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Evaluation of the effects of overdose Acetaminophen toxicity in rats: Ozone, which can be preferred as a complementary therapy

Evaluación de los efectos de la toxicidad por sobredosis de paracetamol en ratas: el ozono, que puede preferirse como terapia complementaria

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ABSTRACT

This study aims to investigate the effects of Ozone therapy used in traditional and complementary medicine on possible toxicity caused by Acetaminophen (APAP). There was no intervention in first group (control). The second group received 150 µg·kg^{-1} day, i.p. for three weeks of ozone, 2 g·kg^{-1} orally single dose APAP to third group, and APAP+Ozone to fourth group. APAP was administered on 21st day of ozone application. Malondialdehyde (MDA) levels, reduced glutathione (GSH) levels, and antioxidant enzyme activities were all measured to assess their contribution to pathogenesis of toxicity in blood tissues. Compared to the control group, the group receiving APAP showed increased MDA levels (P=0.009) and decreased GSH levels (P<0.001), as well as reduced CAT (P<0.001), GSH-Px (P<0.001) and SOD (P<0.001) enzyme activities. However, in the group treated with ozone and APAP, levels of MDA and GSH, as well as the activities of the antioxidant enzymes, were similar to those of the control group, indicating a protective effect of ozone against APAP–induced oxidative stress. In conclusion, the results of the study showed that APAP caused oxidative stress in blood tissue. The present study showed that ozone had potential protective effects against toxicity induced with APAP through various mechanisms in different cellular processes. This may be related to cytoprotective and antioxidant properties of ozone. Ozone can provide a chemical basis for some health benefits against toxicities. Ozone may be protective against APAP–induced oxidative damage. As a result, it was concluded that ozone may be a natural and effective antioxidant that can be used to reduce the toxicity caused by APAP.

Key words: Acetaminophen; antioxidant; ozone; oxidative stress; toxicity

RESUMEN

Este estudio tiene como objetivo investigar los efectos de la ozonoterapia utilizada en la medicina tradicional y complementaria sobre la posible toxicidad provocada por acetaminofen (APAP). No hubo intervención en el primer grupo (control). El segundo grupo recibió 150 µg·kg^{-1} día, i.p. durante tres semanas de ozono, 2 g·kg^{-1} de APAP en dosis única por vía oral al tercer grupo y APAP + Ozono al cuarto grupo. APAP se administró el día 21 de la aplicación de ozono. Se midieron los niveles de malondialdehído (MDA), los niveles de glutatión reducido (GSH) y las actividades de las enzimas antioxidantes para evaluar su contribución a la patogénesis de la toxicidad en los tejidos sanguíneos. En comparación con el grupo control, el grupo que recibió APAP mostró niveles elevados de MDA (P=0,009) y niveles disminuidos de GSH (P<0,001), así como reducción de CAT (P<0,001), GSH-Px (P<0,001) y SOD. (P<0,001) actividades enzimáticas. Sin embargo, en el grupo tratado con ozono y APAP, los niveles de MDA y GSH, así como las actividades de las enzimas antioxidantes, fueron similares a los del grupo de control, lo que indica un efecto protector del ozono contra el estrés oxidativo inducido por APAP. En conclusión, los resultados del estudio mostraron que APAP causaba estrés oxidativo en el tejido sanguíneo. El presente estudio demostró que el ozono tenía potenciales efectos protectores contra la toxicidad inducida con APAP a través de varios mecanismos en diferentes procesos celulares. Esto puede estar relacionado con las propiedades citoprotectoras y antioxidantes del ozono. El ozono puede proporcionar una base química para algunos beneficios para la salud contra las toxicidades. El ozono puede proteger contra el daño oxidativo inducido por APAP. Como resultado, se concluyó que el ozono puede ser un antioxidante natural y eficaz que puede utilizarse para reducir la toxicidad causada por APAP.

Palabras clave: Acetaminofén; antioxidante; ozono; estrés oxidativo; toxicidad

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INTRODUCTION

Due to its analgesic and antipyretic characteristics, one of the most often used drugs is acetaminophen (APAP)(also known as Paracetamol, N–acetyl–p–aminophenol). At prescribed dosages, it is safe and effective, but an overdose can be lethal [1]. The public's worry over APAP hepatotoxicity has led to significant research into the mechanisms underlying its harmful effects. Overall, the pathophysiology of APAP is mostly influenced by mitochondrial dysfunction and the oxidative stress caused by APAP [2]. APAP is a non–steroidal anti–inflammatory drug (NSAID) that works through a different mechanism than other NSAIDs. Although its exact mechanisms of action are unknown, it seems to specifically block the brain's cyclooxygenase. It can thus alleviate pain and fever as a result. It may also prevent the central nervous system from producing prostaglandins. APAP has an antipyretic effect by directly affecting the hypothalamus [3].

