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Original paper

The Effects on Oxidative Systems in Liver Tissues of Systemic Ozone Application after Critical Size Bone Defect Surgery in Rat Mandibles

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Abstract

Examination of the changes that non-toxic doses of antioxidant action ozone used in increasing osteogenic activity by dentists causes on rat liver tissues. The effects of ozone applications that are significant in mandibular bone defect therapies and oral and maxillofacial surgery practice on oxidant / antioxidant system in rat liver tissues are investigated by analyzing malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) parameters. While there were statistically significant reductions in MDA levels and SOD activities after 15 and 45 days long ozone applications on critical mandibular bone defect induced rat livers, statistically significant increases were observed in GSH levels and CAT activities ($P \leq 0.05$). This is the first study where the effects of ozone therapies used in treatment of mandibular bone defect on liver tissue oxidant / antioxidant system in rats are scrutinized with the analysis of biochemical parameters. It was determined that ozone applications did not have any negative effects on liver tissue oxidant / antioxidant system and concurrently had positive effects on liver antioxidant system and to reveal the biochemical mechanism completely, further studies are needed.

Keywords Liver, mandibular bone defect, oxidative stress, ozone, rat

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Introduction

Deterioration of the existing anatomical integrity and continuity of the bone as a result of direct or indirect interventions is a pathological event and called a fracture. The forces that cause the fracture do not only affect the bone tissue, but could also damage surrounding soft tissues such as muscles, tendons, ligaments, veins, nerve packages, skin and even neighboring organs (SCHENK [1]; RHEE & al. [2]). There are several local and general factors that affect bone recovery positively or negatively. Furthermore, recent studies demonstrated that free radicals are also among these factors. It was reported that during bone recovery and especially during the early period where biological interactions are at maximum excessive amounts of free radicals are formed and the recovery process is deteriorated due to the destructive effects of oxidative stress on tissues and cells (SEVINDIK & al. [3]; DUYGULU & al. [4]; GÖKTÜRK & al. [5]; SEVINDIK & al. [6]; SYMONS [7]).

As a response to endogenous and exogenous free oxygen radicals and to protect their integrity, living organisms developed various defense mechanisms via different endogenous antioxidants. Recent studies increasingly investigate the effects of antioxidant therapies on bone metabolism through increasing osteoblastic activity and preventing the osteoclastic activity. The effect of non-toxic doses of ozone, which function as an antioxidant, on bone generation is reported in recent medical studies (DELGADO-ROCHE & al. [8]; EL-SAWALHI & al. [9]; IGLESIAS & al. [10]; MALLOK & al. [11]).

Liver is one of the largest organs in our body, related to almost all systems of the organism and has complex and important functions in the body. Liver has intense metabolic activity that includes detoxification. This process is called biotransformation, in which free radicals could be generated under physiological conditions. Under which case, sufficient antioxidant intake protects the liver against free radical damage. Sufficient amounts of antioxidant intake promote the decrease in the damage caused by free radicals generated during the detoxification process (MONSHOUWER & al. [12]; SELAMOGLU &YILMAZ [13]; YOUSSEF & al. [14]).

In the present study, together with the oxidative damage potentials in mandibular bone defect induced rat liver tissues, the effects of ozone applications on the oxidative damage in liver tissues are analyzed using biochemical parameters. Furthermore, this study is the first research where the changes created by the effects of the ozone application, which was shown to increase osteogenic activity, in liver tissue were scrutinized with the analysis of biochemical parameters.

Materials and Methods

Animals and experimental design

The subjects of the present study included thirty-six healthy male albino Wistar rats (body weight 200-250 g, three to four months old), divided into six groups, six animals each. Subjects were kept under standardized light cycle conditions (12 h light: 12 h dark period) at a temperature of 22 ± 2°C and had free access to food (standard pellet chow diet) and water. These animals were procured from AfyonKocatepe University Experimental Animal Center. Extreme care was paid to follow the ethical standards set by the "Guide for the Care and Use of Laboratory Animals" (COUNCIL [15]), which is closely enforced by AfyonKocatepe University Experimental Animal Ethics Committee.

