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## The protective effects of ozone therapy in a rat model of acetaminophen-induced liver injury

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### ABSTRACT

**Objectives:** Acetaminophen (APAP) overdose may cause acute liver injury. Ozone therapy (OT) is shown to reduce inflammation and necrosis in several entities. Thus, we have designed this study to evaluate the efficacy of OT in a rat model of APAP-induced liver injury.

**Methods:** Twenty-seven Sprague-Dawley rats were divided into three groups: sham, APAP and APAP+OT groups. In the APAP and the APAP+OT groups, liver injury was induced by oral administration of 1 g/kg APAP. The APAP+OT group received a single dose ozone/oxygen mixture (0.7 mg/kg) intraperitoneally 1 h after APAP administration. All animals were killed at 24 hour after APAP administration. Blood samples and liver tissues were harvested to determine liver injury and oxidative stress parameters. Liver tissues and blood samples were obtained for biochemical and histopathological analyses.

**Results:** APAP administration caused necrosis in the liver after 24 h. The degrees of liver necrosis of the APAP group were higher than the other groups (in both  $p < 0.05$ , respectively). In the APAP+OT group, liver antioxidant enzymes activities were significantly higher than the APAP group ( $p < 0.05$ ), but were lower than the sham group ( $p < 0.05$ ). In the sham group, serum neopterin, a marker of cell-mediated immunity, concentrations ( $4.8 \pm 1.2$  nmol/L) were lower than the APAP ( $14.7 \pm 1.4$  nmol/L) and APAP+OT groups ( $7.5 \pm 2.4$  nmol/L) (in both  $p < 0.05$ , respectively).

**Conclusion:** Our results showed that OT prevented liver necrosis in rats and reduced neopterin levels. These findings suggest that the use of OT as an adjuvant therapy which might improve the outcome in APAP induced liver injury.

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## 1. Introduction

Acetaminophen (N-acetyl-p-aminophenol, paracetamol, APAP) is a widely used agent for its analgesic and antipyretic actions. Therapeutic doses of APAP of approximately 4 g daily are considered to be safe while overdose produces severe centrilobular liver injury that can lead to fatal fulminant hepatic failure (Yaman et al., 2011). APAP is eliminated almost entirely as nontoxic glucuronic acid and sulfate conjugates when used at therapeutic doses (Pacifiçi et al., 1988). However, a small proportion of APAP is converted to N-acetyl-p-benzoquinoneimine (NAPQI) by cytochrome P-450 mediated oxidation. This forms NAPQI, which is normally detoxified by conjugation with reduced glutathione (GSH) (Nelson, 1995). At exposure to high dose APAP, much more NAPQI is formed; subsequently the hepatic GSH stores are consumed. Eventually, the remaining NAPQI binds to cellular macromolecules covalently and leads to cell death (Jollow et al., 1973).

Elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities indicate liver damage (Bruss et al., 2004). Necrosis of the liver usually results in leakage of AST and ALT from the hepatocellular membrane into the bloodstream. Although the elevated activities of these enzymes are indicators of hepatocellular damage, they are poor prognostic indicators for the severity of the liver injury or acute liver failure (Huang et al., 2008). Recently, it was shown that serum neopterin, indicator of the liver injury, levels were elevated in a rat model of APAP induced liver injury and that the increased neopterin levels were correlated with the dose of APAP (Demirbas et al., 2011). Thus, serum neopterin levels are regarded as a better prognostic marker for liver injury than the activities of aminotransferases in APAP toxicity.

Administration of a gas mixture comprising ozone/oxygen ( $O_3/O_2$ ) is known as ozone-therapy (OT) (Bocci et al., 2011).  $O_3/O_2$  mixture exhibits various effects on the immune system, such as the modulation of phagocytic activity of peritoneal and alveolar macrophages (Bocci, 2004, 2006a). So far, clinical studies have shown that OT appears useful in conditions ranging from peritonitis, to infected wounds, chronic skin ulcers, initial gangrene, burns and advanced ischemic diseases (Re et al., 2008). Administration of ozone induced a sort of cross-tolerance to free radicals released after hepatic and renal ischemia-reperfusion injury in experimental studies (Ajamieh et al., 2002; Chen et al., 2008). It was also demonstrated that ozone increased antioxidant enzyme activities, such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), preparing the host to face with physiopathological conditions mediated by reactive oxygen species (ROS) (Bocci, 1996, 2006a).

