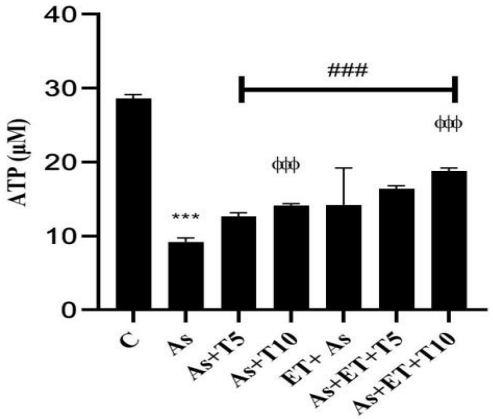

Figure 2. The results of the two-way analysis of variance (ANOVA) for ATP levels in the research groups
C: Control, As: Arsenic, T5: 5 mg/kg of Tribulus terrestris, T10: 10 mg/kg of Tribulus terrestris, ET: Endurance Training
***Significant increase compared to the C group (P = 0.001)
###Significant decrease compared to the As group (P = 0.001)
φφφ Significant increase compared to the As + ET + T5 and As + T5 groups (P = 0.001)

Similarly, training (P=0.001) and T extract consumption of (P=0.001) could significantly decrease the hepatic cytochrome C concentration of the rats poisoned with As.

According to our findings, the interactive effects of training and T extract consumption were significant on the reduction of the liver cytochrome C concentration (P=0.001). In addition, using the T extract at the concentrations of 5 mg/kg (P=0.001) and 10 mg/kg (P=0.001) could significantly reduce the concentration of liver cytochrome C. On the same note, cytochrome C concentration was lower at the dose of 10 mg/kg compared to the dose of 5 mg/kg (P=0.001) (Figure 3).

According to our findings, training (P=0.001) and T extract consumption (P=0.001) had significant effects on the reduction of the MDA concentration in the liver of the rats poisoned with As. In addition, the interactive effects of training and T extract consumption were significant on the reduction of the hepatic MDA concentration (P=0.001). The extract doses of 5 mg/kg (P=0.001) and 10 mg/kg (P=0.001) also had significant effects on the reduction of the hepatic MDA concentration, and the MDA concentration was lower at the dose of 10 mg/kg compared to the dose of 5 mg/kg (P=0.001) (Figure 4).

The obtained results demonstrated that training (P=0.001) and T extract consumption (P=0.001) had significant effects on the reduction of the PAB concentration in the liver tissues of the rats poisoned with As. Furthermore, the interactive effects of training and T extract consumption were significant on the reduction of the PAB concentration (P=0.001). The extract doses of 5 mg/kg (P=0.001) and 10 mg/kg (P=0.001) also had significant effects on the reduction of the liver PAB concentration, while the PAB concentration was lower at the dose of 10 mg/kg compared to the dose of 5 mg/kg (P=0.001) (Figure 5).

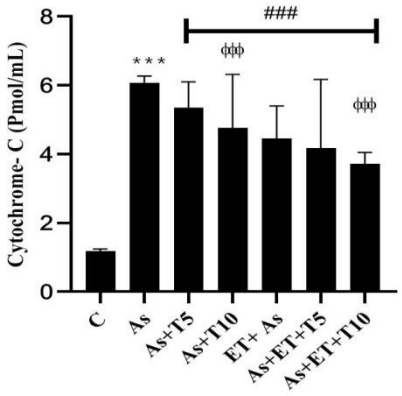

Figure 3. The results of the two-way analysis of variance (ANOVA) for Cytochrome-C levels in the research groups
C: Control, As: Arsenic, T5: 5 mg/kg of Tribulus terrestris, T10: 10 mg/kg of Tribulus terrestris, ET: Endurance Training
***Significant increase compared to the C group (P = 0.001)
###Significant decrease compared to the As group (P = 0.001)
φφφ Significant increase compared to the As + ET + T5 and As + T5 groups (P = 0.001)