Five mm critical size bone defect was induced in the mandibles of the rats in all groups except the control group and biphasic calcium phosphate, which is a synthetic graft material, was placed on the defect region since this material is easily obtainable, has sufficient osteoconductive and de-antigenic properties. Procedures applied to the rats and study groups are listed below:

Group 1: The control group that included healthy rats that underwent no application (sacrificed after 15 days).

Group 2: Only mandibular defect (MD) was induced via surgical procedure (biphasic calcium phosphate graft material was applied immediately after the surgery) (sacrificed after 15 days).

Group 3: After the mandibular defect was induced, 0.6 mg/kg (= 3 mL) ozone (O) was applied once a day by rectal route to the subjects for 15 days (sacrificed after 15 days).

Group 4: The control group that included healthy rats that underwent no application (sacrificed after 45 days).

Group 5: Only mandibular defect (MD) was induced via surgical procedure (biphasic calcium phosphate graft material was applied immediately after the surgery) (sacrificed after 45 days).

Group 6: After the mandibular defect was induced, 0.6 mg/kg (= 3 mL) ozone (O) was applied (together with graft material) once a day by rectal route to the subjects for 45 days (sacrificed after 45 days).

Surgical procedure

All surgical procedures were conducted in Afyon-Kocatepe University, Experimental Animal Center. Experimental animals were anesthetized intraperitoneally using 3 mg/kg xylazine (Rompun 2%, Bayer, Istanbul, Turkey) and 90 mg/kg ketamine HCl (Ketalar, Eczacıbaşı-Warner Lambert, Istanbul, Turkey). After the depth of the anesthesia was controlled by examining eyelid reflexes, operation area was shaved and cleaned with povidone iodine (Batticon, Adeka, Turkey) and the subjects were prepared for surgery by sterile covers in a way that the operation area was totally visible. For local hemostasis, 1 cc 4% articaineHCl + 1/100000 epinephrine HCl (Ultracain DS Forte, Hoechst Marion Roussel, Germany) was injected in the surgical area. A 1 cm incision was applied on angle mandible at a location close to basis mandible and the subcutaneous tissues were incised to reveal the bone surface. Using a 5 mm diameter drill, a standard critical size full level bone defect was induced in the mandibular bone. The area was irrigated with saline solution during the induction of the bone defect to prevent heat and the damage that could be caused by heat on the tissues. After the defect was induced, graft material was placed in the region. When the procedure was completed, subcutaneous tissues and flap were sutured to their original position with 5-0 silk suture and primary closure was obtained.

Ozone application

Rectal ozone application was conducted with methods proposed in the literature (AGRILLO & al. [16]; MARTÍN-BARRASA & al. [17]). Ozone was obtained with ozonosan device. Ozone concentration was set at 3% ozone/oxygen rate the device and applied in 17 µg ozone/mL 0.6 mg/kg doses. Rectal area of the subjects in the ozone application groups were cleaned from fasces and lubricant cream was applied and a 20 mm thin serum pipe was connected to the injection that contained 20 cc ozone and 0.6 mg/kg 3 cc ozone was applied once a day for 15 or 45 days (in different groups).

All subjects were sacrificed after 15 or 45 days based on their groups. All animals were successively sacrificed after the respective test durations with 200 mg/kg sodium pentobarbital anesthesia. After the application, chests of rats were opened, vena cava was incised and 30 mL of 0.9% NaCl was injected into the heart to cleanse the blood. The liver tissues were removed and stored in liquid nitrogen at -80°C until use.

Biochemical analyses

The liver tissues were homogenized in ice with 0.1 M Tris-HCl buffer (pH 7.5; including protease inhibitor, phenylmethylsulfonyl fluoride, 1 mM) at 16,000 rpm and at 4°C for 3 min with a homogenizer (IKA Ultra Turrax T25 basic). MDA, GSH levels and SOD, CAT activities were measured using the homogenates.

Lipid peroxidation assay

Measurement of thiobarbituric acid reactive substances including MDA were conducted by adding thiobarbituric acid to tissue homogenates and measuring the light absorbance at 535 and 520 nm in a spectrophotometer as previously described (UCHIYAMA & al. [18]).