Hepatotoxic metabolites of APAP, which make up about 10% of the total metabolites, are quickly inactivated by glutathione (GSH), preventing the death of hepatic cells [4]. On the other hand, excessive concentrations of toxic liver metabolites, in particular N–acetyl–p–benzoquinone (NAPQI), deplete GSH quickly and covalently alter cellular proteins, causing high concentrations of reactive oxygen species (ROS) to be produced and depleted. As a result, many tissues and cells suffer damage, including mitochondrial damage [5] (FIG. 1).

Recent studies have demonstrated that the use of APAP (acetaminophen) can cause an increase in the formation of free radicals and lipid peroxidation products, such as malondialdehyde (MDA). Furthermore, APAP can lead to toxicity by reducing the levels of important antioxidant enzymes such as glutathione peroxidase (GSH–Px), catalase (CAT), glutathione–S–transferase (GST), superoxide dismutase (SOD), and glutathione reductase (GR) [7, 8].

In experimental studies conducted with APAP, it was stated that oxidative stress developed in organs such as liver, kidney and testicles after APAP applications in rats at different doses 3 g·kg^{-1} body weight (bw) [7], 500 mg·kg^{-1} bw [9, 10]. Besides that, in these studies, folic acid Attempts have been made to reduce or prevent oxidative stress by using substances with known antioxidant activity such as, carvacrol, alpha–tocopherol, etc. In the studies, it was observed that oxidative damage was reduced in the groups in which antioxidant substances were used together with APAP, compared to the groups in which only APAP was applied [7, 9, 10].

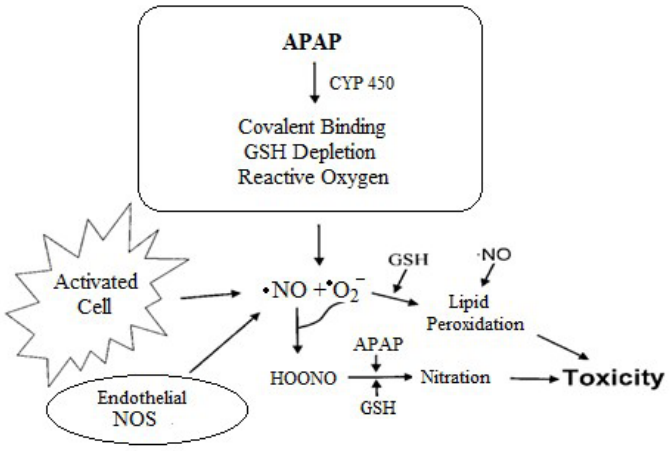

FIGURE 1. Postulated mechanism of APAP–induced toxicity. (HOONO, peroxynitrite; NOS, nitric oxide synthase; CYP 450, cytochrome P450) (Adapted from Jaeschke *et al.* 2002 [6])

Over the past century, it has been demonstrated that medical ozone can treat a wide range of illnesses. In fact, a wide range of research has shown the medicinal benefits of ozone. Ozone, for instance, is known to serve as an antioxidant defense system, a regulator of apoptosis, and has anti–inflammatory characteristics [11, 12]. Medical ozone therapy is the term for an ozone/oxygen gas mixture used in Medicine. The immune system is affected by the ozone/oxygen mixture in a variety of ways, including by regulating peritoneal and alveolar macrophages' phagocytic activity. In addition, systemic ozone therapy in cases of COVID–19 appears to be useful in controlling inflammation, stimulating immunity, and as antiviral activity and protection from acute coronary syndromes and ischemia–reperfusion injury, so it is recommended as a new immunotherapy methodology [13]. Ozone therapy seems to help treat conditions, including infected wounds, peritonitis persistent skin ulcers, burns, early gangrene, and severe ischemia disorders, according to clinical research so far. Ozone has also been shown to elevate the antioxidant enzymes activities, including CAT, SOD, and GSH–Px, as well as prepare the host to handle physiopathological circumstances brought on by ROS. Excessive generation of free radicals and formation of lipid peroxide in target tissues of inflammation are considered as the most common factors implicated in tissue damage in cellular toxicity. Thus, a state of oxidative preconditioning such that achieved with controlled ozone therapy may potentially be able to readjust the redox imbalance in APAP toxicity and attenuate the progression of the disease. [14, 15, 16].

While there are various studies available on the chemical structure, properties, and health effects of ozone, there are limited studies on its potential antioxidant effects [17]. Due to the lack of research on the antioxidant properties of ozone, it is important to investigate whether ozone has a protective effect against the toxicity caused by pain relievers and antipyretic drugs like APAP. This approach has been deemed useful as it could help in understanding the potential benefits of ozone therapy in preventing or reducing oxidative stress caused by APAP and other similar drugs, which could be beneficial for medical research purposes.