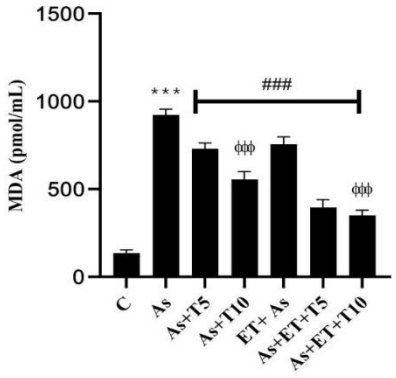

Figure 4. The results of the two-way analysis of variance (ANOVA) for MDA levels in the research groups
C: Control, As: Arsenic, T5: 5 mg/kg of Tribulus terrestris, T10: 10 mg/kg of Tribulus terrestris, ET: Endurance Training
***Significant increase compared to the C group (P = 0.001)
###Significant decrease compared to the As group (P = 0.001)
φφφ Significant increase compared to the As + ET + T5 and As + T5 groups (P = 0.001)

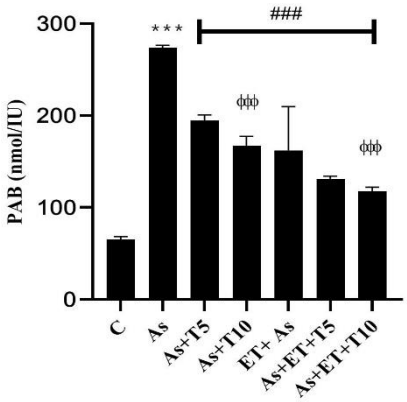

Figure 5. The results of the two-way analysis of variance (ANOVA) for PAB levels in the research groups
C: Control, As: Arsenic, T5: 5 mg/kg of Tribulus terrestris, T10: 10 mg/kg of Tribulus terrestris, ET: Endurance Training
*** ***Significant increase compared to the C group (P= 0.001)
###Significant decrease compared to the As group (P = 0.001)
φφφ Significant increase compared to the As + ET + T5 and As + T5 groups (P = 0.001)

Discussion

The results of the present study showed that As poisoning had significant effects on increasing the concentrations of MGMT, cytochrome C, MDA, and PAB, as well as the reduction of ATP, in the liver tissues of the rats. Researchers have proposed a strong link between increased As and higher levels of superoxide, peroxides, hydroxyls, and glutathione. In other words, increased ROS has been shown to disrupt the oxidant-antioxidant balance and cause damage to biological macromolecules (e.g., membrane lipids, proteins, and DNA), thereby leading to the increased oxidation of membrane lipids and MDA and the exacerbation of oxidative stress (e.g., prooxidant/antioxidant balance index) (18). This process leads to the activation of the caspase cascade, increased cytochrome C oxidase, cytochrome C release, and induction of cell death (1,2). According to the literature, the higher uptake of As, nickel, and other heavy metals with the increased binding of hydroxyl radicals to position four or eight of purine bases leads to an increment in the 8-hydroxydeoxyguanosine (8-OHdG) levels, as well as the reduction of O-6 methylguanine DNA methyltransferase (MGMT), DNA regeneration damage, mitochondrial membrane protein transcription defects (e.g., ATP synthase), and mitochondrial dysfunction in the ATP production to continue the vital cellular process (18-20). The increased MGMT following As poisoning could be due to the fact that the response to acute oxidative damage caused by heavy metals is dose-dependent, which has been confirmed in the review study conducted by Hu et al. (2020). In addition, high and acute doses of As increase oxidative stress, while the subsequent chronic poisoning with As and oxidative damage that disrupts the DNA structure may not provide comprehensive data in this regard.

In response to low and chronic doses, nuclear respiratory factor 1 and 2 are also activated (1). In the present study, AT reduced the concentrations of MGMT, cytochrome C, MDA, and PAB, while increasing hepatic ATP concentration in the As-poisoned rats. Studies have indicated that the induction of ROSs after exercise is a key pathway for biological cellular mechanisms. However, increased excessive oxidative stress after exercise may have detrimental effects on cells. The level of the oxidative stress induced by exercise depends on exercise duration and intensity (21). On the other hand, prolonged and regular exercise causes compensatory mechanisms against oxidative stress, thereby increasing enzymatic and non-enzymatic antioxidants, reducing carbonyl protein, MDA, lipid peroxidation, and 8-OHdG, and increasing the transcription of GPx, SOD, and catalase, which are the proteins responsible for DNA regeneration and mitochondrial repair (21).

