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Effect of ozone and melatonin on oxidative stress parameters and enzymatic factors of the liver and kidney in mice with busulfan-induced injury

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ABSTRACT

Background: The aim of this study was to investigate the effects of ozone and melatonin interventions on anti-oxidant effects and enzymatic parameters in the liver and kidneys of mice experiencing busulfan-induced injury, a common chemotherapy-related condition known to severely affect these organs. ***Materials and Methods:*** The research included 24 male mice were randomly assigned in four groups of six mice each: a control group without any intervention, busulfan group, busulfan with melatonin group, and busulfan with ozone group. This experimental setup spanned seven days. After a 35-day period, blood, liver and kidney tissues were extracted for the assessment of various parameters, such as super oxide dismutase (SOD), catalase, total antioxidant capacity (TAC), malondialdehyde, and tissue creatinine. ***Results:*** Busulfan administration resulted in a decrease in SOD, catalase, and TAC levels, along with an increase in malondialdehyde levels in both the kidneys and liver. Additionally, Busulfan led to elevated ALT levels and decreased AST in liver tissue, as well as increased BUN and tissue creatinine in the kidneys. However, the introduction of ozone and melatonin ameliorated these effects across all parameters. Notably, ozone exhibited a stronger impact on catalase, TAC, and malondialdehyde in both organs. Moreover, ozone showed pronounced effects on ALT and AST in the liver and on creatinine in the kidneys. ***Conclusion:*** Both melatonin and ozone show promise in ameliorating Busulfan-induced injury. Interestingly, ozone appears to exhibit greater efficacy in enhancing kidney and liver function in such cases.

INTRODUCTION

Cancer, a major cause of death globally, is often treated with chemotherapy. The impact of chemotherapy on enhancing and extending the lifespan of cancer patients is indisputable. However, this treatment frequently causes serious health issues such as cognitive impairments, early menopause, heart conditions, bone and joint disorders, secondary cancers, disruption of the blood-testis barrier, and kidney diseases [1, 2]. The liver and kidneys are crucial for drug and toxin metabolism and elimination, as well as for maintaining homeostasis. Therefore, injury to the liver and kidneys can be very harmful. Kidney failure can occur for various reasons, such as chemotherapy, shock, infection, surgery, or antibiotic use. Similarly, liver failure can be caused by the consumption of certain drugs or toxic chemicals [3, 4]. Research has demonstrated that oxidative stress is a significant factor in causing liver and kidney injuries, so antioxidants may be effective in healing these injuries [5, 6].

Chemotherapy-induced nephrotoxicity is a major complication that reduces the effectiveness of treatment [7]. Chemotherapy agents like Busulfan can cause various kidney-related disorders, including kidney tissue inflammation, papillary necrosis, urinary changes, hemorrhagic cystitis, acute tubular necrosis, and infarction [7, 8]. Another disorder related to Busulfan is hepatic veno-occlusive disease, the occurrence of which varies depending on the dose of Busulfan consumed [9].

Before bone marrow transplantation, Busulfan is one of the most commonly prescribed chemotherapy drugs. Chemotherapy drugs, including Busulfan, damage deoxyribonucleic acid (DNA), leading to the induction of aging and, in some cases, apoptosis. This medication possesses the capability to trigger apoptosis in germ cells. Busulfan belongs to the class of alkylating agents and is a synthetic derivative of dimethane-sulfonate [10]. It is one of the cytotoxic drugs, and when it is hydrolyzed, it releases methane sulfonate groups. These methane sulfonate groups finally turn into methanesulfonic acid and

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tetrahydrofuran, causing DNA alkylation and preventing DNA replication and ribonucleic acid (RNA) translation [11]. Alkylation also causes cross-linking in DNA, ultimately preventing the proliferation of cancer cells and inducing cell apoptosis through the P53 pathway [12].