In the present study, the activities of MDA in the plasma of rats, GSH levels in blood tissue, and antioxidant enzymes like CAT, SOD and GSH–Px were investigated to determine effects of Ozone therapy used in traditional and complementary medicine on possible toxicity caused by APAP.

MATERIALS AND METHODS

Animals and working order

For this study, Wistar–Albino male rats (*Rattus norvegicus*) aged 3 months and weighing between 250–300g were obtained from the Firat University Laboratory Animals Breeding Unit. Ethical approval was obtained from the Firat University Animal Experiments Local Ethics Committee (Protocol No: 2022/17–1). The rats were housed in air–conditioned rooms with a fixed temperature of 25±2°C and 60–65% humidity, with a 12/12 hour dark/light cycle and standard conditions. The rats were provided with standard rat food (pellets) and tap water *ad libitum* throughout the study. The experimental procedures were performed at the Firat University Experimental Research Center, Turkey.

Experimental protocol

In this study, with seven rats apiece, four groups of rats were formed: The 1st group was the control group (No application has been made), 2nd group: The group that received ozone was administered intraperitoneally for three weeks at a dose of 150 µg·kg^{-1} day, while APAP was applied at a dose of 2 g·kg^{-1} as a single dose and orally in 3th group, and 4th group: The group that received APAP (2 g·kg^{-1} bw as a single dose, orally)+ozone (150 µg·kg^{-1} day for three weeks, i.p.). The ozone to be used in this study was obtained from the ozone generator (Humazona®, Humares, Germany). Ozone was applied to all rats once a day for three weeks according to their body weight. APAP was dissolved following in physiological saline (0.9% NaCl), and it was applied on the 21st day of ozone application. One day after the APAP application, the rats were decapitated to detect acute effects. The amount of APAP and ozone used in this study was determined based on previous studies [18, 19]. In the blood tissue, the levels of MDA and GSH, as well as the activity of antioxidant enzymes like CAT, GSH-Px, and SOD, were measured spectrophotometrically by UV-VIS spectrophotometer (Thermo Scientific, Genesys 10S UV-VIS Spectrophotometer, USA).

Biochemical studies

Plasma was collected by centrifuging by refrigerated centrifuge (NF NUVE NF800R, Turkey) blood samples in EDTA tubes at 1,630 G for 15 min. MDA, a marker of lipid peroxidation, was measured in plasma. For the measurement of GSH and GSH-Px, whole blood was used. Blood samples with EDTA plasma separation were washed three times in saline solution which a mixture of sodium chloride (NaCl-salt) and water (0.9% NaCl). Then, erythrocyte hemoglobin (Hb) levels, CAT and SOD activities were assessed.

In this study, the levels of MDA in tissue samples were determined using a spectrophotometer device with a modified method based on Placer *et al*. [20]. The method involves the interaction between thiobarbituric acid (TBA) and MDA, which is a by product of lipid peroxidation. The GSH levels were measured using a method reported by Ellman *et al*. [21], which is based on the spectrophotometric determination of the yellow color formed when 5,5'dithiobis 2-nitrobenzoic acid (DTNB) is added to sulfhydryl groups. The CAT activity was determined using the method of Aebi [22], which involves the spectrophotometric determination of the rate of hydrogen peroxide (H_2O_2) degradation by the CAT enzyme, as H_2O_2 has the ability to absorb light at 240 nm. The GSH-Px activity was determined using the Beutler method [23], which involves the oxidation of GSH to oxidized glutathione (GSSG) using H_2O_2 catalyzed by GSH-Px. The rate of GSSG formation was measured using the GR reaction. The SOD activity was determined using a modified method by Sun *et al.* [24], which involves the reduction of nitroblue tetrazolium (NBT) by the superoxide anion produced by the xanthine xanthine oxidase system, and the color of the reduction product was evaluated as SOD activity. Hemoglobin concentrations were determined using the Drabkin method developed by Frankel *et al*. [25].

Statistical analysis

Using the SPSS 22 software, the statistical significance between various groups was assessed. To determine if the raw values of all the measured parameters showed a normal distribution, the Shapiro-Wilk normality test was employed. The test's results showed that all of the parameter values did. One-way analysis of variance (ANOVA) was used to evaluate group differences based on the results of this test, and the post hoc Tukey test was employed to compare the two groups. The mean and standard error of the mean (mean ± SEM) were used to derive all values. The findings obtained in this study were represented by the mean and standard error. *P*-values less than 0.05 were considered significant statistically.