GSH assay

The results were reported in nmol/g wet tissue. Reduced glutathione assay based on the spectrophotometric Ellman's method was used to measure liver homogenate GSH concentrations (ELLMAN [19]).

SOD assay

Total reduction of nitro blue tetrazolium by the superoxide anion produced by xanthine and xanthine oxidase was used for the measurement of SOD activity (JOLITHA & al. [20]). Unit SOD activity was defined as the quantity of protein inhibiting the rate of NBT reduction by 50%, and the results were reported as units per milligram protein.

Protein assay

Lowry's method was used to determine the total protein content of the liver tissue homogenate samples (LOWRY & al. [21]).

Determination of CAT activity

Aebi's method was used to measure CAT activity (AEBI & al. [22]) by determining H_2O_2 rate constant k (dimension: s-1, k) (initial concentration 10 mM) indicated by the absorbance at 240 nm in a spectrophotometer (CASADO & al. [23]). The activity was reported as k (constant rate).

Statistical analyses

Statistical analyses were conducted with SPSS 21.0 for Windows software. Shapiro-Wilk test was utilized to determine normal distribution of data. Data were summarized with median values (min – max) since the data did not demonstrate a normal distribution. Inter-group comparisons were conducted with Kruskal-Wallis test. Dual comparisons were conducted with Conover method after the Kruskal-Wallis test. Level of significance was accepted as 0.05 in all conducted ($P \leq 0.05$).

Results and discussion

Changes observed in biochemical parameters such as MDA, GSH levels and SOD, CAT activities in rat liver tissues as a result of ozone (O) application in mandibular defected (MD) rats for a period of 15 and 45 days are presented in (Tables 1 and 2).

MDA levels in mandibular defected (MD) groups statistically significantly increased when compared to other groups in both 15 and 45-day application groups (P<0.05). There were statistically significantly decreases in MDA levels in MD + O groups compared to MD groups (P<0.05). GSH levels in liver tissues of MD groups did not change statistically significantly when compared to control group in both 15 and 45-day application groups (P > 0.05) (Tables 1 and 2). There were statistically significantly increases in GSH levels in MD + O groups compared to control and MD groups (P < 0.05). SOD activities in MD groups did not change statistically significantly when compared to control group in both of 15 and 45-day application groups (P > 0.05). There were statistically significant decreases in SOD activities in MD + O groups compared to MD and control groups (P<0.05). CAT activity in MD group did not change statistically significantly when compared to control group in the 15-day group (P>0.05). But, there were statistically significantly decreases in CAT activity in the 45-day group (P < 0.05). CAT activity in MD + O group significantly increased compared to control and MD groups (P < 0.05).

Studies reported that ozone therapies, which have stimulating impact on osteogenic activity, have very low toxic effects (MALLOK & al. [11]; AGRILLO & al. [16]; MARTÍN-BARRASA & al. [17]; DELGADO-ROCHE & al. [24]). There are several studies in the literature that exhibited the biological effects of ozone and there are also studies that scrutinized its effects on removal of oxidative stress. Studies further reported that it had positive impact on bone defect recovery (MALLOK & al. [11]; AGRILLO & al. [16]; MARTÍN-BARRASA & al. [17]; DELGADO-ROCHE & al. [24]; BOCCI [25]; GUVEN & al. [26]). Findings of the present study also determined that ozone therapies had oxidative stress removal effects in liver of rats with bone defects.

In literature, it was observed that recovery periods were assessed based on various lengths of recovery periods in studies that scrutinized mandibular defect recovery (BULBUL & al. [27]; RASUBALA & al. [28]). Following the fracture, certain biological events affect the recovery process negatively, especially during the few initial weeks. Previous studies demonstrated that oxidative stress occurred during recovery from fracture and ROS was one of the factors that affected fracture recovery negatively (DUYGULU & al. [4]; SYMONS [7]; ZHU & al. [29]). In our study, it was also identified that mandibular fracture defect was a factor inducing oxidative stress that increases

Table 1. Changes in MDA, GSH levels and SOD, CAT activities in liver tissues caused by application of ozone in mandibular bone defected rats during 15 days

