Original Research

Ozone therapy ameliorates paraquat-induced lung injury in rats

Umit Kaldirim[1], Bulent Uysal[2], Ramazan Yuksel[2], Enis Macit[3], Yusuf E Eyi[1], Mehmet Toygar[4], Salim K Tuncer[1], Sukru Ardic[1], Ibrahim Arziman[1], Ibrahim Aydin[5], Yesim Oztas[6], Yildirim Karslioglu[7] and Turgut Topal[2]

[1]Department of Emergency Medicine Gulhane Military Medical Academy, 06010 Etlik, Ankara, Turkey; [2]Department of Physiology, Gulhane Military Medical Academy, 06010 Etlik, Ankara, Turkey; [3]Department of Toxicolgy, Gulhane Military Medical Academy, 06010 Etlik, Ankara, Turkey; [4]Department of Forensic Medicine, Gulhane Military Medical Academy, 06010 Etlik, Ankara, Turkey; [5]Department of Biochemistry, Sarikamis Military Hospital, Sarikamis, 36500 Kars, Turkey; [6]Department of Medical Biochemistry, Faculty of Medicine, Hacettepe University, Sihhiye 06100, Ankara, Turkey; [7]Department of Pathology, Gulhane Military Medical Academy, 06010 Etlik, Ankara, Turkey

Corresponding author: Bulent Uysal. Email: buysal@gata.edu.tr

Abstract

Paraquat (PQ) overdose can cause acute lung injury and death. Ozone therapy (OT) was previously demonstrated to alleviate inflammation and necrosis in various pathologies. We therefore hypothesized that OT has ameliorative and preventive effects on PQ-induced lung damage due to anti-inflammatory and antioxidants properties. Sprague-Dawley rats ($n = 24$) were separated into three groups: sham, PQ, and PQ+OT groups. 15 mg/kg PQ was administered intraperitoneally in PQ and PQ+OT groups to induce experimental lung injury. One hour after PQ treatment, PQ+OT group was administered a single dose of ozone–oxygen mixture (1 mg/kg/day) by intraperitoneal route for four consecutive days. The animals were sacrificed on fifth day after PQ administration. Blood samples and lung tissues were collected to evaluate the inflammatory processes, antioxidant defense and pulmonary damage. Serum lactate dehydrogenase (LDH) and neopterin levels, tissue oxidative stress parameters, total TGF-β1 levels, and histological injury scores in PQ+OT group were significantly lower than PQ group ($P<0.05$, PQ vs. PQ+OT). Total antioxidant capacity in PQ+OT group was significantly higher than PQ group ($P < 0.05$, PQ+OT vs. PQ). These findings suggest that outcome in PQ-induced lung injury may be improved by using OT as an adjuvant therapy.

Keywords: Paraquat, lung injury, ozone therapy, neopterin, TGF-β1, oxidative stress

***Experimental Biology and Medicine* 2014; 239: 1699–1704. DOI: 10.1177/1535370214543060**

Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridylium dichloride; PQ) is one of the most frequently used bipyridylium quaternary ammonium herbicides.[1] When ingested accidentally, PQ produces extensive damage in kidneys, liver, and especially in lungs.[2] PQ lung toxicity occurs due to damage to type I and II pneumocytes in alveolar epithelium and bronchiolar cells. Eventually, clinical signs such as edema, inflammation, hemorrhage, hypoxemia, fibrosis, and disseminated intravascular coagulation may be seen.[3]

The first report on PQ poisoning in a human was presented in 1960s.[4] Later, a number of deaths due to PQ poisoning have been reported. These deaths were due to accidental ingestion of PQ or suicide attempts. Although hepatotoxicity, cardiotoxicity, and neurotoxicity are prominent in cases of PQ poisoning, respiratory arrest due to irreversible lung damage is the main cause of mortality.[5,6]