Our group recently reported that OT had an ameliorative effect in reducing oxidative stress in caustic esophageal burn, necrotizing enterocolitis, methotrexate induced intestinal injury and acute necrotizing pancreatitis in experimental rat models (Güven et al., 2008, 2009; Kesik et al., 2009; Uysal et al., 2010). In our previous study, it was also shown that OT had an ameliorative effect on APAP induced renal injury (Demirbag et al., 2010), although APAP hepatic overdose is known to cause especially acute liver injury rather than renal

injury. Therefore, we designed this experimental study to evaluate the efficacy of OT in the liver injury caused by APAP overdose in rats.

## 2. Methods

### 2.1. Animals

Adult male Sprague-Dawley rats (Health Sciences Institute, Gulhane Military Medical Academy, Ankara, Turkey), weighing 200–250 g each, were used in our study. They were randomly assigned into three groups containing nine rats each: sham, APAP and APAP+OT groups. Before the experiment, all animals were fed standard rat chow and water ad libitum and were kept in an air-conditioned room at 21 °C, with a 12 h:12 h light:dark cycle and were handled humanely, in accordance with the European Union Directive 609/86 for care and use of laboratory animals. Animals were fasted for 12 h before APAP treatment. This project was approved by the Experimental Ethics Committee of Gulhane Military Medical Academy, Ankara, Turkey.

### 2.2. Surgery and experimental protocol

The acetaminophen (APAP) and APAP+OT groups of animals were given as single dose 1 g/kg body weight of APAP (Ordu Ilac Fabrikasi, Ankara, Turkey) suspended in hot distilled water was administered by gastric tube. Rats in the sham group received distilled water by gastric tube. All animals were sacrificed, under light diethyl ether anesthesia, at time points of 24 h after APAP treatment. Whole blood was drawn from the heart. The abdomen was opened, and livers were removed and cleaned. Liver tissue samples were stored in 10% formalin solution for histological analysis. The remaining liver tissues were immediately frozen in liquid nitrogen and stored in a deep freezer at –80 °C until all assays.

### 2.3. Ozone treatment

OT was performed immediately after the induction of liver injury. After 1 h the induction of liver injury, the rats in the APAP+OT group were administered  $O_3/O_2$  mixture at a dose of 0.7 mg/kg via intraperitoneal route.  $O_3$  was generated by the ozone generator (OZONOSAN Photonik 1014, Hansler GmbH, Nordring 8, Iffezheim, Germany), allowing control of the gas flow rate and  $O_3$  concentration in real time by a built-in UV spectrometer. The  $O_3$  flow rate was kept constant at 3 L/min representing concentration of 60 mg/mL and gas mixture of 97%  $O_2$  + 3%  $O_3$ . Tygon polymer tubes and single-use silicon-treated polypropylene syringes (ozone resistant) were used throughout the reaction to ensure containment of  $O_3$  and consistency of concentrations.

### 2.4. Serum and tissue preparations

Whole blood samples were collected into tubes without anti-coagulant. The serum fraction was obtained by centrifugation ( $2000 \times g$ , 10 min, 4 °C) after storing the whole blood at room temperature for ~1 h. All sera were stored at –80 °C until

all assays. The frozen liver tissues were homogenized in phosphate buffer solution (pH 7.4) by means of a homogenizer (Heidolph Diax 900; Heidolph Elektro GmbH, Kelheim, Germany) on an ice cube. Homogenates were centrifuged at 14 000 rpm in 4 °C for 10 min. The supernatants were used for entire assays. The protein content of liver homogenates was measured by the method of Lowry et al. (Lowry et al., 1951) using bovine serum albumin as the standard.