Groups / Parameters	MDA (nmol/gwt)	GSH (nmol/gwt)	SOD (U/g protein)	CAT (U/g protein)
Control	280 (270-290)^{b}	1855 (1750-1870)^{b}	36.1 (33.1-38)^{a}	32.8(31.6-34.5)^{b}
MD	379 (304-408)^{a}	1846 (1794-1871)^{b}	36 (29.5-39)^{a}	33.1(24.5-38.1)^{b}
MD+O	258 (228-288)^{c}	2100 (1192-2206)^{a}	14.1 (11.6-15.5)^{c}	47.9(42.5-50.4)^{a}

Data are expressed Median (Min-Max) of six animals. gwt; gram wet tissue. Different letters in columns are significant P<0.05.

Table 2. Changes in MDA, GSH levels and SOD, CAT activities in liver tissues caused by application of ozone in mandibular bone defected rats during 45 days

Groups / Parameters	MDA (nmol/gwt)	GSH (nmol/gwt)	SOD (U/g protein)	CAT (U/g protein)
Control	284 (270-290)^{b}	1850 (1820-1870)^{b}	35.6 (33-37.5)^{a}	32.4(31.6-33.5)^{b}
MD	419 (408-421)^{a}	1449 (1420-1999)^{b}	36.4 (31.7-41.5)^{a}	22.4(18.2.-16.2)^{c}
MD+O	234 (204-278)^{c}	2445 (2288-2544)^{a}	10.9 (10.1-12.5)^{c}	53.2(52.6-58.3)^{a}

Data are expressed Median (Min-Max) of six animals. gwt; gram wet tissue. Different letters in columns are significant P<0.05.

lipid peroxidation. Fifteen and forty-five-day long ozone applications resulted in similar changes in liver tissue oxidant / antioxidant systems in both time periods.

In empirical studies conducted with rats, it was shown that ROS induced damage was higher in post-fracture 7th and 14th days and it started to decrease during the fourth week (ZHU & al. [29]; YELER & al. [30]). Göktürk et al. also reported that increased radical production deteriorated fracture recovery in examinations conducted on post-fracture 22nd day in their study that investigated the effects of ROS on fracture recovery (GÖKTÜRK & al. [5]). Thus, for the fracture to mend completely, early periods are of extreme significance (GÖKTÜRK & al. [5];YELER & al. [30]). For this purpose, to examine the effects of ozone on ROS and possible tissue damage during the early period in the present study, we considered it appropriate to assess the recovery processes on 15th and 45th days. The present study was conducted to demonstrate the degree that bone recovery processes and treatment procedures would affect rat liver tissue with respect to biochemical changes as well.

Under normal conditions, healthy living organism has an antioxidant defense mechanism to protect itself against the effect of free radicals. When this existing system is insufficient, examination of the oxidant / antioxidant system would show theextend of the damage caused by ROS on rat liver tissue and the level of suppression of this situation by the counter-defense mechanism. In our study, we utilized liver MDA, GSH, SOD and CAT parameters to examine the action of mandibular defect model induced by open surgery method and ozone applied for the treatment of this defect model on oxidant / antioxidant system.

Functional criteria were the basis of the preference for the liver since it is a target of changes in metabolism. Free radical generation that could be induced by defects and lesions plays a key role in the damage and function loss in tissues and organs. Lipid peroxidation, resulting from oxidative injury of saturated and unsaturated lipids, is widely used as a marker of oxidative damage induction in rats suffering from traumatic damage induced physiological stress. In the present study, MDA levels increased more in mandibular defected rat liver tissues when compared to the control group. These findings strongly suggested that defected bone caused generalized oxidative stress in rat liver. Thus, it could be argued that changes in the metabolic process could have caused elevated ROS production, while other factors such as enzymatic inhibition or genotoxic damage could have been effective (SELAMOGLU TALAS & al. [31]). When ROS overruns endogenous protective systems, exogenous antioxidant agents must be delivered. Ozone treatments resulted in decreases in MDA levels in the liver of mandibular defected rats in both 15 and 45 day groups. Thus, research on new antioxidants as potential substances is an active field of medicinal chemistry. It is well known that the most important factor for the development of metabolic disorders is ROS. When the factor of oxidative stress is met, cellular defense system activity is increased (DUYGULU & al. [4]; YELER & al. [30]; SELAMOGLU TALAS & al. [31]; PETROVICH & al. [32]; STAN & al. [33]). However, the findings of the current study indicated that there were no statistically significant differences between the SOD and CAT activities and also GSH levels of mandibular defected rat liver stress levels in both 15 and 45 day periods when compared to the control groups.

