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Research Article

Effects of Ozone Treatment in Endotoxin Induced Shock Model in Rats

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Abstract

Objective: This study was focused on effects of ozone application on vital and biochemical parameters in rat model of endotoxin-induced shock. **Materials and Methods:** Lipopolysaccharide (LPS; 10 mg kg^{-1}, i.p.) induced shock model was used in male Wistar albino rats. Three different doses of ozone were used for this study (0.1, 0.3 and 1.0 mg kg^{-1}, i.p.). Tail-cuff method was preferred for measurements of systolic blood pressure and heart rate. Plasma nitric oxide (NO), total antioxidant capacity (TAC), asymmetric dimethylarginine (ADMA), aspartate transaminase (AST), alanine transaminase (ALT) and lactate dehydrogenase (LDH) levels were detected as biochemical parameters. **Results:** Decline in systolic blood pressure and increment in heart rate of rats were observed an hour after injection of LPS. Ozone application did not possess any significant improvement on impaired systolic blood pressure and heart rate. Increased plasma levels of NO and decreased TAC levels with endotoxemic shock were reversed by ozone treatment. No significant effect on augmentated plasma levels of ALT by endotoxemia was observed with ozone application. On the other hand ADMA, AST and LDH levels were not changed with endotoxemia or ozone application. **Conclusion:** These results suggested that ozone treatment reversed the LPS-induced changes inplasma NO and TAC levels, but not other vital and biochemical parameters in rat model of endotoxin induced shock.

Key words: Endotoxemic shock, ozone, nitric oxide, total antioxidant capacity, asymmetric dimethylarginine, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, rat

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sepsis represents a serious medical condition characterized by systemic inflammation. Septic shock occurs when condition leads to vascular hyporeactivity, hypotension, myocardial dysfunction and multiple organ failure. Physiopathology remains to be poorly understood and recent therapeutic approaches are not sufficient enough to reduce high mortality rates.

Lipopolysaccharide (LPS), a component of the Gram-negative bacteria cell wall that has been released during septicemia is a major cause of septic shock in humans and it has been used for the development of experimental septic shock model in animals[1-4]. Inflammatory signals as a consequence of LPS-stimulated immunity can activate the secretion of pro-inflammatory and anti-inflammatory mediators in order to maintain an equibrium[5]. A loss of balance between those mediators may occur with over-production of endogenous pro-inflammatory mediators such as oxygen radicals and nitric oxide (NO)[6,7]. Over-released NO by the activation of inducible isoform of nitric oxide synthase (iNOS) may cause arterial hypotension during septic shock. Excessively produced NO and superoxides causes increased level of nitrogen radicals which are considered as strong cytotoxic agent[8-10]. Nitrosative stress can cause various structural modifications in proteins, lipids and nucleic acids[11]. Changes in total anti-oxidant capacity are known as an indicator forim balance between oxidizing and anti-oxidizing agents[12]. An endogen molecule-asymmetric dimethylarginine (ADMA) is a non-selective competitive inhibitor of NOS and an increase of ADMA levels in plasma have been observed in sepsis[13].

Ozone is an unstable, ring-structural triple oxygen molecule can be used for medical use in a mixture of oxygen (95% minimum concentration)-ozone (5% maximum concentration)[14]. While ozone reacts with essential fatty acids at pshysiological pH conditions, hydroxy-hydroperoxides arises spesifically. These molecules also called as ozone peroxides are thought to be responsible for physiological properties of ozone which have low tendency to participate in radical reactions, interact with sulfhydryl groups. Thus, they induce antioxidant enzyme expression in order to reconstitute oxidative balance[15,16]. Ozone has a bactericidal, virucidal and fungicidal effect and also it renovates systemic homeostasis. It has been shown that ozone/oxygen mixture has various effects on immune system[17]. Ozone application may induce an increment at antioxidant enzyme expression[18]. It has been suggested that ozone application has a protective effect on septic injury of lungs in addition to antibiotic therapy[19]. Studies have been carried out for effects of ozone on sepsis but its benefits can not be manifested clearly[20-23].