The specific feature of lung damage in PQ poisoning is fibrosis of the lungs that becomes symptomatic nearly 5 days after PQ exposure.[6] The pathology in PQ toxicity results from excessive formation of reactive oxygen species such as superoxide anion.[7] To date, a large amount of therapeutic agents have been used in order to treat or alleviate PQ toxicity.[8,9] Since one of the main mechanisms of PQ toxicity is excessive production of reactive oxygen species, novel therapeutic approaches aim to halt the process of oxygen radical formation. Our group had previously reported the beneficial effects of medical ozone therapy (OT) in caustic esophageal burn, necrotizing enterocolitis, paracetamol-induced renal injury, acute necrotizing pancreatitis, and methotrexate-induced intestinal injury in experimental rat model.[10–14] Also, beneficial effects of OT in various diseases such as chronic skin ulcers, initial gangrene, burns, infected wounds, peritonitis, and advanced ischemic diseases were previously reported.[15]

Copyright © 2014 by the Society for Experimental Biology and Medicine

Ozone–oxygen gas mixture has also been shown to modulate the phagocytic activity of peritoneal and alveolar macrophages.[16–19] Additionally, OT demonstrated beneficial effects against hepatic and renal ischemia-reperfusion injury in experimental studies.[20,21] It was also shown that OT raised the antioxidant defense supplied by the activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT). OT helps to eliminate deleterious effects of reactive oxygen species produced in various pathologies by increasing the antioxidant defense.[19,22] However, OT has not been previously evaluated for its effects in PQ toxicity. Under the light of previous reports, we considered that OT may have potentially beneficial effects against the lung injury due to PQ toxicity. We therefore hypothesized that OT has ameliorative and preventive effects on PQ-induced lung damage due to anti-inflammatory and antioxidant properties. So, we aimed to observe the outcome of OT in a rat model of PQ toxicity.

Materials and methods

Experimental design

All procedures were approved by the Institutional Committee on the Care and Use of Animals (Issue; 13/48, 8 March 2013). Twenty-four female Sprague-Dawley rats (200–250 g in weight) were randomly split into three groups (sham, PQ, and PQ+OT) involving eight animals per group. Animals were fed standard rat chow and water *ad libitum* and housed in cages with controlled temperature and 12-h light/dark cycle for at least one week before the experiment.

Induction of lung injury

PQ (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was administered intraperitoneally to rats at a single dose of 15 mg/kg in 1 mL saline. The animals were kept in their cages during the recovery period. Water and food were available *ad libitum*.

Ozone treatment

The rats in the PQ+OT group were administered ozone–oxygen mixture at a dose of 1 mg/kg daily *via* intraperitoneal route by means of a syringe resistant to ozone; the first dose beginning 1 h after the administration of PQ. Ozone (O_3) was produced by an O_3 generator (OZONOSAN Photonik 1014, Hansler GmbH, Nordring 8, Iffezheim, Germany). The flow-rate of the oxygen entering to O_3 generator was kept constant at 3 L/min to generate a concentration of 60 μg/mL of ozone. The concentration of ozone in gas mixture produced by generator was monitored in real time by a built-in UV spectrometer. The ozone gas mixture was composed of 97% O_2 + 3% O_3.

Sample collection

All animals were anesthetized with ketamine (85 mg/kg) and xylazine (12.5 mg/kg) on fifth day after induction and their thoracic cavities were opened. Blood samples and lung tissues were collected for laboratory analysis. Blood samples were centrifuged, and serum was separated and stored at −80°C until laboratory analysis. Harvested lung tissue samples were portioned into two; one was immediately stored at −80°C for biochemical analysis of antioxidant enzyme activity and tissue lipid peroxidation levels. The rest was immersed in formalin solution for histological evaluation. All animals were killed *via* decapitation at the end of the sample collection.

Biochemical analysis of serum samples

Lactate dehydrogenase (LDH) was measured by Olympus AU-2700 autoanalyzer using commercial kits (Olympus, Hamburg, Germany) and was expressed as units per liter (U/L).

Total antioxidant capacity (TAC) assays were performed by the method defined by Celik et al.[23] Serum TAC levels were given as mmol Trolox Equivalent/L.

Serum neopterin concentrations were measured by a high-pressure liquid chromatography (Agilent Technologies 1200 Series System, Santa Clara, CA, USA) system with a fluorescence detector as described previously.[24]

Biochemical analysis of lung tissues

The tissues were homogenized in phosphate buffer (pH 7.4) on ice by a homogenizator (Heidolph Diax 900; Heidolph Elektro GmbH, Kelhaim, Germany). The supernatant was separated for biochemical assays. The protein amount in the tissue homogenates was determined by the Lowry method.[25] The below mentioned biochemical assays were analyzed by the ELISA plate reader, (BioTek, FLx800) spectrophotometrically.