An imbalance between antioxidants and oxidants causes oxidative stress [13], and antioxidants should be used to reduce its effects [14]. In recent years, there has been significant focus on the role of melatonin as a potent and highly effective antioxidant that counters the effects of free radicals. Melatonin, one of the secretions of the pineal gland, effectively regulates various physiological processes. This hormone has a neuro-hormonal function, regulating reproduction, immunity, and temperature. Additionally, it affects cell proliferation, growth, and differentiation [15]. In addition to the role of melatonin receptors, it has been shown that melatonin or its metabolites are capable of neutralizing free radicals [16].

Moradpour *et al*. showed that melatonin affected oxidative stress and improved liver damage induced by radiofrequency electromagnetic radiation [17]. Treatment with melatonin led to an increase in the frequency of lymphocytes in breast cancer patients [18]. Ozone is also used as a potential inducer of the antioxidant response in cases of oxidative stress and inflammation caused by some diseases or drugs, such as chemotherapy drugs [19]. The role of ozone in the treatment of acute skin wounds, infectious wounds, ischemic diseases, and joint problems has also been mentioned [20]. Under strict control, ozone is able to stimulate the antioxidant system and can help deal with ischemia-reperfusion injuries (IRI) [21]. Low doses of ozone can exert beneficial effects by stimulating the antioxidant response and inducing metabolic adaptations related to oxidative stress [22]. Since Busulfan, as an anticancer drug, can induce disorders in the liver and kidneys, it is desirable to explore treatments that can exert protective effects in these vulnerable tissues with minimal side effects. Drawing upon reports highlighting the roles of ozone and melatonin in modulating the antioxidant system and treating various diseases, this study aims to explore the impact of ozone and melatonin on Oxidative damage and enzymatic traits of the liver and kidneys of mice with Busulfan-induced injury.

MATERIALS AND METHODS

Study subjects

The study involved 24 male mice with a mean age and weight of 4-6 weeks and 25-30 grams, respectively. The mice were housed under standardized environmental conditions (temperature: 24 ± 3°C, with a 12-hour duration of light-darkness.) and were given a typical meal and regular water intake. A period of 7 days was allotted for the mice to acclimate to their new environment [23]. Four groups of six mice were randomly assigned to the animals. A control group receiving intraperitoneal injections of physiological serum for seven days. The second group received an intraperitoneal dose of 30 mg/kg Busulfan on the first day [24]. The Busulfan with melatonin group received the same Busulfan dose on the first day, along with a daily intraperitoneal injection of melatonin at 10mg/kg for seven days [23]. The Busulfan with ozone group received the initial Busulfan dose, along with daily intraperitoneal injections of ozone at 10mg/kg for seven days [23]. The mice underwent anesthesia after breeding for 35 days, and tissue samples from the liver and kidneys were collected for the measurement of study variables.

Study design

After measuring the mice's weight, mix an appropriate amount of busulfan powder with dimethyl sulfoxide (Sigma B2635, Iran). Next, 0.1 ml of the solution, which meets the required dose, was injected intraperitoneally into the mice, along with 0.1 ml of distilled water [25]. To prepare melatonin (Sigma M5250), the mice were first weighed, and then an appropriate quantity of melatonin was diluted in 1% ethanol based on their weight. Subsequently, the diluted melatonin solution was injected intraperitoneally into the mice [23]. The ozone was prepared from pure oxygen using the Gardina MC80F device (Gardina MC80F, Iran), which contained approximately a 3% ozone/oxygen gas mixture. Ozone concentration was assessed by measuring ultraviolet rays at a wavelength of 254 nm. The ozone dosage was calculated based on the mice's weight, and it was administered at 4 mg/kg in a single dose [23]. The combination of ketamine (75 mg/kg) and xylazine (10 mg/kg), was used to anesthetize mice after 35 days of breeding. Afterward, they were euthanized, and finally dissected, and liver and kidney tissues were collected.