This study examine deffects of ozone treatment on vital and biochemical parameters in endotoxin-induced shock model of rats. In this manner, blood pressure, heart rate, plasma levels of NO, ADMA, TAC, ALT, AST and LDH were measured and compared between study groups of animals.

MATERIALS AND METHODS

Chemicals: Lipopolysaccaride of *Escherichia coli* (O111:B4, L4130) was obtained from Sigma. The LPS was dissolved in saline (5 mg mL^{-1}). Ozone/oxygen gas mixture was produced by OZONOSAN® ozone generator. All chemicals were obtained from Sigma.

Animals and experimental design: Male Wistar albino rats (250-300 g) were used in experiments. Animals were kept at 12 h light/12 h dark cycle and allowed free access to tap water and standard rat cow. The study was approved by Gazi University Local Ethics Committee for Animal Experiments (G.Ü.ET-11.045). Rats were obtained from Kobay Inc. Experimental Animal Laboratory. About 36 rats were divided into 6 groups. After that, protocols for each group were applied (Fig. 1):

- Control group: Saline (i.p.)
- LPS group: LPS (10 mg kg^{-1}, i.p.)
- Treatment-1 group: LPS (10 mg kg^{-1}, i.p.)+O_2/O_3 gasmixture (0.1 mg kg^{-1}, i.p.)
- Treatment-2 group: LPS (10 mg kg^{-1}, i.p.)+O_2/O_3 gasmixture (0.3 mg kg^{-1}, i.p.)

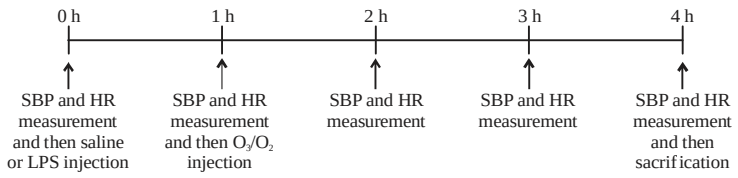

Fig. 1: Experimental protocols, SBP: Systolic blood pressure, HR: Heart rate, LPS: Lipopolysaccaride

- Treatment-3 group: LPS (10 mg kg^{-1}, i.p.)+O_2/O_3 gasmixture (1.0 mg kg^{-1}, i.p.)
- Ozone group: O_2/O_3 gasmixture(1.0 mg kg^{-1}, i.p.)

Heart rates (HRs) and systolic blood pressures (SBPs) of rats were measured and then saline or LPS injections were performed intraperitoneally. After an hour, vital parameters were measured again and oxygen/ozone gas mixtures were injected at predetermined doses. The HR and SBP measurements were performed until 4 h of LPS injection with intervals of an hour. At the end of the experimental procedures, all rats were anesthetized with thiopental sodium (60 mg kg^{-1}, i.p.) and then blood samples were collected from hearts of animals. Blood samples were taken into tubes containing EDTA, centrifuged at 10000 rpm for 10 min to extract their plasma. Plasma samples were kept at -80°C.

Blood pressure measurement: Blood pressure and heart rate measurements performed with non-invasive tail-cuff device (Non-invasive indirect blood pressure system for rats, NIBP200A, COMMAT, BIOPAC®, Turkey) in pre-warmed and restrained rats. At least five measurements were made for each rat and theme an values were estimated as the systolic blood pressure.

Biochemical examination: For the measurement of NO, ADMA and TAC concentrations, blood samples (1.5 mL) were withdrawn from the arterial catheter in ice-cold tubes containing EDTA (50 µg mL^{-1} blood) at the time points described above. Samples were centrifuged (10.000 rpm; 4°C, 10 min) and their plasma was separated to be stored at -80°C until analysis process.

Plasma nitrite/nitrate levels were measured as a marker for NO production. Spectrophotometric method based on the Griess reaction[24] was used for that manner. We also modified it for 96-well plates.

The TAC levels in plasma were measured by using a previously-described method[25] based on the reduction of Cu^{+2} to Cu^{+} by antioxidants in the plasma. Neocuproine was used as a chromogenic agent and colored complex was detected spectrophotometrically at 455 nm.

The ADMA levels were measured by using ELISA kits (Immunodiagnostic A.G., Germany) according to the manufacturer's instructions.