Malondialdehyde (MDA) as an indicator of lipid peroxidation was measured by the thiobarbituric acid (TBA) reaction method.[26] The color produced after the reaction to TBA with malondialdehyde (MDA) was measured at 535 nm and MDA levels were expressed as nanomole per milligram of protein (nmol/mg protein).

SOD activity was determined by the nitroblue tetrazolium (NBT) method.[27] In this method, NBT was reduced to blue formazan by O_2^-, and the color was measured 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 SOD activity was expressed as units per gram of protein (U/g protein).

GSH-Px activity was measured by coupling GSH-Px activity to the oxidation of NADPH by glutathione reductase.[28] The oxidation of NADPH was followed at 340 nm at 37°C by a spectrophotometer for 5 min. The activity was the slope of the curve formed by mmol of NADPH oxidized per minute. GSH-Px activity was presented as U/g protein.

Total TGF-β levels in lung tissue were measured by enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (Bender MedSystems GmbH, Vienna, Austria). Lung total TGF-β1 levels were expressed as picogram per gram of protein (pg/g protein).

TGF-β1 immunoassay kit has the minimum detectable dose of <15.6 pg/mL.

Histological analysis of lung tissues

Tissue specimens were fixed in formalin for 24 h, then embedded in paraffin and cut into 5 μm sections. Slides were stained with hematoxylin and eosin (H&E) and examined under a light microscope. Each slide was evaluated by an expert investigator blinded to the experiment. Injury in lung tissue sections was scored according to the following histological parameters: alveolar epithelial injury, edema, hemorrhage, and inflammation as previously described.[29] Histological parameters were graded according to 4-grade scale quantitatively as follows. Grade 0: no changes; Grade 1: minimal to mild change; Grade 2: moderate change; Grade 3: severe change. Mean injury scores were calculated for each parameter. The extension of histological changes was assessed as focal or diffuse (Figure 1).

Statistical analysis

Results were expressed as median ± standard deviation (SD). Differences among the groups were evaluated by Kruskal–Wallis test. Dual comparisons among groups were done by Mann-Whitney U test. $P < 0.05$ was considered statistically significant. All analyses were performed by the Statistical Package for the Social Sciences (SPSS) (Software version 11.0, SPSS Inc. Chicago, IL, USA).

Results

Animals

In the study, all animals in the groups survived until the end of the experiment.

Biochemical analysis of serum samples

Serum LDH levels. These LDH levels in the PQ group were significantly higher than those of the sham and the PQ+OT groups ($P < 0.05$ PQ vs. the other groups) indicating lung damage. In PQ+OT group, LDH values were decreased when compared with the PQ group. But there was statistically significant difference when compared with sham group (Table 1).

Total antioxidant capacity. Serum TAC values in the PQ and PQ+OT groups were significantly lower than the sham group ($P < 0.05$). TAC values in PQ+OT group were significantly higher than PQ group, but lower than the sham group ($P < 0.05$) (Table 2).

Table 1 Biochemical parameters and TGF-β levels (median ± SD)

Groups	Neopterin (nmol/L)	LDH (U/L)	TGF-β (pg/g protein)
Sham	5.48 ± 2.11	167.00 ± 107.93	35.81 ± 6.62
PQ	14.99 ± 5.53*	579.00 ± 274.24*	268.71 ± 77.08*
PQ+OT	4.38 ± 2.44†	332.00 ± 164.41*†	178.88 ± 61.47*,†

Data are expressed as median ± SD. PQ: paraquat; OT: ozone therapy.
*$P < 0.05$ Statistically significant from sham group.
†$P < 0.05$ Statistically significant from PQ group.

TGF-β levels. Lung tissue total TGF-β levels in the PQ group were significantly higher when compared with the other groups ($P < 0.05$). The TGF-β levels were significantly decreased in the PQ+OT group compared with PQ group ($P < 0.05$). But, there was statistically significant difference compared to sham group (Table 1).