Measurment of malondialdehyde, SOD, TAC, catalase, ALT, AST, ALP, BUN, and creatinine

Biochemical experiments were conducted by freezing samples in liquid nitrogen stored at -80°C. The samples intended for biochemical tests were frozen in liquid nitrogen and subsequently stored at -80°C until the time of experiments. The centrifuge (CFP-15000, Iran) was used to centrify homogenized tissues (in 1 ml of 0.9% NaCl solution on ice) for 10min at 4 °C at 1500 rpm [26]. Levels of malondialdehyde, Superoxide Dismutase (SOD), Total Antioxidant Capacity (TAC), and Catalase were assessed. The assessment was conducted using diagnostic ELISA kits from ZellBio GmbH, Germany.

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The assays were performed by the spectrophotometric method in both liver and kidney tissues . To evaluate liver function, mild anesthesia using ether was administered to obtain blood samples from the rats' hearts [26]. Following centrifugation of the blood samples at 3500 rpm, serum samples were isolated. These samples were then transported to the laboratory for measuring the levels of Alanine Transaminase (ALT), Aspartate Transaminase (AST), and Alkaline Phosphatase (ALP). Finally Blood Urea Nitrogen (BUN) and creatinine levels in the serum were assessed as renal parameters using a commercial kit from Pars Azmoon Co., Tehran, Iran, with an automatic analyzer from Hitachi, Ltd., Tokyo, Japan.

Statistical analysis

All calculated quantitative parameters were analyzed using SPSS version 24 software with ANOVA and Mann-Whitney post-hoc tests. Mean and standard deviation with significance level 0.05 was used to present the results.

RESULTS

Examination of renal parameters

The study findings revealed that Busulfan caused a significant decrease in SOD levels in comparision to the control group (P<0.001), but in the melatonin and ozone groups this reduction was not statistically significant (P=0.063 and P=0.079, respectively). In contrast to the control group, difference was significant when compared to the Busulfan group (P<0.001).

In acordance with catalase levels, both the Busulfan (P<0.001) and melatonin (P<0.001) groups exhibited a substantial reduction in comparison to the control group. This parameter showed a similar increase as the one in control group, but did not show statistically significant (P=0.097). The catalase levels of the busulfan group differed significantly from those of both the melatonin and ozone groups (P<0.001).

Renal tissue TAC levels were decreased in both the busulfan (P<0.001) and melatonin groups (P=0.001) compared with the control group. Compared to the ozone group, TAC levels did not experience significant reductions (P=0.057).

In kidney tissue, there was no significant difference in malondialdehyde levels between the busulfan and melatonin groups. (P=0.175). Nonetheless, within each group, the rise in this parameter was notable in comparision to the value in control group (P<0.001). There was a significant difference in this parameter between the ozone and busulfan groups (P<0.001). There is no significant difference in the kidney tissue levels of malondialdehyde when compared to the malondialdehyde levels in the control group (P=0.062).

BUN and creatinine significantly elevated within the Busulfan group, demonstrating a notable difference when contrasted to the values in control group (P=0.001). The BUN levels in the melatonin and ozone groups were similar to those in their control group (P>0.05), but both groups exhibited a significant decrease in comparision to the BUN level in Busulfan group (P=0.002 and P=0.001, respectively). The levels of creatinine were significantly higher in the Busulfan group than those in control groups (P<0.001). Furthermore, the creatinine levels in the busulfan group and the other ozone group decreased significantly (P=0.005) (figure 1).