## 2.5. Biochemical analysis

### 2.5.1. Biochemical parameters

Serum ALT and AST activities were measured with a spectrophotometric technique by the Olympus AU-2700 auto-analyzer (Olympus, Hamburg, Germany) using commercial kits and were expressed as U/L.

### 2.5.2. Measurement of serum neopterin

Serum neopterin levels were measured with a high performance liquid chromatography (HPLC) device (Agilent Technologies 1200 Series System, Santa Clara, CA, USA), using the method defined by Alrashed et al. (2002). In brief, to 0.4 mL serum, 0.1 mL 2 M trichloroacetic acid was added and the mixture was left in an ice bath for 10 min. The precipitated protein was removed by centrifugation at  $2000 \times g$  for 10 min. 20  $\mu$ L of the supernatant was filtered through a 0.2  $\mu$ m filter and then injected into the chromatographic system. Separation of neopterin was achieved with a 250  $\times$  4.6 mm I.D. Allsphere ODS-2, C18 RP column with a particle size of 5  $\mu$ m (Alltech, Deerfield, IL, USA) fitted with a 10  $\times$  4.6 mm I.D. Allsphere ODS-2 guard column (Alltech, Deerfield, IL, USA) using 0.015 M phosphate buffer (pH 6.4) as mobile phase (isocratic elution) at a flow rate of 0.8 mL/min. The areas of peaks detected by fluorescent detector (Ex: 353 nm; Em: 438 nm) were used for quantification. Serum neopterin levels were expressed as nmol/L.

### 2.5.3. Measurement of serum nitrite/nitrate (NO<sub>x</sub>)

Serum NO<sub>x</sub> levels were detected by means of an ion chromatograph (Dionex ICS-1000, Sunnyvale, CA, USA). Before NO<sub>x</sub> analysis, serum samples were passed through 0.45  $\mu$ m pore membrane nitrocellulose filters. Anion and guard columns (AS-9HC/AG-9HC, CS12A/CG12A, Sunnyvale, CA, USA) and automated suppression were used. NO<sub>x</sub> levels were quantified using separate standard solutions for each ion and expressed as mg/L (Koca et al., 2010).

### 2.5.4. Measurement of oxidant stress parameters

The level of tissue MDA, lipid peroxidation marker, was measured with the thiobarbituric acid (TBA) reaction method (Ohkawa et al., 1979). This method was used to obtain a spectrophotometric measurement of the color produced during the reaction to TBA with MDA at 535 nm. The calculated MDA levels were expressed as mmol/g protein.

Liver SOD activity was assayed using the nitroblue tetrazolium (NBT) method described by Durak et al. (1993). In this method, NBT was reduced to blue formazan by superoxide anion, which has a strong absorbance at 560 nm. One unit (U) of SOD is defined as the amount of protein that inhibits the

rate of NBT reduction by 50%. The estimated SOD activity was expressed as U/g protein.

Tissue GPx activity was measured using the method described by Paglia and Valentine (1967). Briefly GPx activity was coupled with the oxidation of NADPH by GSH reductase. The oxidation of NADPH was spectrophotometrically followed at 340 nm and 37 °C, the absorbance was recorded for 5 min. The activity was the slope of the lines as mmol of NADPH oxidized per minute. GPx activity was presented as U/g protein.

## 2.6. Histological evaluation of liver injury

This processing consisted of specimens' fixation in 4% buffered neutral formalin solution for 24 h, embedding in paraffin wax, slicing sections at 5  $\mu$ m of thickness and staining them with hematoxylin and eosin (H&E). Slides were examined under a light microscope. Each slide was independently evaluated and scored, while the examiner was unaware of the group to which the specimen belonged. In liver sections, injury was scored using the parameters such as sinusoidal dilatation (0–4), inflammatory cell infiltration (0–6), hemorrhage (0–6) and necrosis (0–8). The area size affected from edema, cell infiltration, hemorrhage and ballooning are taken into account to evaluate the liver injury. The scores for each histological parameter were summed up, with a maximum score of 24.