RESULTS AND DISCUSSIONS

FIG 2-6 present the levels of MDA and GSH, and the activity of CAT, GSH-Px and SOD enzymes in the blood tissue of rats in both control and experimental groups. In the group treated with APAP, there was a significant increase in MDA levels (P=0.009) and a significant decrease in GSH levels (P<0.001) as well as CAT (P<0.001), GSH-Px (P<0.001), and SOD (P<0.001) activities when compared to the control group. No statistically significant differences were observed when compared to the control group that only received ozone treatment. However, when compared to the APAP group, the group treated with ozone and APAP had MDA and GSH levels, as well as CAT, GSH-Px and SOD activities, that were closer to the control group. Compared to the control group, it was determined that the values of all parameters in the APAP + ozone applied group approached the control group values and the difference was statistically insignificant (P>0.05). The APAP group had a 15.55% increase in MDA levels, a 15.94% decrease in GSH levels, a 31.67% decrease in CAT activity, a 20.63% decrease in GSH-Px activity, and an 11.14% decrease in SOD activity compared to the control group. MDA level (P=0.002) decreased while GSH level (P=0.043), CAT (P=0.012), GSH-Px (P=0.038), SOD (P=0.002) activities increased in the APAP+ozone applied group compared to the APAP applied group (FIGS. 2, 3, 4, 5, and 6).

APAP is an over-the-counter drug known for its 'analgesic' and 'antipyretic' properties, used alone or in combination with other substances, and widely used Worldwide. Overdose of APAP may cause severe toxicity and even organ failure in many tissues and organs, especially the liver, in experimental animal models and humans. Consumption of high doses of APAP is toxic to many organisms. However, it is safe to use at therapeutic doses as it will be eliminated as non-toxic conjugates of sulfate and glucuronic acid, where it will be bioconverted [26].

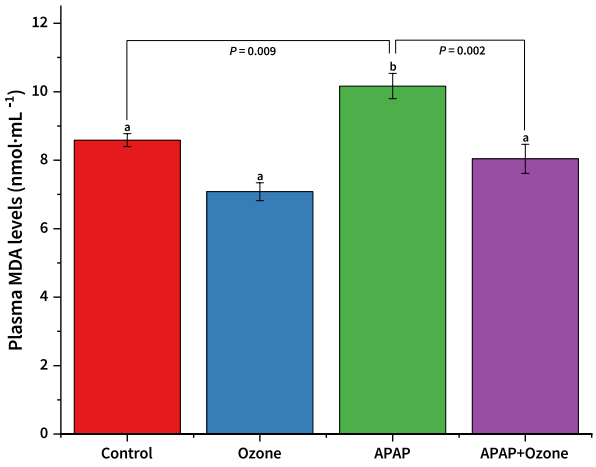

FIGURE 2. Effects of ozone on plasma MDA in rats treated with APAP. a,b: Different letters show statistical differences between columns (groups). *P*<0.05

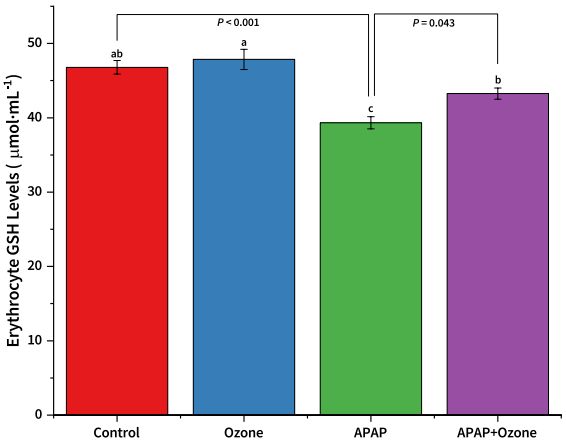

FIGURE 3. Effects of ozone on erytrocyte GSH levels in rats treated with APAP. a,b,c: Different letters show statistical differences between columns (groups). *P*<0.05

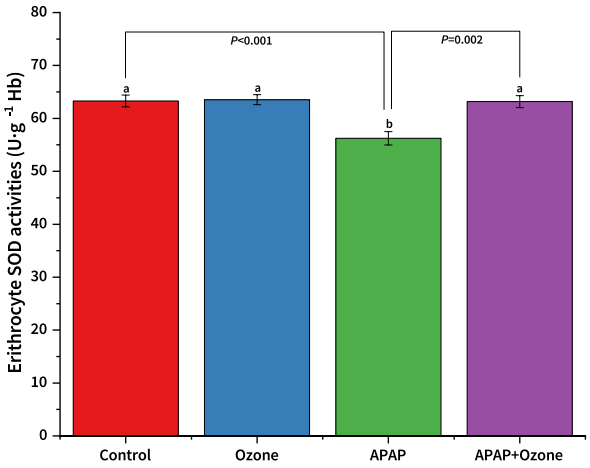

FIGURE 6. Effects of ozone on erytrocyte SOD activities in rats treated with APAP. a,b: Different letters show statistical differences between columns (groups). *P*<0.05