Serum neopterin levels. These levels were significantly increased in the PQ group ($P < 0.05$). PQ+OT group had significantly decreased levels when compared with the PQ group ($P < 0.01$) (Table 1).

Tissue lipid peroxidation levels. PQ group had significantly higher mean MDA levels when compared with that of other groups ($P < 0.05$). The MDA levels were significantly lower in PQ+OT group compared with PQ group ($P < 0.05$) (Table 2).

Tissue antioxidant enzyme activities. Tissue SOD activity in the PQ and PQ+OT groups were significantly lower than the sham group ($P < 0.05$, PQ). The tissue SOD activities in the PQ+OT group were significantly higher than the PQ group ($P < 0.05$) (Table 2).

Mean tissue GSH-Px activities were significantly lower in the PQ and PQ+OT groups compared to the sham group ($P < 0.05$). The GSH-Px activity was significantly higher in the PT+OT group than the PQ group, but still lower than the sham group ($P < 0.05$) (Table 2).

Histological Analysis of Lung Tissues. In histological analysis, there were no changes indicating lung injury in the sham-operated group, while all animals in the PQ group showed severe degree of lung injury with marked mesothelial proliferation, hemorrhage, and leukocyte infiltration. Lung injury parameters such as hemorrhage, edema, leukocyte infiltration, and especially mesothelial proliferation in PQ+OT group were less than the PQ group ($P < 0.05$) (Table 3).

Discussion

This study is the first report on administration of ozone in PQ-induced experimental lung injury. In this study, OT had an ameliorative effect on oxidative stress parameters in the lung tissue and normalized the serum LDH levels in correlation with histological tissue injury findings. In addition, OT modulated the serum neopterin levels. All these findings demonstrated that OT had a beneficial effect against PQ-induced lung injury.

The decrease in tissue MDA and GSH-Px levels, and increase in SOD levels revealed that OT significantly ameliorated oxidative stress as strengthening the antioxidant defense. Previous studies have shown that ozone increased activities of antioxidant enzymes such as GSH-Px, SOD, and CAT which help the organism fight against destructive effects of reactive oxygen species.[19,22] GSH-Px values were expected to be increased in terms of a strong antioxidant defense, but our values were decreased. Normally GSH-Px is activated by the presence of H_2O_2, which is a product of SOD enzyme and the substrate of GSH-Px.[30] We think that

Table 2 Tissue lipid peroxidation and antioxidant enzyme levels (median ± SD)

Groups	MDA (mmol/mg protein)	SOD (U/g protein)	GSH-Px (U/g protein)	TAC (mmol Trolox Equivalent/L)
Sham	0.21 ± 0.04	610.22 ± 185.55	62.46 ± 12.26	2,07 ± 0.28
PQ	0.32 ± 0.03*	272.48 ± 79.30*	20.77 ± 4.86*	0.89 ± 0.31*
PQ+OT	0.26 ± 0.04*†	367.45 ± 80.36*†	32.36 ± 4.12*†	1.62 ± 0.16†

Data are expressed as median ± SD. PQ: paraquat; OT: ozone therapy.
*$P < 0.05$ Statistically significant from sham group.
†$P < 0.05$ Statistically significant from PQ group.

Table 3 Histological injury scores for the lung tissue of the groups (median [min−max])

Groups	Edema	Focal hemorrhage	Extensive hemorrhage	Mezothelial reaction
Sham	0 (0−1)	0 (0−0)	0 (0−0)	0 (0−0)
PQ	3 (2−3)*	3 (2−3)*	3 (2−3)*	3 (2−3)*
PQ + OT	1 (0−2)*†	1 (1−2)*†	1 (0−1)†	1 (0−1)†

*$P < 0.05$ Statistically significant from sham group.
†P < 0.05 Statistically significant from PQ group.

the levels of H_2O_2 may have been insufficient to activate GSH-Px, which may be the main reason of the decrease in GSH-Px levels.