Antioxidant enzyme activities such as SOD in the liver did not change in MD group when compared to control in this study. However, ozone treatments resulted in decreases in SOD activities in mandibular defected rat liver in 15 and 45 day periods. It was considered that mandibular bone defect induced oxidative stress with increased MDA levels impressed the SOD activities. Consequently, SOD activities did not change in mandibular defected rat livers in both 15 and 45 day periods. Similarly, CAT activity did not change in the livers of mandibular defect group in the 15-day group. However, it was observed that CAT activity decreased in the 45-day group. CAT activity decreased in the MD group due to the increased oxidative stress after 45 days.

Mandibular bone defect increased oxidative stress. Ozone treatments caused decreases in MDA levels in mandibular defect rat livers in 15 and 45 day groups due to oxidative stress inhibition. As a result of ozone treatment, SOD activities decreased along with the decreasing oxidative stress in both 15 and 45 day groups. In contrast, GSH levels and CAT activities increased as a result of ozone application due to the repression of the oxidative stress.

These findings suggest that ozone might play an important role in maintaining antioxidants systems as an antagonist substance in traumatic bone defect induced stress in rat liver. New antioxidant therapy approaches using more stable, less toxic, and easily accessible biomaterials that could repair bone defects and stimulate new bone formation attract great interest (MARTÍNEZ-SÁNCHEZ & RE [34]; PECORELLI & al. [35]). Current study findings show that ozone therapies could regulate mandibular bone defect-induced stress-related changes in rat liver. Ozone applications could contribute to antioxidant defense systems in rat tissues as demonstrated in certain studies (MALLOK & al. [11]; AGRILLO & al. [16]; MARTÍN-BARRASA & al. [17]; BOCCI [25]; GUVEN & al. [26]; BOCCI [36]; BOCCI & al. [37]; IGLESIAS & al. [38]; TRAVAGLI & al. [39]; BOCCI [40]; BOCCI & al. [41]). Oxidative factors such as bone defects might significantly increase oxidative cellular damage. Adequate antioxidant defense systems including therapeutic agents might prevent lipid peroxidation. Ozone has antioxidant

properties and is a scavenger of free radicals, thus preventing tissue damage (MALLOK & al. [11]; DELGADO-ROCHE & al. [24]; IGLESIAS & al. [38]). We could argue that exposure to mandibular bone defect induces an increase in ROS in the rat liver.

Epidemiological study findings indicated protective and preventive effects of ozone therapies against various mandibular defects and their ability to reduce oxidative injury and to enhance exerted antioxidant properties (MALLOK & al. [11]; AGRILLO & al. [16]; MARTÍN-BARRASA & al. [17]; DELGADO-ROCHE & al. [24]; IGLESIAS & al. [38]). The present study findings demonstrated that ozone applied after mandibular defect was induced minimized the damage to the liver of mandibular bone defect induced rats. Ozone has antioxidant effects against mandibular defect-induced oxidative stress in rat liver. The findings of the present study were parallel that of similar studies in the literature (DUYGULU & al. [4]; GÖKTÜRK & al. [5]; EL-SAWALHI & al. [9]; MALLOK & al. [11]; MONSHOUWER & al. [12]; YOUSSEF & al. [14]; DELGADO-ROCHE & al. [24]; IGLESIAS & al. [38]).

Conclusion

As a result, we conclude that mandibular bone defect results in an increase in oxidative damage in rat liver. This increase in oxidation plays a role in mandibular defect-induced rat liver damage. And as a result, it could be concluded that novel therapeutic agents such as ozone provide a decrease in bone damage induced oxidative stress in rat liver.

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