Plasma ALT, AST and LDH activities were determined using commercial kits adapted to a COBAS c501 autoanalyzer (Roche Diagnostics, Germany).

Plasma levels of biomarkers were plotted as a percentage of pre-shock levels (0 min).

Statistical analysis: Results were expressed as "Mean±standard error of the mean". Blood pressure measurements and heart rates of all groups were compared with control group using repeated measurements two-way analysis of variances (ANOVA) followed by Bonferroni test. Biochemical data were compared with control group by Student's t-test. The p-values lower than 0.05 are considered as statistically significant.

RESULTS

Systolic blood pressure measurements and heart rate values of all groups measured according to experimental protocol (Fig. 1) are presented on Fig. 2a and b. The SBP and HR values of all groups were similar with results obtained from early measurements before the experimental procedure. Inendotoxemic shock group (81.23±7.62, 83.35±8.32 and 79.76±4.43; respectively), mean SBP measurements were significantly decreased when compared with the control group (109.81±1.51, 110.23±2.41 and 110.43±3.27,

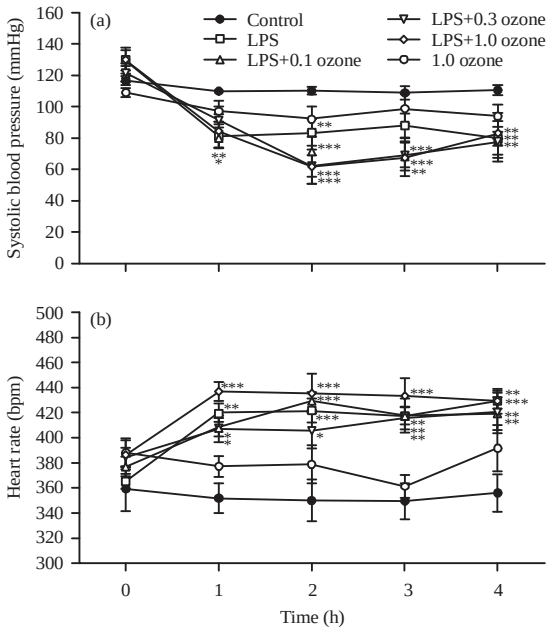

Fig. 2(a-b): Effect of ozone treatment on systolic blood pressure and heart rate in endotoxin-induced shock model in rats. Decrease on SBP values by LPS injection were not changed by ozone treatment, (a) Increases on HR values by LPS injection were not changed by ozone treatment and (b) *Difference from control (*p<0.05, **p<0.01 and ***p<0.001) (repeated measures two-way ANOVA; *post-hoc* Bonferroni test)

respectively) at the 1, 2 and 4 h. Also significant increase on mean HR values at the 1-4 h was observed following LPS injection (420.31±7.23, 422.00±9.52, 417.58±6.79 and 429.53±9.73, respectively) when compared with the control group (352.15±12.08, 350.26±16.65, 349.75±14.41 and 356.15±15.05, respectively). These impaired hemodynamic parameters induced by LPS injection did not altered by ozone applications. Ozone injection at 1 mg kg^{-1} dose, per se, did not make any effect on SBP or HR values.

In LPS (10 mg kg^{-1}, i.p.) applied group, plasma NO levels were significantly higher than the control group. In the ozone-alone group, NO levels were measured significantly lower than the control group. Ozone treatment groups at 0.3 and 1.0 mg kg^{-1} ozone doses were not different from control group (Fig. 3).

There is no statistically significant difference between ADMA levels in all groups (Fig. 4). Plasma TAC levels in LPS (10 mg kg^{-1}, i.p.) group was significantly low when compared to the control group. The TAC levels in ozone-alone group were not different than the control group. In endotoxemic shock groups, it is observed that decreased TAC levels by LPS application were significantly elevated to the control levels while medical ozone treatment applied (Fig. 5). Plasma ALT levels in LPS (10 mg kg^{-1}, i.p.) group were significantly higher than the control group and ozone treatment did not reversed ALT levels (Fig. 6). There is no statistically significant difference in AST and LDH levels between groups (Fig. 7, 8).