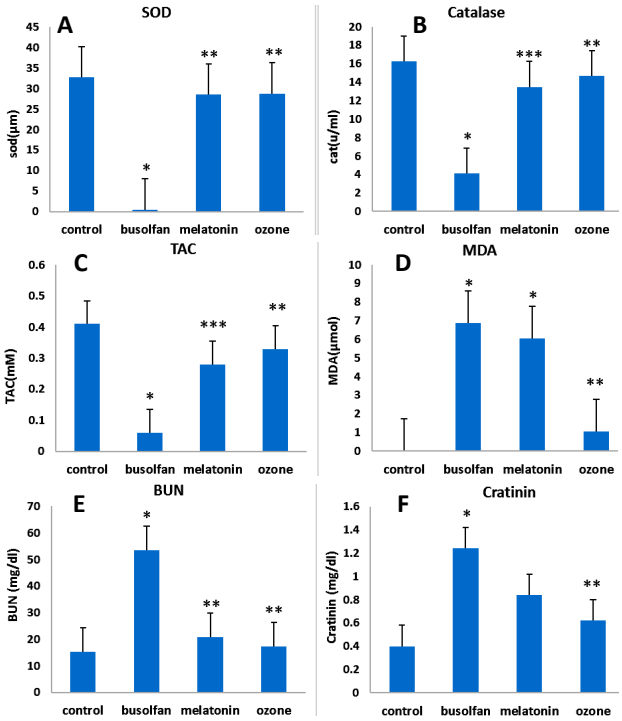

Figure 1. The mean levels of **(A):** super oxide dismutase (SOD), **(B):** catalase (CAT), **(C):** total antioxidant capacity (TAC), **(D):** malondialdehyde (MDA), **(E):** blood urea nitrogen (BUN), and **(F):** creatinine (CREAT) in the kidneys tissue of the mice in control, busulfan intervention, melatonin+busulfan intervention, and ozone+busulfan intervention groups SOD, catalase and TAC levels of kidneys tissue in melatonin and ozone group were higher than Busulfan group (P < 0.001). Malondialdehyde, BUN and creatinine levels of kidneys tissue in ozone group, showed a significant decrease compared to Busulfan group (P < 0.001).

*: Comparison with control group and significance level is 0.05.
**: Comparison with busulfan group and significance level is 0.05.
***: Comparison with control group and buslfan group and significance level is 0.05.

Examination of liver parameters

The study indicates that busulfan was found to be effective in decrease liver SOD levels, which was significantly higher than those observed in the control group (P<0.001). SOD was similar to the control group in both the melatonin and ozone groups (P=0.100 and P=0.270, respectively). Moreover, this characteristic was significantly

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elevated in the two groups when compared to that of the busulfan group (P<0.001).

Catalase levels in liver tissue decreased considerably in the busulfan group (P<0.001) and the Melatonin group (P=0.008) in comparision to the control group. Compared to the control group, there was no significant reduction in the ozone group's catalase (P=0.214). The catalase levels in the busulfan group were significantly lower than those in both the melatonin and ozone groups. (P<0.001).

Busulfan and melatonin TAC levels were found to be significantly reduced from the control group (P<0.001 and, P=0.007 respectivly). The TAC in the ozone group showed an increase that was similar to the control group's (P=0.340). Furthermore, TAC levels were significantly higher in the ozone and melatonin groups than in those of the Busulfan group (P<0.001).

Malondialdehyde levels in liver tissue were found to be significantly higher in the busulfan and melatonin groups than in their control group (P<0.001) while not significantly different in the ozone group (P=0.152). Additionally, malondialdehyde levels were significantly lower in the melatonin and ozone groups than those in their busulfan group (P<0.001).

The ALT level saw an increase in both the Busulfan and melatonin groups when compared to the control group. (P<0.001), while the ozone group did not display any significant difference (P=0.090). In both the melatonin and ozone intervention groups, the ALT levels were significantly lower in the Busulfan group (P<0.001).

The AST level in the liver tissue of the busulfan group was significantly lower than that of their control group (P=0.009). Compared to the control and the busulfan groups, the melatonin intervention resulted in an increase in AST levels (P=0.001). The difference in the AST levels in liver tissue between the ozone group and the Busulfan group was considerable (P<0.001). This variation was insignificant when compared to the control group (P=0.106). The ALP level in liver tissue did not exhibit different between the groups examined (P>0.05) (figure 2).