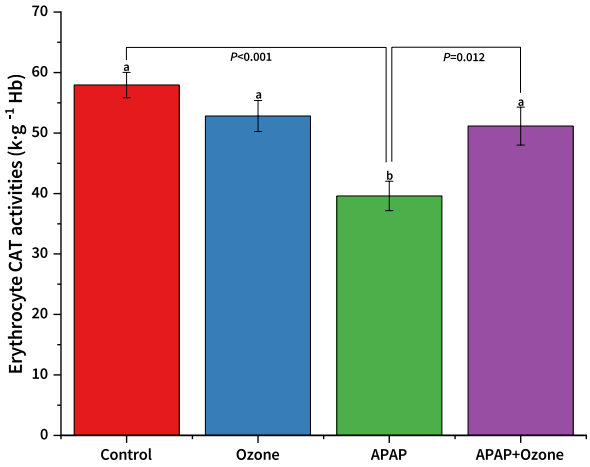

FIGURE 4. Effects of ozone on erytrocyte CAT activities in rats treated with APAP. a,b: Different letters show statistical differences between columns (groups). *P*<0.05

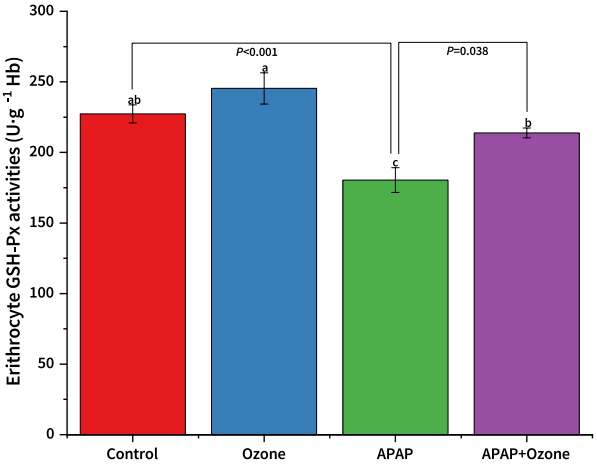

FIGURE 5. Effects of ozone on erytrocyte GSH–Px activities in rats treated with APAP. a,b: Different letters show statistical differences between columns (groups). *P*<0.05

When APAP is used in therapeutic doses, a small part is excreted unchanged, while a large part is excreted by biotransformation in the liver and, to a certain extent, in the kidneys. It is excreted as APAP sulfate and glucuronide conjugation, but a small portion is converted to a reactive metabolite, NAPQI. NAPQI is formed during the metabolism of APAP by the cytochrome P450 (CYP450) system in the liver. After an overdose of APAP, this harmful metabolite primarily causes liver damage. When APAP is used in therapeutic doses, some NAPQI occurs, and this resulting metabolite is detoxified by GSH. Since NAPQI mainly tends to bind to structures containing the –SH group, NAPQI rapidly binds especially to cellular GSH while forming and is detoxified in this way. However, after excessive doses of APAP use, an excessive amount of NAPQI occurs in the cell, and when the decreased GSH levels cannot meet the increased amount of NAPQI, NAPQI, a reactive metabolite of APAP, binds to other –SH–containing cellular proteins, causing suppression of mitochondrial respiration and development of cellular damage [27, 28, 29].

MDA is one of the parameters indicating lipid peroxidation. In many experimental studies, it has been reported that APAP causes lipid peroxidation, which is characterized by high MDA levels both in plasma and in many tissues and organs, even at different doses [9, 10, 30]. Najafizadeh *et al.* [9] in their study investigating the protective effect of carvacrol against APAP–induced kidney damage in male rats, determined that MDA levels, which are an indicator of lipid peroxidation, increased significantly after a single dose of 500 mg·kg^{-1} APAP administration. Researchers explained this situation by APAP overdose depleting antioxidant enzymes and increasing the concentration of MDA, the second messenger of free radicals, in kidney and liver tissue.

In this study, the data showed that the APAP group had a significantly higher MDA level in the plasma than the control group. It was demonstrated that APAP produces many free radicals, which the body's antioxidant defense mechanism could not withstand. However, ozone application reversed the increased MDA level to the control level, which suggests that ozone may be effective in reducing free radicals, blocking lipid peroxidation, and preventing oxidative damage to membrane lipids in rat plasmas. The findings of the present investigation show that an APAP overdose may cause

oxidative stress in blood tissue. The blood of the rats in the group that received APAP treatment at a dose of 2 g·kg^{-1} showed substantial biochemical alterations, including elevated levels of oxidative stress (GSH levels, MDA levels, GSH–Px, CAT, and SOD activities). Treatment with ozone improved the dose of biochemical degradation in groups of rats receiving APAP. In the APAP–treated group, the content of lipid peroxidation and thiobarbituric reagents, which are biomarkers of oxidative stress, significantly increased. Rapid creation of free radicals leads the antioxidant defense system to become depleted, which then allows lipids to oxidize. Protein oxidation, cellular damage, and cell death were all started by lipid peroxidation. However, it was shown that ozone could reverse APAP–induced toxicity in different animal models and with applications at different doses.