Bocci et al.[31] previously showed the increase of glutathione levels in erythrocytes due to ozone and declared the possibility of similar action in hepatocytes to increase the detoxification capacity of these cells. Also, it has been shown that OT demonstrates beneficial effects against free radicals released after experimental hepatic ischemia-reperfusion injury.[20,21]

Neopterin is a catabolic product of monocytes/macrophages upon stimulation with interferon-γ and serves as a cellular immunity marker.[32] It has been shown that neopterin levels provide information about the state of cell-mediated immunity as well as monitoring the disease progression.[32] In our study, neopterin values of PQ+OT group were decreased, when compared with PQ group. This result implies that OT decreased monocyte/macrophage recruitment and/or activation in lung and/or other target tissues by preventing target tissue injury. That is to say, the decrease of the neopterin levels is not a cause, but likely a result of the reduction in monocyte/macrophage recruitment to necrotic tissue. Also, these actions of OT are supported by the results of pathological scoring, indicating decreased leukocyte infiltration into lung tissue.

Intracellular enzymes such as LDH may leak into the bloodstream and then be detected in serum in pathological conditions involving cellular injury and necrosis. Serum levels of these enzymes may also be used to predict the extent of tissue injury. OT significantly prevented lung injury, as evidenced by reduction in LDH levels in animals in PQ+OT group. It seems likely that OT achieves this by preventing pneumocyte necrosis, thus reducing plasma membrane leakage of LDH into the bloodstream. When compared to lung tissue in PQ group, OT demonstrated amelioration of injury and inflammation processes in PQ+OT group. OT probably prevented lung injury by modulating the antioxidant defense system, improving O_2 delivery and increasing release of vascular nitric oxide.[33]

It was previously reported that ozone exposure altered the levels of cytokines like TNF-α, TGF-β, IFN-c, and IL-8 involving the inflammatory processes.[34–37] In our study, we found decreased TGF-β cytokine levels similar to the results of these studies mentioned above. We think that OT may be altering the expression pattern of TGF-β. Also, decrease of neopterin values may be related to the altered levels of these same cytokines, especially interferon. Previously, it has been shown that there is a correlation between neopterin and TGF-β levels in various pathological conditions.[38] Also, it has been shown that TGF- β is a potent neutrophil chemoattractant that is released in several lung diseases, along with interleukin-8 (IL-8) and leukotrienes.[39,40]

Further studies are needed to explain the possible mechanisms of action of OT in PQ toxicity. OT may presumably show some of its beneficial effects in injured lung tissue by modulating inflammatory pathways via altering the expression pattern of genes encoding cytokines, antioxidant enzymes, etc.

In conclusion, OT may have possible ameliorative effects by decreasing oxidative stress and improving antioxidant mechanisms. These findings encourage for the future inclusion of OT in the management schemes of PQ-induced lung injury.

Limitations of the study

One of the important limitations of our study is lack of molecular analyses such as immunohistochemical staining and western blotting analyses, and another limitation was that we could not study the enzymes or isoenzymes specific to lung tissue.

Author contributions: UK, RY, SKT, and SA conducted the experiments. BU performed ozone treatment, the production of ozone, and ELISA analysis. EM supplied critical reagents. YEE and IA performed the English redaction of the article. MT homogenized the tissue samples and conducted daily animal care. IA and YO analyzed the biochemical parameters. YK analyzed the histopathological changes and provided photos. TT performed statistical analyses and photoshop study for the histopathological figure.