Examination of the kidney and liver weight parameters

The study results indicated no significant difference in mean kidneys weight among the Busulfan, melatonin, ozone, and control groups in this study (P>0.05). Compared to the control group, busulfan injection resulted in lower mean liver weight (P<0.001). Moreover, the intervention groups for melatonin and ozone showed significantly higher mean liver weights than those for busulfan (P<0.001). Moreover, the average liver weight of the melatonin group was significantly distinct from that of their control group (P=0.016), whereas the difference between the ozone and control groups was not significant (P=0.962) (table 1).

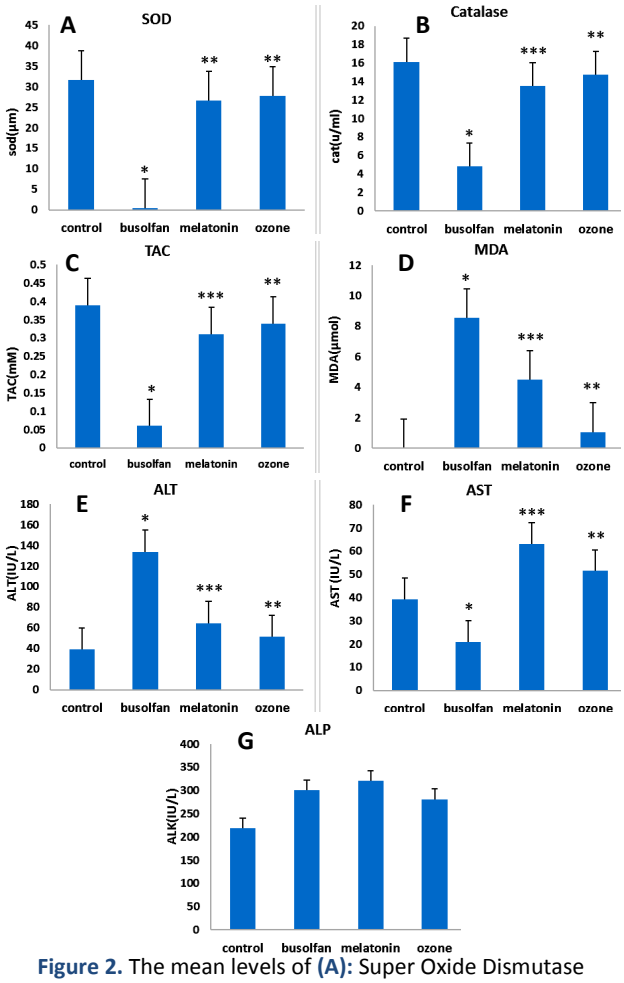

Figure 2. The mean levels of **(A):** Super Oxide Dismutase (SOD), **(B):** Catalase (CAT), (C): total antioxidant capacity (TAC), **(D):** malondialdehyde (MDA), **(E):** alanine transaminase (ALT), **(F):** aspartate transaminase (AST), and **(G):** alkaline phosphatase (ALP) in the liver tissue of the mice in control, busulfan intervention, melatonin+busulfan intervention, and ozone+busulfan intervention groups. ALT level in Busulfan and melatonin groups increased (P < 0.001). In melatonin and ozone groups, the ALT level decreased (P < 0.001). The AST level of liver tissue in Busulfan group decreased (P = 0.009). In melatonin and ozone groups, the AST level increased (P = 0.001 and P<0.001).

*: Comparison with control group and significance level is 0.05.
**: Comparison with busulfan group and significance level is 0.05.
***: Comparison with control group and buslfan group and significance level is 0.05.

Table 1. Comparison of kidney and liver weight (in miligrams) in the studied groups.