A significantly lower level of GSH was detected in rat erythrocytes treated with APAP. APAP is enzymatically degraded from uptake by the CYP450 system to the toxic intermediate NAPQI. The endogenous antioxidant decreased GSH conjugates with NAPQI to become a non–toxic product at therapeutic doses. An APAP overdose causes the synthesis of significant amounts of hazardous metabolites, which the generation of ROS and lower the level of GSH. In this instance, NAPQI's direct interaction with cellular proteins results in cellular harm and death [31]. Ozone is a product whose mechanism of action is not clear yet, which prevents cell damage by clearing ROS and increasing the level of depleted GSH. In the group treated with APAP+ozone, a dose–dependent increase in GSH level was observed when ozone was applied.

In rat tissues that have been exposed to APAP, antioxidant enzymes like SOD, CAT, GSH–Px, and GR are markedly inhibited in their activity. ROS production and oxidative stress are brought on by inhiting the antioxidant enzyme system. Superoxide dismutation to H_2O_2, which is converted by CAT to molecular oxygen and water, is catalyzed by SOD, an essential enzyme. Increased superoxide serves as a trigger for the production of ROS that causes inflammatory cells. The elimination of ROS is greatly aided by the decreased activity of GSH–Px and GR, two of GSH's dependent enzymes. In GSH–Px, harmful oxides like H_2O_2 and other ones are taken out of tissues. Due to their decreased activity, these enzymes cannot remove H_2O_2 and instead produce dangerous hydroxyl radicals [7, 8].

According to our findings, ozone therapy dramatically increased the antioxidant enzymes' reduced activity in the APAP+Ozone group. Previous studies showing that antioxidant therapy significantly reduced APAP–induced toxicity also corroborated the obtained findings [9, 10, 30]. Abdulrazzaq *et al*. [30] taking acetaminophen (APAP)–induced toxicity in rats as both an in vivo and in vitro model, they aimed to investigate the mitigating effects of ascorbic acid, alpha lipoic acid and silymarin on APAP–induced oxidative stress and cellular damage, and applied APAP at a dose of 2,800 mg·kg^{-1}. Researchers detected significant changes in MDA, GSH levels and SOD activities. Researchers found that after antioxidant applications, these changes approached the control group data.

It has been observed in many studies that the effects of APAP, which has been proven to cause plasma and tissue damage even at different doses, are tried to be eliminated or reduced with substances and compounds with different properties [7, 8, 9, 10]. Olaniyi and Agunbiade [10] showed that the administration of α–tocopherol in rats after treatment with APAP (500 mg·kg^{-1} bw) preserved testicular function and reduced oxidative stress in plasma and testicular tissue. The reason for this was explained by its antioxidative effect and increasing pituitary–gonadotropic hormone secretion.

Suhail and Ahmad [32] determined that a nontherapeutic toxic dose (250 mg·kg^{-1}) of APAP increased osmotic fragility while significantly decreasing red cell GSH content and (Na^+, K^+)–ATPase enzyme activity in albino rats in vivo. Vitamin E supplementation to drug–treated rats effectively almost normalized GSH content (Na^+, K^+)–ATPase activity and osmotic fragility. The findings show that APAP administration at hazardous doses causes metabolic and membrane changes that predispose red blood cells to hemolysis and that antioxidant vitamin E has a protective effect on these alterations.

Rats, mice, and rabbits have been used in the majority of in vivo research showing how antioxidant defense is altered. Rats receiving APAP (dose of 750 mg·kg^{-1} body weight/day) for seven days experienced kidney toxicity, oxidative kidney diseases, and a decline in plasma and kidney GSH levels, as well as plasma SOD activity [33]. In the study to look at the effects of APAP (dose of 1 g·kg^{-1} body weight) on the rat kidney's oxidatively damaged lipids and proteins, it was shown that APAP lowered GSH level [34]. In studies with doses of 15 to 3,500 mg·kg^{-1} body weight and trial durations of one to eight weeks or even 70 days, the chance that it may cause liver damage with a notable change in the antioxidant status of both the low dosage administered over time and the high dosage and brief exposure is increased [35, 36, 37]. In rabbits exposed to APAP (dose of 1 g·kg^{-1} body weight) for nine days, a substantial drop in liver and serum GSH levels was seen [38].

Mechanism of antioxidant defense elements like total antioxidant capacity (TAC) activities, CAT, GR, SOD, GST, GSH–Px, total thiol (T–SH), non–protein thiol (NP–SH), protein thiol (P–SH), reduced/oxidized glutathione (GSH/GSSG) ratio have also been determined to point to the involvement along with the GSH level. The activities of CAT, SOD, GSH, SOD, GSH/GSSG, TAC and cellular thiol levels, such as total thiol (T–SH), non–protein thiol (NP–SH), protein thiol (P–SH) decreased after being treated with APAP, showing that the antioxidant system has been compromised [36, 38, 39, 40].