## 2.7. Statistical analysis

All the statistical analyses were performed by using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) statistical package. Differences among groups were analyzed by one-way ANOVA and post hoc Bonferroni test or Mann Whitney U test where appropriate. The results were expressed as mean  $\pm$  standard deviation (SD) and median (minimum–maximum). A probability level of <0.05 was considered statistically significant for overall comparisons.

## 3. Results

No deaths were observed in all groups of rats that were given APAP or distilled water during 24 h period. All laboratory parameters belong to all experimental groups are shown in Table 1. Serum ALT and AST activities in the APAP group were significantly higher than those of the sham and the APAP+OT groups (in both,  $p < 0.05$ ). A nearly 18-fold increase in serum ALT and nearly 3-fold increase in serum AST levels were observed in the APAP group rats (in both,  $p < 0.05$ ) compared to the sham group rats.

In the APAP group, serum neopterin concentrations ( $14.7 \pm 1.4$  nmol/L) were significantly increased compared to other groups (in both,  $p < 0.05$ ) (Table 1). OT administration reduced the neopterin concentrations significantly ( $7.5 \pm 2.4$  nmol/L) ( $p < 0.05$ ) but still higher than the sham group ( $4.8 \pm 1.2$  nmol/L) ( $p < 0.05$ ) (Table 1).

Serum NO<sub>x</sub> levels ( $7.08 \pm 1.39$  mg/L) in the APAP group were significantly increased compared to other groups (in both,  $p < 0.05$ ). NO<sub>x</sub> levels of the APAP+OT group ( $4.44 \pm 1.45$  mg/L) were significantly lower than the APAP group and were also higher than the sham group ( $3.40 \pm 1.33$  mg/L) ( $p < 0.05$ ).

**Table 1 – Laboratory parameters belong to sham, APAP and APAP+OT groups.**

Parameters	Groups		
	Sham (n=9)	APAP (n=9)	APAP+OT (n=9)
ALT (U/L)	67 ± 17	1183 ± 245 <sup>a</sup>	105 ± 49 <sup>a,b</sup>
AST (U/L)	140 ± 24	491 ± 147 <sup>a</sup>	208 ± 42 <sup>a,b</sup>
Neopterin (nmol/L)	4.8 ± 1.2	14.7 ± 1.4 <sup>a</sup>	7.5 ± 2.4 <sup>a,b</sup>
MDA (mmol/g protein)	0.66 ± 0.16	1.74 ± 0.44 <sup>a</sup>	1.05 ± 0.14 <sup>a,b</sup>
SOD (U/g protein)	647 ± 201	262 ± 89 <sup>a</sup>	388 ± 84 <sup>a,b</sup>
GPx (U/g protein)	59.4 ± 10.4	21.4 ± 4.7 <sup>a</sup>	34.7 ± 3.5 <sup>a,b</sup>
NOx (mg/L)	3.40 ± 1.33	7.08 ± 1.39 <sup>a</sup>	4.44 ± 1.45 <sup>a,b</sup>

APAP, acetaminophen; OT, ozone therapy; ALT, alanine aminotransferase; AST, aspartate aminotransferase; MDA, malondialdehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; NOx, nitrite/nitrate.

Data are expressed as mean ± standard deviation.

<sup>a</sup>  $p < 0.05$  Statistically significant from sham group.

<sup>b</sup>  $p < 0.05$  Statistically significant from APAP group.

Liver MDA levels ( $1.74 \pm 0.44$  mmol/g protein) in the APAP group were significantly higher than other groups indicating increased hepatocellular oxidative damage (in both,  $p < 0.05$ ). Liver MDA levels were significantly decreased in the APAP+OT group ( $1.05 \pm 0.14$  mmol/g protein) compared to the APAP group ( $p < 0.05$ ).