Group	Kidney weight (mg)			Liver weight (mg)		
	Mean ± SD	P-value *	P-value **	Mean ± SD	P-value *	P-value **
Control	329.6 ± 221.6	-	-	1666.6 ± 137.4	-	-
busulfan	161.0 ± 3.2	0.127	-	1030.8 ± 41.2	< 0.001	-
Melatonin +busulfan	207.8 ± 30.9	0.355	0.913	1422.8 ± 130.5	0.016	< 0.001
Ozone+ busulfan	240.2 ± 34.7	0.607	0.691	1632.4 ± 114.3	0.962	< 0.001

Data are expressed as mean ± SD (standard deviation) in miligrams.
* Comparison with control group and significance level is 0.05.
** Comparison with Busulfan group and significance level is 0.05.

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DISCUSSION

Busulfan is a popular choice for chemotherapy treatment, particularly in cases of chronic leukemia and ovarian cancer, as well as in bone marrow transplantation for cancer patients. This drug causes major health risks in the body and in many organs, including the liver and kidneys [11, 27]. In this study, Busulfan was employed to create a model of liver-kidney injury. Currently, researchers are exploring methods to mitigate the toxic effects of Busulfan in cancer patients. Some approaches include the use of substances like melatonin and ozone.

Our results demonstrated that the administration of ozone and melatonin in the kidneys reduced BUN and serum creatinine levels compared to the Busulfan group. Moreover, the treatment caused a reduction in ALT and an increase in overall AST relative to the busulfan group, while there was no discernible differences were observed between ALP value in the busulfan and control groups. Ozone and melatonin treatments also significantly enhanced the antioxidant status in both the liver and kidneys. Additionally, improvements in liver weight were observed in mice.

Busulfan disturbs the balance of oxidative stress in cells, leading to disruption as one of its effects. The imbalance between reactive oxygen species (ROS) and antioxidant production is responsible for this disorder. The TAC also contains non-enzymological substances such as glutathione, vitamins A and C, various food antioxidants, SOD, catalase enzymes, and GPX. These components work to prevent the uncontrolled increase of ROS within the cell. Busulfan interacts with glutathione and nicotinamide adenine dinucleotide phosphate (NADPH), exacerbating oxidative stress. Additionally, it alters cell membrane fluidity and permeability, ultimately leading to structural and functional damage by producing malondialdehyde [28, 29].

The findings from this study showed Mice that received Busulfan had an increase in malondialdehyde production and reduced SOD, TAC, and catalase levels in their liver and kidney. Additionally, the study found that by administering ozone, the malondialdehyde levels decreased and minimized the harmful impact of busulfan by control SOD and catalase levels of liver and kidney. Consistent with these findings, Buyuklu *et al*. indicated antioxidant functions of ozone by elevating factors such as SOD and catalase in the heart. Additionally, ozone reduced malondialdehyde levels, thus positively impacting heart function [28]. Taheri Moghadam *et al*. concluded that the testicular's quality was significantly diminished by the use of Busulfan. So that different factor including sperm characteristic, catalase , SOD, and also TAC were controled while levels of malondialdehyde increasing. Furthermore, the use of ozone and melatonin was found to have significant benefits in improving testis quality as well as parameters for sperm, malondialdehyde values, and antioxidant activity [30], which aligns with the findings of the current study regarding kidney tissue. Additionally, the results revealed that melatonin decreased malondialdehyde levels, increased catalase levels alongside SOD levels, and mitigated the toxic effects of Busulfan.

Melatonin's anti-inflammatory properties and ability to shields cells from free radicals damage are among the powerful antioxidant properties found in most natural compounds [31]. Consistent with the results of the present study, the findings of Kurhaluk's study indicate that melatonin exerted protective effects on the liver, kidneys, and muscle tissues exposed to ethanol poisoning. It achieved this by preventing severe lipid peroxidation processes in both the early stages (conjugated diene production) and the final stages (malondialdehyde level) [32]. In another study conducted by Kurhaluk, it was concluded that melatonin influenced the low oxidative stress induced by lipopolysaccharide in the liver, kidneys, and muscle of mice. Furthermore, melatonin was found to modulate the states caused by oxidative stress [33]. Moradpour *et al*. reported that melatonin decreases liver damage induced by radiotherapy [17]. Similarly, Samei et al. demonstrated that melatonin increases the frequency of lymphocytes in breast cancer patients [18].