The increased ability to regenerate DNA and MGMT following ROS-induced damage is directly associated with increased total antioxidant capacity, mitochondrial SOD, protein kinase, and catalase (22). However, the protein activation responsible for DNA

regeneration may be dependent on the type and duration of exercise, as well as the baseline levels of oxidative stress. To date, no studies have investigated the effects of exercise on the markers associated with the liver tissue. In a report, 16 weeks of moderate-intensity combined training was reported to significantly increased the total antioxidant capacity and DNA oxidative damage, while no significant effects were observed on DNA repair markers in healthy middle-aged men (22). Furthermore, eight and 20 weeks of voluntary training with a cycling wheel did not have a significant effect on increasing the antioxidant profile of elderly rats, while eight weeks of voluntary training increased GPx, *SOD* gene expression, catalase, kinase protein, and MGMT. In another study, 20 weeks of optional training could significantly improve the expression of antioxidants and DNA repair capacity compared to eight weeks of optional training (23).

According to the current research, the consumption of the T extract significantly reduced the concentrations of MGMT, cytochrome C, MDA, and PAB, while increasing the hepatic ATP concentration of the rats poisoned with As. Notably, the effects of the T extract were dose-dependent, and the dose of 10 mg/kg had a more significant impact compared to the dose of 5 mg/kg on increasing the ATP concentrations and decreasing the hepatic concentrations of cytochrome C, MDA, and PAB.

Previous studies have indicated that T plant species modifies oxidant-antioxidant systems through the mechanism of increasing antioxidants such as SOD, GPX, and catalase (10). However, the effects of various doses of T on the cell cycle function and DNA remain unclear. In addition, high doses of T aqueous extract have been reported to alter the basic structure of chromatin and impair the process of DNA repair and proliferation (24), while the consumption of T may also reduce 8-OHdG by inhibiting the inhibition mechanism of prostaglandins, increasing the activity of nitric oxide and cyclooxygenase 2 (COX-2), clearing MDA, and increasing the expression of SOD, GPX, and catalase, which increase DNA repair protein levels. Increased NAD following the use of T has also been shown to activate the cellular charge pathways to express the metabolic proteins of the mitochondrial membrane, thereby improving the mitochondrial function and metabolism of sugars, fats, and insulin (25, 26).

In terms of the effects of T on the research variables of the present study, studies have demonstrated that 40 and 80 mg/kg of T aqueous extract could disrupt the cell division cycle, while the doses of 10 and 20 mg/kg have no significant impact on the cell division and cell differentiation of human lymphoma cells (24). On the other hand, using 100, 200, and 300 mg/kg of dose-dependent T for eight days has been observed to increase SOD, glutathione reductase, GPx, and the proteins linked to liver fatty acids in the kidney tissues of rats with mercury poisoning (10). In addition, 5 and 10 mg/kg of T extract has been shown to reduce oxidative stress, repair DNA, and increase the antioxidants in neurons (25).

According to the results of the present study, the interaction between training and T significantly decreased the concentrations of MGMT, cytochrome C, MDA, and PAB, while increasing the hepatic concentration of the liver ATP in the As-poisoned rats. In this regard, a review of the literature indicated that the consumption of T plant species along with exercise is of interest to athletes as it could improve their athletic performance. In addition, the T plant species contains steroid-like substances, saponins, and alkaloids, which could enhance the metabolism, increase the muscle tone and energy substrates, and activate the protein synthesis pathways in athletes (9). ET has been reported to increase antioxidants and decrease carbonyl protein, MDA, lipid peroxidation, and 8-OHdG depending on the severity and type of training through reducing oxidative stress, thereby increasing the proteins responsible for DNA regeneration and mitochondrial repair (21, 22). Depending on the dose and basic oxidative damage and through the oxidant-antioxidant system modulation mechanism (10) and inhibition of prostaglandins, the consumption of the T plant species has been shown to increase nitric oxide activity and COX-2, decrease MDA, and increase SOD, GPX, and catalase, thereby reducing 8-OHdG.

According to the literature, increased NAD has been reported to improve mitochondrial function, cellular metabolism regulation, and ATP synthesis regulation (25, 26). Due to the uncertainty regarding the effects of As on MGMT in the present study, the reduction of MGMT

following training and T extract consumption may be attributed to the dose-dependent function of As.

Considering the affectability of 8-OHdG and NRFs following adaptation to training and T extract consumption, one of the limitations of the present study was the lack of measurement of these variables. Therefore, it is recommended that in addition to examining various doses of As, the concentrations of the two proteins be measured in using different approaches. Another limitation of our research was the control of possible injuries during training, as well as the control of potential stressors on the rats during the exercise.

Conclusion

According to the results, AT and the use of T extract could synergistically reduce oxidative stress and improve the concentration of ATP in the liver tissues. However, further investigations are required regarding the impact of similar research interventions on MGMT.

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