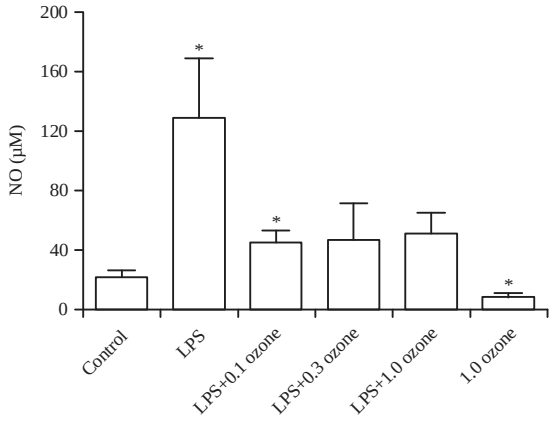

Fig. 3: Effect of ozone treatment on plasma NO level in endotoxin-induced shock model in rats. Endotoxemic shock-induced increase in NO level was decreased by ozone at 0.3 and 1.0 mg kg^{-1} doses. The NO levels in ozone-alone group were lower than control group. *Difference from control (*p<0.05) (student t test)

DISCUSSION

Septic shock is a syndrome characterized by high cardiac output and reduced peripheral vascular resistance. Reduction in peripheral vascular resistance leads to irreversible hypotension that is non-responsive to vasoconstrictors[26,27]. In septic and endotoxic shock, vascular hyporesponsiveness against vasopressors and increment at plasma levels of vasodilatory molecules such as NO have been demonstrated by many of experimental studies[28-35]. Due to unequal hyporeactivity between different vascular beds leads to redistribution of the cardiac output and impaired tissue perfusion that may cause multiple organ failures[30]. In terms of

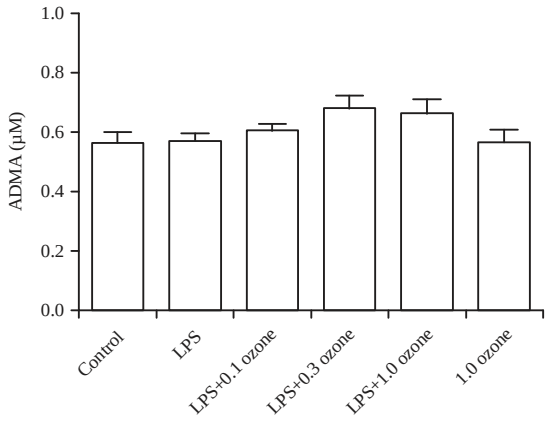

Fig. 4: Effect of ozone treatment on plasma ADMA level in endotoxin-induced shock model in rats. The ADMA levels did not change by administration of LPS or ozone treatment

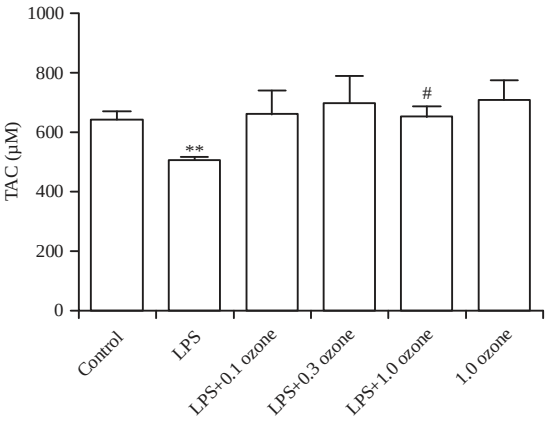

Fig. 5: Effect of ozone treatment on plasma TAC level in endotoxin-induced shock model in rats. Endotoxemic shock-induced decrease in TAC level was reversed with all doses of ozone treatment. *Diffence from control (**p<0.01) (student t test)

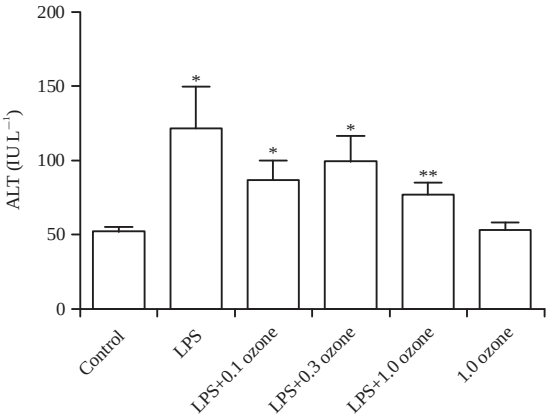

Fig. 6: Effect of ozone treatment on plasma ALT level in endotoxin-induced shock model in rats. Endotoxemic shock-induced increases in ALT levels were not reversed by ozone treatment. *Diffence from control (*p<0.05 and **p<0.01) (student t test)