The cellular antioxidant system helps minimize the damage caused by ROS. Generally, using some antioxidant agents may be beneficial to minimize the toxic side effects that may occur in overdose of some drugs [41]. The toxicity in the study may occur due to oxidative stress caused by the high dose of APAP and damage to the cell membrane. The fact that ozone applied together with APAP reduces MDA levels and increases antioxidant enzyme activities means that there is protection against oxidative damage.

Antioxidants have been used to alleviate the side effects or oxidative stress that may occur during the therapeutic use of many agents, such as APAP. In this context, they determined that cinnamon [42], *Terminalia arjuna* [43], nobiletin [44], metformin [45] prevented or alleviated the oxidative stress caused by APAP in different doses and different tissues. Reserachers explained this with a decrease in lipid peroxidation or changes in antioxidant enzymes. Antioxidant enzymes, such as SOD, GSH–Px and CAT, act as free radical scavengers. Güvenç *et al*. [44] in their study examining the effects of Nobiletin on acetaminophen–induced hepatorenal toxicity in rats, determined an increase in MDA levels, a decrease in glutathione levels, and changes in antioxidant enzyme activities, similar to the results in our study, even when APAP was administered at a toxic dose of 1,000 mg·kg^{-1}, and they emphasized that APAP causes oxidative stress. They also concluded that nobiletin may be a beneficial substance that protects against APAP–induced toxicity through antioxidant and anti–inflammatory mechanisms. Aycan *et al*. [46], in rats with APAP–induced liver toxicity, determined

a reduction in GSH-Px activity. Interestingly, APAP administration has been reported to significantly reduce SOD, GSH-Px and CAT activities. Compared to the control group, APAP treatment in this study increased MDA levels while lowering GSH levels, CAT, SOD, and GSH-Px activity. Notably, the APAP+ozone group had higher enzyme activity following ozone treatment. Free radicals are a primary source of energy for SOD and GSH-Px antioxidant enzymes, including superoxide anion, radical hydroxyl, and H_2O_2 [7, 8]. After APAP overdose, MDA increased and CAT, SOD and GSH-Px activity decreased, possibly due to increased free radical formation. The mechanism of the effects of ozone on antioxidant enzyme activities is not fully understood. It is probably due to an increase in CAT, SOD, and GSH-Px activity by reducing hydroxyl radical formation. The decrease in GSH levels in the study is attributed to the decrease in NADPH and using APAP or its formed metabolites to form a conjugate with GSH and facilitate its excretion from the body. The decrease in GSH-Px activity in the APAP-treated group may be due to the decrease in the availability of substrate (GSH) and also to ROS-induced changes in protein structure. Antioxidant enzymes like CAT and GSH-Px are the enzymes that form the first line of defense against ROS, and a decrease in these enzyme activities was determined with the APAP application. The reduction of CAT and GSH-Px activities by oxidative stress caused by APAP may be due to the inhibition effect of ROS caused by APAP on these enzymes.

Eroğlu *et al.* [47], in their study aiming to figure out the benefits of medical ozone and L-carnitine therapy for APAP-induced (dose of 1 g·kg^{-1} single dose, orally) kidney damage, determined that APAP toxicity causes oxidative damage in the examined kidney tissues and that L-carnitine and/or ozone (0.7 mg·kg^{-1} i.p.) applications for protective purposes levels MDA, a product of lipid peroxidation showed that it decreased tissue GSH levels with GSH-Px antioxidant enzyme activity. Ucar *et al.* [48], in their study in which they examined using N-acetylcysteine (NAC) and NAC+ozone treatment, may have some protective qualities (0.7 mg·kg^{-1} day intraperitoneally for five days) combination against APAP (1 g·kg^{-1} orally)-induced nephrotoxicity, found that NAC and NAC+ozone therapy applications significantly reduced MDA and TNF-α levels, while those that decreased after APAP application. They determined that it increased IL-10 levels and GSH-Px activities. As in a few examples above, APAP and ozone applications are encountered. However, there are differences in both APAP and ozone application doses. In our study, ozone was administered intraperitoneally for three weeks at a dose of 150 µg·kg^{-1} day, while APAP was treated at a dose of 2 g·kg^{-1} as a single dose and orally.

Ozone is used to treat a wide range of clinical disorders, both on local skin conditions and systemic conditions, and has several advantages in the treatment of various diseases, primarily through the manipulation of the immune system and of the oxidant/antioxidant balance. The primary feature of ozone therapy is the anticipation of the healing process, which is associated with the rise in endogenous growth factors and the improvement in the oxygenation of the affected area. Ozone has a physiological effect, but it also has the ability to oxidize substances, which renders bacteria inactive. This substance's toxicity is dependent on the amount of ozone present; at greater concentrations, it can exacerbate the immune system and have negative effects, while at lower concentrations, it can reduce inflammatory reactions and boost the production of antioxidant enzymes. Overall, ozone has been utilized as a therapy with success for a variety of medical illnesses, but it must be administered in the right dose range and concentration to prevent any potential side effects from high concentration toxicity [49].