The present study also showed that ozone have a grater impact on malondialdehyde concenteration in liver and kidney tissues compared to melatonin. Ozone treatment led to levels of catalase and TAC similar to the control group and performed even better than melatonin. However, ozone and melatonin both elevated SOD levels in liver and kidney tissues. In support of these findings, the study found that ozone administration to diabetic mice reduced oxidative stress markers and increased renal SOD and catalase as antioxidants [34]. Ozone therapy has the potential to mitigate chemotherapy-induced toxicity by modulating free radicals and antioxidants [35].

Studies have demonstrated that in mice undergoing kidney transplantation, ozone can reduce inflammatory reactions and oxidative stress damage. Furthermore, ozone has been linked to bolstering the antioxidant system and suppressing inflammatory reactions [36].

Because of its alkylating nature, Busulfan destroys stem cells and induces cell death by generating free radicals. Consequently, Busulfan appears to impact the liver and kidneys, particularly through damage inflicted by oxidative stress [37, 38]. Studies have indicated that ozone under strict control can stimulate the antioxidant system and prepare to deal with the damage caused by IRI [21, 39]. Hence, ozone has the capacity to regulate cellular defense systems

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against oxidative stress, thereby promoting recovery and control of the damage inflicted by Busulfan on liver and kidney cells.

In a study investigating the effect of melatonin against methotrexate-induced hepatotoxicity in mice, the results showed that melatonin significantly increased ALT, AST, and ALP levels in mice with methotrexate-induced hepatotoxicity [40]. In this study, it was shown that the ALT and AST levels of the ozone and melatonin groups were significantly different in comparision to the busulfan group. However, the ALP levels did not exhibit notable differences between groups. Examining the beneficial effects of ozone in acetaminophen-induced hepatotoxicity in mice revealed that ozone has a positive impact on improving liver enzymes affected by acetaminophen-induced hepatotoxicity [41]. Our results also demonstrated a significant difference in ALT and AST levels between the ozone and Busulfan groups. ALP levels remained the same with no significant differences in the busulfan group.

Consistent with the results of our study, ozone's protective effect on mice induced nephropathy showed significant effects on serum BUN and creatinine levels, as well as the degree of tubular necrosis, compared to the contrast media group [42]. Investigating the melatonin effect in mice showed melatonin's protective effects on BUN and creatinine levels, and on kidney damage [43].

CONCLUSION

According to the findings of this study, both melatonin and ozone show promise in ameliorating Busulfan-induced injury in the liver and kidneys of mice while regulating cellular defense systems against oxidative stress. However, ozone demonstrated greater efficacy in improving catalase , TAC, creatinine, and malondialdehyde parameters, indicating a more significant functional effect on these organs. Further investigations are necessary to comprehensively understand the precise mechanisms underlying the beneficial effects of ozone on cellular function and other organs, particularly in reducing the various adverse effects of Busulfan on liver and kidney tissues and its impact on their function.

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***Conflict of interest*:** The authors of the present study declare no conflict of interest with other people or organizations that would inappropriately influence this work.

***Ethical consideration*:** This study was approved by the Animal Research Ethics Committee of Jundishapur University of Medical Sciences, Ahvaz, Iran, with the code IR.AJUMS.ABHC.REC.1400.119.

***Authors' contribution*:** All authors have made substantial contributions as following: R.D: The conception and design of the study, analysis and interpretation of data, methodology, and drafting the article or revising it critically for important intellectual content; T.S.: The conception and design of the study, acquisition of data, and writing the original draf; M.T.M.: The conception and design of the study, project administration, methodology, supervision.

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