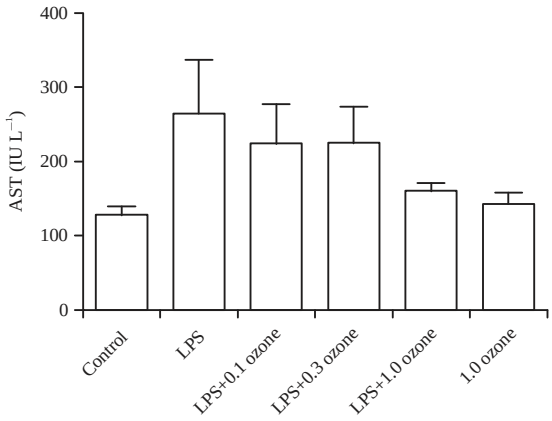

Fig. 7: Effect of ozone treatment on plasma AST level in endotoxin-induced shock model in rats. The AST levels did not change by administration of LPS or ozone treatment

the success of the treatment, vascular hyporesponsiveness is still a formidable subject[26].

The NO arising from iNOS is considered as an important molecule that may be responsible from vasodilation occurred in sepsis[36,37]. Excessive amount of NO derived from iNOS activity turns into peroxynitrite molecules reacting with superoxides, which leads to production of highly reactive nitrogen radicals. These radicals may trigger cellular toxicity leadind to tissue injury by reacting key cellular components such as proteins[8-11].

It has been demonstrated that ozone preconditioning reversed the NO enhancement in endotoxemic shock[38]. In a

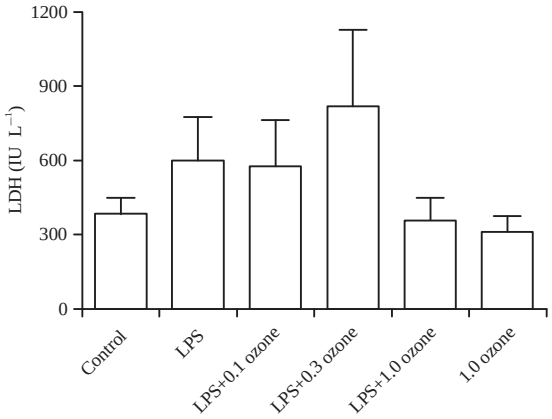

Fig. 8: Effect of ozone treatment on plasma LDH level in endotoxin-induced shock model in rats. The LDH levels did not change by administration of LPS or ozone treatment

septic shock model induced by cecal ligation and puncture (CLP) method, ozone preconditioning (1.2 mg kg^{-1}, single dose) stabilized plasma nitrite/nitrate levels, mean arterial pressure and vascular reactivity and these healing effects were equivalent to selective iNOS inhibitor, L-canavanine[39]. Oztas *et al.*[40] observed that inhibition of iNOS reduces therapeutic efficacy of ozone in acute necrotizing pancreatitis, suggesting that iNOS inhibition may mediate effect of ozone on NO levels.

In this study, ozone treatment at 0.3 and 1.0 mg kg^{-1} doses decreased LPS-induced augmentation in NO levels but did not ameliorate systolic blood pressure and heart rate[41]. Improvement at NO levels due to treatment was not sufficient enough to reverse LPS-induced worsening on hemodynamic parameters that cen be influenced by many other factors. These results suggest that NO and its metabolites, per se, may not be responsible for reduction in the systolic blood pressure and vascular hyporeactivity at early phase of endotoxemic shock. In addition, further reduction of NO levels by only ozone application (8.12±2.79) suggests that ozone may not act only by iNOS inhibition but also with contributions of constitutive ones.