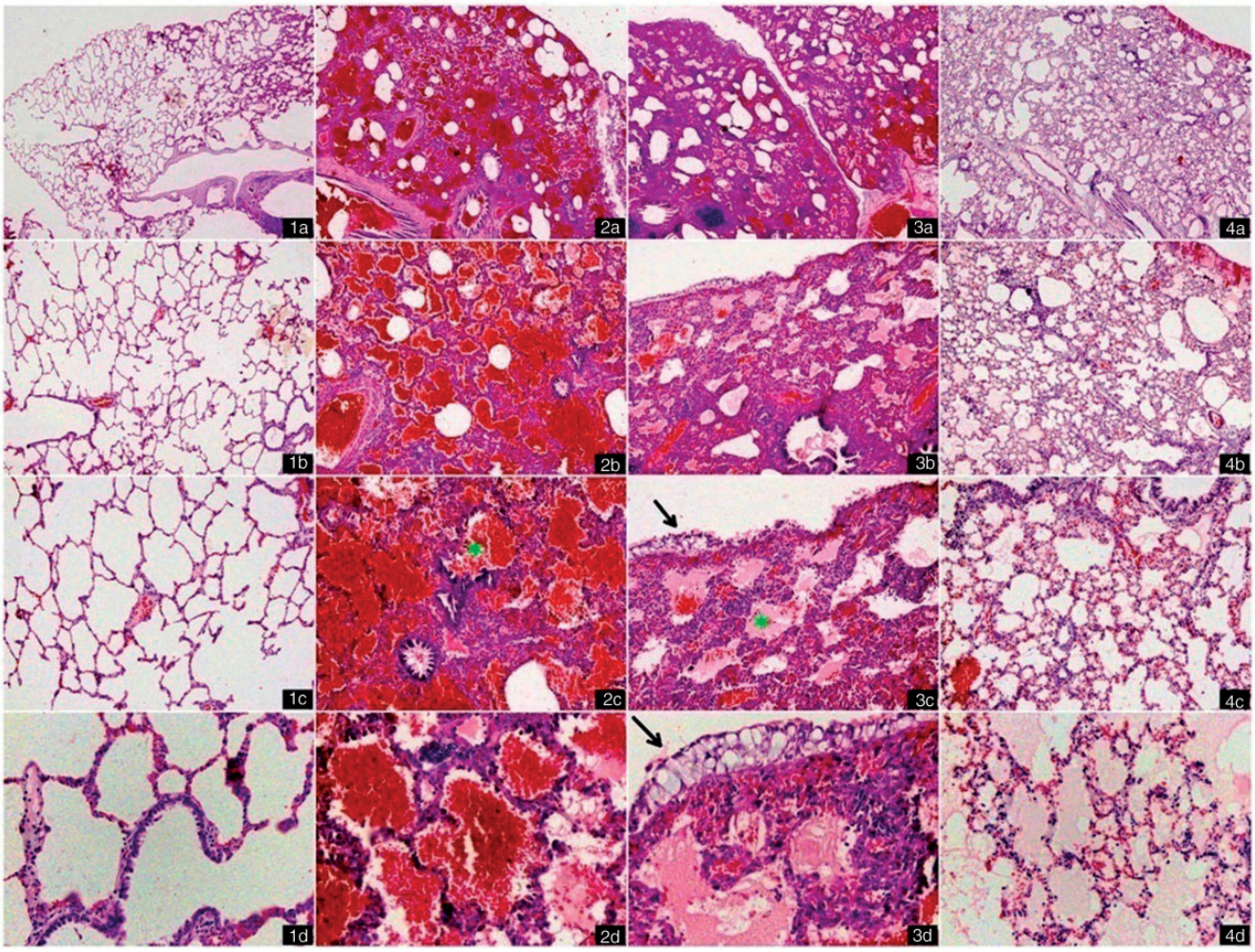

Figure 1 Comparative histological views from sham (leftmost column), paraquat (PQ) (next two columns), and PQ + OT (rightmost column) groups. Rows of the image mosaic from the top to the bottom represent the increasing magnification powers of ×40, ×100, ×200, and ×400, respectively. In the sham group, all the structures of pulmonary parenchyma and bronchial tree are of normal appearance. On the contrary, massive alveolar hemorrhage throughout the parenchyma and/or extensive alveolar edema (asterisks), as well as prominent mesothelial proliferation with reactive atypia (arrows) are observed in PQ group (2 and 3, a–b–c–d). In PQ+OT group, on the other hand, we have noticed that most of the serious effects of PQin lung have been ameliorated, except some degree of pulmonary edema (4, a–b–c–d). (H&E stain). (A color version of this figure is available in the online journal.)