Tissue SOD activities in the APAP ( $262 \pm 89$  U/g protein) and the APAP+OT ( $388 \pm 84$  U/g protein) groups were significantly higher than the sham group (in both,  $p < 0.05$ ). Tissue SOD activities were significantly higher in the APAP+OT group than the APAP group ( $p < 0.05$ ).

Liver GPx activities in the APAP group ( $21.4 \pm 4.7$  U/g protein) were significantly lower than those in the sham group ( $59.4 \pm 10.4$  U/g protein) ( $p < 0.05$ ). GPx activities were significantly higher in the APAP+OT group ( $34.7 \pm 3.5$  U/g protein) than the APAP group and were also lower than the sham group ( $p < 0.05$ ).

The histological scores of liver injury in all rat groups are shown in Table 2. Sample views of the histological examination of sham, APAP and APAP+OT group livers are shown in Fig. 1. All animals in the APAP group showed severe liver injury with marked central necrosis, hemorrhage and leukocyte infiltration confirming the hepatotoxicity. In APAP+OT group, liver injury parameters such as necrosis and leukocyte infiltration were much lower than the APAP group ( $p < 0.05$ ).

#### 4. Discussion

According to our knowledge, this is the first report concerning the administration of OT in experimental APAP-induced hepatotoxicity. OT has an ameliorative effect on oxidative stress indices of liver tissue in correlation with histopathological findings. In addition, OT modulated serum neopterin concentrations and normalized serum ALT and AST activities which are indicative of liver injury. All these findings demonstrated an amelioration of liver injury by means of OT administration.

Decreased tissue MDA (as an oxidative stress marker) levels and increased GPx and SOD activities revealed that OT positively modulated oxidative stress and strengthened the antioxidant defense system. It was previously reported that OT stimulated antioxidant enzyme activities, such as GPx, SOD and CAT, preparing the organism to fight against the

detrimental effects of ROS (Bocci, 1996, 2006a). O<sub>3</sub> was previously reported to increase glutathione levels in erythrocytes and it may show the same action in hepatocytes, thus increasing the detoxification capacity of these cells (Bocci et al., 1993).

Superoxide anion can react with nitric oxide (NO) to produce the powerful oxidant peroxynitrite (Yaman et al., 2010); indeed, many of the biological effects attributed to NO are actually mediated by peroxynitrite, as it is a strong tissue oxidant and reacts with most biological molecules (Pacher et al., 2007). Vascular peroxynitrite formation occurs early in the pathophysiology of APAP induced liver injury and may be relevant for later hepatocellular events (Knight et al., 2001). In our study, we showed that serum NOx levels were increased with APAP administration. However, serum NOx levels in APAP+OT group were lower than those of the APAP group. This may imply that OT shows a beneficial effect decreasing excessive NOx production in the injured liver tissue.

Neopterin is a marker associated with cell-mediated immunity and is produced in monocytes/macrophages via stimulation of interferon- $\gamma$ . It has been shown that neopterin levels not only provide knowledge about the state of cell-mediated immunity but also allow monitoring of disease progression (Kaufmann et al., 1998). In the present study, a decreased neopterin concentration in the APAP+OT group implies that the administration of O<sub>3</sub> decreased monocyte/macrophages recruitment and/or activation in liver tissue and ultimately, prevented liver tissue injury. These actions of OT are supported with findings of decreased leukocyte infiltration in the histological slides of the APAP+OT group when compared with the APAP group.

OT significantly improved liver functions, as evidenced by reduction in ALT and AST activities in the animals with liver necrosis. It seems likely that OT achieve by reducing leakage of AST and ALT from hepatocellular plasma membrane into the bloodstream via preventing liver necrosis. OT decreased liver injury and inflammation. OT probably prevented liver necrosis by modulating the antioxidant defense system, improving O<sub>2</sub> delivery and increasing release of vascular nitric oxide (Bocci, 2006b). It was previously reported that exposure to ozone altered levels of inflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Paulesu et al., 1991), transforming growth factor- $\beta$  (TGF- $\beta$ ) (Bocci, 1994), interferon- $\gamma$  (IFN- $\gamma$ ) (Bocci and Paulesu, 1990), and interleukin (IL)-8 (Bocci et al., 1998).