The ADMA is one of the naturally occurring methyl arginine that results from proteolysis of post-translationally and irreversibly methylated tissue proteins. This endogen molecule inhibits NOS enzyme to decrease NO production in order to regulate of endothelial cell responses. Elevation of plasma ADMA levels are stated to be correlated with severity of organ failure and considered to be an early predictor for survival in septis patients[13,38,42-44]. In macrophages that exposed to

endotoxin, incubation with ADMA is reported to reduce NO production and iNOS expression[38].

In this study we examined that whether endogen ADMA levels increase and regulate NO that was over-released from endothelial and immune cells and cause robust hypotension. However, there was no difference in plasma ADMA levels between groups at the 4 h. While considering methylated proteins that contain clusters of asymmetrically methylated arginine residues are ubiquitous and highly abundant proteins in normal conditions to balance NO levels[42], similar plasma ADMA levels between groups in our experiment may be result from inadequate up-regulation of proteolytic enzymes to over-release ADMA at the 4 h of endotoxin challenge.

In endotoxemic shock, one of the underlying pathology is inadequate antioxidant defense due to excessive and extensive oxidative stress. Because of ozone being a highly reactive molecule itself, it can be assumed that ozone may be hazardous under these conditions. However, ozone molecules turn into ozone peroxides immediately after they enter to the body by reacting with unsaturated fatty acids. Ozone peroxides do not act as free radicals, but they can induce production of antioxidant enzymes[16]. Stimulative effect of ozone on antioxidant system has been demonstrated in numerous pathologies that involve disturbance in oxidant/anti-oxidant balance[45-49]. In addition, many researchers have agreed that ozone applications may improve antioxidant defense in septic or endotoxemic shock models by pre-treatment process[20,50,51].

In this study, ozone treatment reversed LPS-induced decrease of total antioxidant capacity at 0.1, 0.3 and 1.0 mg kg^{-1} doses. These results may suggest that ozone application might have a therapeutic effect in endotoxemic shock because of compensating antioxidant enzyme depletion. Endotoxemic shock is often an unpredictable condition due to its acute nature and rapid onset. This condition makes therapeutic effect of ozone more valuable than protective one.

Serum transaminases (AST and ALT) and LDH levels are often being measured as markers for cell injury. Transaminases are widely found throughout the body. The AST is detected early in heart, liver, skeletal muscles and kidneys and has both mitochondrial and cytoplasmic forms. However, ALT is found primarily in the liver and kidneys and has only cytoplasmic form. Despite ALT and AST are both exist in liver and their plasma levels are elevated in the liver diseases that affects cell integrity, ALT is thought to be more specific for hepatic injury because of being at the higher levels in the cytosol of the liver than other tissues[52,53]. Thus, isolated exaggerated ALT activities alone compared to AST levels may indicate hepatocellular damage[54]. Serum LDH elevations, unspecificially, occur in various diseases such as myocardial infarction, hemolysis and disorders of liver, kidneys, lung and muscles[53].

Ozone preconditioning reported to reduce elevated serum ALT and AST levels related to hepatic injury induced by hepatic ischemia[49] and radiation[55]. Rodriguez *et al.*[56] demonstrated that septic shock induction by intraperitoneal injection of fecal material elevated plasma ALT and AST levels of rats at 12 h. While they performed ozone/oxygen preconditioning 24 h before septic shock induction, they were able to reduce both ALT and AST levels significantly. In endotoxemic shock model, histochemical evolution demonstrated that 24 h after LPS injection, levels of LDH in liver, kidney, lungs and heart samples were increased significantly while compared with control group and ozone preconditioning for 10 consecutive days have decreased LDH levels only in liver tissue[21].

It is found that only ALT levels were increased in endotoxemic shock induced by LPS injection and this increment was not reversed by ozone treatment. The AST and LDH levels did not show any difference between groups. This may be resulted from time period variables among induction of endotoxic/septic shock and measurement of blood ALT, AST or LDH levels between our study and previous ones.

CONCLUSION

In conclusion ozone treatment ameliorates NO and TAC levels but fail to compensate blood pressure, heart rate, ALT levels at the early phase of endotoxemic shock. This failure may be overcomed by supporting ozone treatment with fluid resuscitation.

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