FUNDING

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

REFERENCES

1. Dinis-Oliveira RJ, Duarte JA, Sanchez-Navarro A, Remiao F, Bastos ML, Carvalho F. Paraquat poisonings: mechanisms of lung toxicity, clinical features, and treatment. *Crit Rev Toxicol* 2008;**38**:13–71
2. Onyeama HP, Oehme FW. A literature review of paraquat toxicity. *Vet Hum Toxicol* 1984;**26**:494–502
3. Minakata K, Suzuki O, Oh-ishi S, Hayashi I, Saito S, Harada N. Acute-phase reactant proteins and antioxidants in rats intoxicated chronically with paraquat. *J Toxicol Environ Health* 1995;**44**:29–41
4. Bullivant CM. Accidental poisoning by paraquat: report of two cases in man. *Br Med J* 1966;**1**:1272–3
5. Fairshter RD, Wilson AF. Paraquat poisoning: manifestations and therapy. *Am J Med* 1975;**59**:751–3
6. Smith P, Heath D. The pathology of the lung in paraquat poisoning. *J Clin Pathol Suppl* 1975;**9**:81–93
7. Suntres ZE. Role of antioxidants in paraquat toxicity. *Toxicology* 2002;**180**:65–77
8. Gocgeldi E, Uysal B, Korkmaz A, Ogur R, Reiter RJ, Kurt B, Oter S, Topal T, Hasde M. Establishing the use of melatonin as an adjuvant therapeutic against paraquat-induced lung toxicity in rats. *Exp Biol Med* 2008;**233**:1133–41
9. Park HK, Kim SJ, Kwon do Y, Park JH, Kim YC. Protective effect of quercetin against paraquat-induced lung injury in rats. *Life Sci* 2010;**87**:181–6
10. Demirbag S, Uysal B, Guven A, Cayci T, Ozler M, Ozcan A, Kaldirim U, Surer I, Korkmaz A. Effects of medical ozone therapy on acetaminophen-induced nephrotoxicity in rats. *Ren Fail* 2010;**32**:493–7
11. Guven A, Gundogdu G, Sadir S, Topal T, Erdogan E, Korkmaz A, Surer I, Ozturk H. The efficacy of ozone therapy in experimental caustic esophageal burn. *J Pediatr Surg* 2008;**43**:1679–84
12. Guven A, Gundogdu G, Vurucu S, Uysal B, Oztas E, Ozturk H, Korkmaz A. Medical ozone therapy reduces oxidative stress and intestinal damage in an experimental model of necrotizing enterocolitis in neonatal rats. *J Pediatr Surg* 2009;**44**:1730–5
13. Uysal B, Yasar M, Ersoz N, Coskun O, Kilic A, Cayc T, Kurt B, Oter S, Korkmaz A, Guven A. Efficacy of hyperbaric oxygen therapy and medical ozone therapy in experimental acute necrotizing pancreatitis. *Pancreas* 2010;**39**:9–15
14. Kesik V, Uysal B, Kurt B, Kismet E, Koseoglu V. Ozone ameliorates methotrexate-induced intestinal injury in rats. *Cancer Biol Ther* 2009;**8**:1623–8