One of the strongest known oxidants is ozone, which has a standard redox potential of +2.07 V [50]. As a result, there have been questions about its application because it can cause free radicals and associated diseases. Ozone is recognized nowadays for its paradoxical activity, which involves increasing the antioxidant qualities of structures impacted by its diseases despite the fact that it serves as an oxidizing molecule. Due to its low antioxidant system and ability to oxidize organic substances, ozone has harmful effects on tissue cells, especially those in the respiratory tract. Ozone has medicinal benefits that are dose-dependent. Moreover, acquiring the right concentration of ozonation products is essential to prevent toxicity. When ozonation products overwhelm the antioxidant system, adverse effects eventually happen [51].

Ozone therapy has been extensively researched for many years in human medicine; however, ozone therapy in veterinary medicine is still in the early stages of development. Ozone's antibacterial, immunostimulant, and antioxidant properties are the basis for its medical use. Particularly, no residues are discovered in tissues and biological fluids following the injection of O_3 to evoke its antibacterial, antiviral, antifungal, anti-yeast, and antiprotozoal activities. Ozone may therefore offer a chance to stop the spread of antibiotic resistance. Many details about how ozone works whether administered locally or systemically are yet unknown. Before some of the ozone applications utilized in humans or investigated in vitro and in rat models, a comprehensive grasp of the mechanisms involved in ozone therapy should be understood [52].

The dose of a substance is one of the most critical factors in determining whether that substance can be beneficial or harmful. In fact, substances known as antioxidants can be toxic at high doses, while substances known to have oxidant effects can show antioxidant effects at low doses. It is very interesting that in a previous study, a study was conducted in the opposite direction to the one we performed. In the study by Van der Zee *et al.* [53], they stated that APAP (10–20 mM) protects human red blood cells (erythrocytes) against a variety of oxidative stress mechanisms. They underlined the need for protection of ozone (10 µmol·min^{-1})-induced damage can be explained by the direct scavenging reaction between APAP and ozone and that APAP seems to be an effective scavenger of radicals produced in secondary reactions. In all cases, they speculated that the protective effect of acetaminophen might be accompanied by APAP's covalent attachment to membrane proteins. In this study, the plasma MDA concentration and antioxidant enzyme activities approaching the control group values after ozone application show that ozone helps lessen oxidative stress and damage brought on by APAP. As a result, it was concluded that ozone might be an effective antioxidant in reducing the side effects of APAP, an analgesic and antipyretic drug.

While many scientists argue that ozone therapy is effective in the field of health, there are also many scientists and scientific studies claiming that this form of treatment is not scientific and its benefit has not been proven, and on the contrary, it is harmful. However, the appropriate application method to be preferred by providing patient-specific dose selection in ozone therapy draws attention as an important method in preventing many different pathogenesis or supporting existing treatments. As the chemist Paracelsus said, "it is the dose that separates the medicine from the poison". Therefore, in order for any drug or active substance to be called "harmful, ineffective or effective", it must first be investigated at what stage of the disease, at what dose and at what frequency it was given.

Considering all these, we think that many new studies should be done in the evaluation of the effects of ozone therapy. More comprehensive studies should be planned by completing the deficiencies in our study. In this context, studies involving different tissues and different doses can be planned, and changes on more biomarkers should be evaluated than in the present study.

CONCLUSION

In conclusion, the results of the study showed that APAP caused oxidative stress in blood tissue. The present study showed that ozone had potential protective effects against toxicity induced with APAP through various mechanisms in different cellular processes. This may be related to cytoprotective and antioxidant properties of ozone. Ozone can provide a chemical basis for some health benefits against toxicities. Ozone may be protective against APAP–induced oxidative damage. As a result, it was concluded that ozone may be a natural and effective antioxidant that can be used to reduce the toxicity caused by APAP.

Conflict of interests

No conflicts of interest for all authors are declared.

Credit author statement

Emre Kaya was involved in the design of the study, animal applications, laboratory experiments, and evaluation of results. Seval Yilmaz was involved in the animal applications, laboratory experiments, and evaluation of results. Feyza Aksu and Ahmet Kavakli took part in obtaining ozone and applying it to experimental animals.

Ethics approval and consent to participate

The Firat University Animal Studies Local Ethics Committee accepted the experiments (Protocol No: 2022/17–1), which were carried out strictly in compliance with the Experimental Animal Ethics Committee's Guiding Principles.

Disclosure statement

The authors state that there are no interests at odds with one another.

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