**Table 2 – The degree of liver necrosis belongs to sham, APAP and APAP+OT groups.**

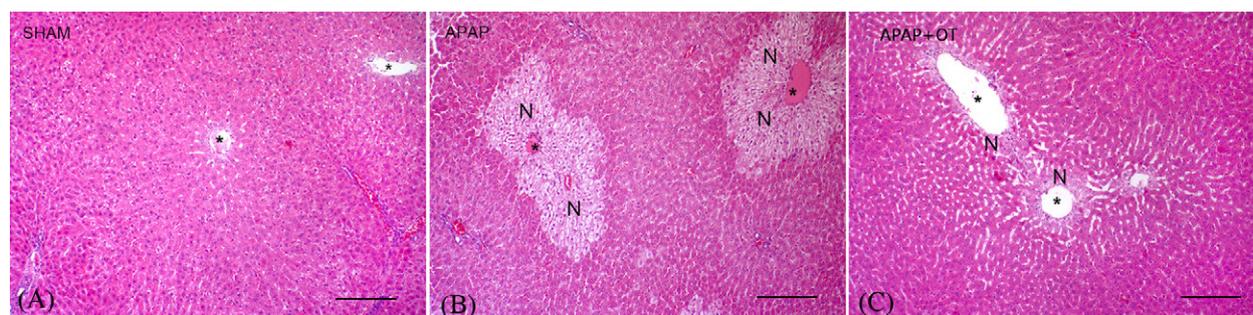
Groups	Sinusoidal dilatation	Hemorrhage	Leukocyte infiltration	Necrosis	Total score
Sham	0 (0–1)	0 (0–1)	0 (0–1)	0 (0–0)	0 (0–3)
APAP	3 (2–4) <sup>a</sup>	4 (3–7) <sup>a</sup>	4 (2–7) <sup>a</sup>	6 (3–7) <sup>a</sup>	17 (10–25) <sup>a</sup>
APAP+OT	2 (1–3) <sup>a</sup>	2 (1–3) <sup>a,b</sup>	1 (1–3) <sup>a,b</sup>	2 (1–4) <sup>a,b</sup>	7 (4–13) <sup>a,b</sup>

APAP, acetaminophen; OT, ozone therapy.

Data are expressed as median (minimum–maximum).

<sup>a</sup>  $p < 0.05$  Statistically significant from sham group.

<sup>b</sup>  $p < 0.05$  Statistically significant from APAP group.



**Fig. 1 – Representative photographs are showing the histological changes in liver tissues. (A) Sham group: Normal central vein and hepatic parenchyma. (B) APAP group: Extensive necrosis, mild sinusoidal dilatation and ballooning in hepatic parenchyma. (C) APAP+OT group: Mild necrosis and moderate sinusoidal dilatation (H&E, scale bar for all images represent 200  $\mu\text{m}$  length). N: necrosis, \*: hepatic central vein.**

Gene and protein expression studies will better illuminate the action of OT in combating NAPQI, degradation product of APAP, toxicity. The effect of OT on hepatocyte mitochondrial integrity may be evaluated to understand the role of OT against NAPQI toxicity with the molecular studies. OT presumably may show some of its beneficial effects in the injured liver tissue by modulating inflammatory pathways via altering the expression pattern of genes encoding cytokines and antioxidant enzymes.

In conclusion, APAP-induced hepatotoxicity in rats was ameliorated by OT, possibly through decreasing oxidative/nitrosative stress and improving the antioxidant defense system. These findings are promising for future inclusion of OT in the management schemes of APAP induced liver injury. However, further studies are required to better understand the effects of OT in APAP induced liver injury.

### Conflict of interest

The authors declare that there are no conflicts of interest.

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