15. Re L, Mawsouf MN, Menendez S, Leon OS, Sanchez GM, Hernandez F. Ozone therapy: clinical and basic evidence of its therapeutic potential. *Arch Med Res* 2008;**39**:17–26
16. Bocci V, Zanardi I, Travagli V. Has oxygen-ozonetherapy a future in medicine? *J Exp Integr Med* 2011;**1**:5–11
17. Oter S, Korkmaz A. Relevance of hyperbaric oxygen to ozone therapy. *Arch Med Res* 2006;**37**:917–8; author reply 19
18. Bocci V. Ozone as Janus: this controversial gas can be either toxic or medically useful. *Mediators Inflamm* 2004;**13**:3–11
19. Bocci VA. Scientific and medical aspects of ozone therapy. State of the art. *Arch Med Res* 2006;**37**:425–35
20. Ajamieh H, Merino N, Candelario-Jalil E, Menendez S, Martinez-Sanchez G, Re L, Giuliani A, Leon OS. Similar protective effect of ischaemic and ozone oxidative preconditionings in liver ischaemia/reperfusion injury. *Pharmacol Res* 2002;**45**:333–9
21. Chen H, Xing B, Liu X, Zhan B, Zhou J, Zhu H, Chen Z. Similarities between ozone oxidative preconditioning and ischemic preconditioning in renal ischemia/reperfusion injury. *Arch Med Res* 2008;**39**:169–78
22. Bocci V. Does ozone therapy normalize the cellular redox balance? Implications for therapy of human immunodeficiency virus infection and several other diseases. *Med Hypotheses* 1996;**46**:150–4
23. Celik SE, Ozyurek M, Guclu K, Apak R. Differences in responsivity of original cupric reducing antioxidant capacity and cupric-bathocuproine sulfonate assays to antioxidant compounds. *Anal Biochem* 2012;**423**:36–8
24. Alrashed M, Abougoush M, Akgul EO, Erbil MK. Detection method of serum and urine neopterin levels by high performance liquidchromatography and clinical applications. *Gulhane MJ* 2002;**44**:273–77
25. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265–75
26. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979;**95**:351–8
27. Durak I, Yurtarslanl Z, Canbolat O, Akyol O. A methodological approach to superoxide dismutase (SOD) activity assay based on inhibition of nitroblue tetrazolium (NBT) reduction. *Clin Chim Acta* 1993;**214**:103–4
28. Paglia DE, Valentine WN. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J Lab Clin Med* 1967;**70**:158–69
29. Yamanel L, Kaldirim U, Oztas Y, Coskun O, Poyrazoglu Y, Durusu M, Cayci T, Ozturk A, Demirbas S, Yasar M, Cinar O, Tuncer SK, Eyi YE, Uysal B, Topal T, Oter S, Korkmaz A. Ozone therapy and hyperbaric oxygen treatment in lung injury in septic rats. *Int J Med Sci* 2011;**8**:48–55
30. Comhair SA, Erzurum SC. The regulation and role of extracellular glutathione peroxidase. *Antioxid Redox Signal* 2005;**7**(1–2): 72–9
31. Bocci V, Luzzi E, Corradeschi F, Paulesu L, Rossi R, Cardaioli E, Di Simplicio P. Studies on the biological effects of ozone: 4. Cytokine production and glutathione levels in human erythrocytes. *J Biol Regul Homeost Agents* 1993;**7**:133–8
32. Kaufmann P, Tilz GP, Demel U, Wachter H, Kreijs GJ, Fuchs D. Neopterin plasma concentrations predict the course of severe acute pancreatitis. *Clin Chem Lab Med* 1998;**36**:29–34
33. Bocci V. Is it true that ozone is always toxic? The end of a dogma. *Toxicol Appl Pharmacol* 2006;**216**:493–504
34. Bocci V, Paulesu L. Studies on the biological effects of ozone 1. Induction of interferon gamma on human leucocytes. *Haematologica* 1990;**75**:510–5
35. Bocci V, Valacchi G, Corradeschi F, Fanetti G. Studies on the biological effects of ozone: 8. Effects on the total antioxidant status and on interleukin-8 production. *Mediators Inflamm* 1998;**7**:313–7
36. Bocci V, Luzzi E, Corradeschi F, Silvestri S. Studies on the biological effects of ozone: 6. Production of transforming growth factor 1 by human blood after ozone treatment. *J Biol Regul Homeost Agents* 1994;**8**:108–12
37. Paulesu L, Luzzi E, Bocci V. Studies on the biological effects of ozone: 2. Induction of tumor necrosis factor (TNF-alpha) on human leucocytes. *Lymphokine Cytokine Res* 1991;**10**:409–12
38. Grüngreiff K, Reinhold D, Ansorge S. Serum concentrations of sIL-2R, IL-6, TGF-beta1, neopterin, and zinc in chronic hepatitis C patients treated with interferon-alpha. *Cytokine* 1999;**11**(12): 1076–80
39. Tylman M, Bengtson JP, Hyllner M, Bengtsson A. Release of PMN elastase, TGF-beta1 and neopterin during blood storage; unfiltered versus filtered blood. *Transfus Apher Sci* 2006;**35**(2): 97–102
40. Bühling F, Thölert G, Kaiser D, Hoffmann B, Reinhold D, Ansorge S, Welte T. Increased release of transforming growth factor (TGF)-beta1, TGF-beta2, and chemoattractant mediators in pneumonia. *J Interferon Cytokine Res* 1999;**19**(3): 271–8

(Received March 5, 2014, Accepted June 6, 2014)