Original Article
Hyperbaric oxygen treatment ameliorates lung injury in paraquat intoxicated rats

Raziye Akcılar[1], Aydın Akcılar[2], Hasan Şimşek[1], Fatma Emel Koçak[3], Cengiz Koçak[4], Gündüz Yümün[5], Zeynep Bayat[6]

[1]Department of Physiology, Faculty of Medicine, University of Dumlupınar, Kütahya, Turkey; [2]Faculty of Medicine, University of Dumlupınar, Experimental Animal Research Center, Kütahya, Turkey; [3]Department of Biochemistry, Faculty of Medicine, University of Dumlupınar, Kütahya, Turkey; [4]Department of Pathology, Faculty of Medicine, University of Dumlupınar, Kütahya, Turkey; [5]Department of Cardiovascular Surgery, Faculty of Medicine, University of Namık Kemal, Tekirdağ, Turkey; [6]Department of Biochemistry, Faculty of Arts and Sciences, University of Dumlupınar, Kütahya, Turkey

Abstract: Paraquat (PQ) is an agrochemical agent commonly used worldwide, which can cause acute lung injury (ALI) and death. Hyperbaric oxygen treatment (HBOT) is a therapeutic method, but the mechanisms of the protective effect of HBOT on ALI remain elusive. The purpose of this study was to evaluate the effect of HBOT on acute lung injury induced by PQ in rats. Wistar Albino rats (n=21) were separated into three groups of seven animals each: control (C), PQ, and PQ + HBOT groups. 20 mg/kg PQ was administered intraperitoneally in PQ and PQ + HBOT groups to induce experimental lung injury. Three days after PQ treatment, PQ + HBOT group was administered 100% O_2 at 2.0 ATA for 1 hour per day, for five consecutive days. At the end of the study, lung tissue was obtained for determining total oxidant status (TOS), total antioxidant status (TAS), oxidative stress index (OSI) and histopathological determination. *Tumor necrosi*s factor-alpha (TNF-α), interleukin 6 (IL-6), basic fibroblast growth factor (bFGF), transforming growth factor *(TGF)-β1 mRNA* levels were assessed by quantitative reverse transcription-polymerase chain reaction. In addition, the inducible nitric oxide synthase (iNOS) level in the plasma was determined. Plasma iNOS, OSI, tissue *TNF-α, TGF-β1 and bFGF mRNA* levels, and histological injury scores in PQ + HBOT group were significantly lower than PQ group. TAS level in PQ + HBOT group was significantly higher than PQ group. The findings suggest that HBOT could effectively ameliorate PQ-induced lung injury in rats.

Keywords: Paraquat, lung injury, hyperbaric oxygen treatment, oxidant, antioxidant

Introduction

Paraquat (PQ) is an agrochemical agent commonly used worldwide, which is allied to potential risks of intoxication. Paraquat, a widely used herbicide, is well known to exhibit oxidative stress and lung injury. Paraquat a highly toxic herbicide causes fatal pulmonary damage. Although liver, kidney, heart and central nervous system are affected, lung damage and pulmonary fibrosis are the most widespread injuries and the usual causes of death [1, 2]. It is pathologically characterized by lung edema, hemorrhage, interstitial inflammation, and bronchial epithelial cell proliferation. Respiratory failure as a result of lung injury is the most common cause of death from PQ [3, 4].

Hyperbaric oxygen treatment (HBOT) is a therapy based on high concentrations of partial oxygen pressure inside a compartment known as hyperbaric chamber. The oxygen used in the chamber is pure and is present at a pressure of two atmospheres [5, 6]. Exposure to HBOT increases the dissolved oxygen content in blood, stimulates fibroblastic activity and collagen production, decreases the inflammatory response, and limits oedema, which improves the microvasculature [7]. HBOT has been reported to be an effective therapy for peripheral vascular disorders, crushing injury, compartment syndrome, acute traumatic peripheral ischemia [8, 9] and improving chronic wounds healing [10]. Various mechanisms, including the ability to improve tissue oxygenation, metabo-

lism, and microcirculation of ischemic tissues, have been suggested to account for the beneficial effects of HBOT [8, 9].

HBOT is an established modality in the treatment of many disorders although their effects and functions are still unclear. Although HBOT has been studied on different lung injury models, the physiological effects and usefulness of HBOT on lung injury has not been well elucidated. The aim of the present study was to examine the effect of HBOT on paraquat-induced lung injury in rats and whether HBOT could prevent lung tissue injury induced by paraquat by measuring oxidant parameters, such as total oxidative status (TOS), oxidative stress index (OSI), and antioxidative parameters such as total antioxidant status (TAS) in the tissue samples of rats.

Methods and methods

Animals

Twenty-one adult male Wistar albino rats (Dumlupınar University Experimental Animal Laboratory, Kütahya, Turkey) weighing 250-300 g were used. All rats were kept under environmentally controlled conditions in an air-conditioned room at 21°C, with appropriate humidity and a 12 h:12 h light:dark cycle and were fed standard rat chow and water ad libitum. This project was approved by the Dumlupınar University Ethics Committee of Animal Care and Usage, Kütahya, Turkey.

Experimental design

The rats were randomly divided into three groups, 7 rats each:

Group 1: served as controls and the rats were injected intraperitoneally with saline (0.9% NaCl). Group 2: designated PQ was injected intraperitoneally daily with PQ at a single dose of 20 mg/kg body weight. Group 3: designated PQ + HBOT, was injected intraperitoneally daily with PQ at a single dose of 20 mg/kg body weight and was administered 100% O_2 at 2.0 ATA for 1 hour per day, for five consecutive days, three days after PQ treatment.

Lung injury was induced by paraquat as previously described [11]. Paraquat dichloride (methyl violgen, Sigma) was dissolved in saline (0.9% NaCl) and animals were given 20 mg/kg intraperitoneal injection in each case.

Hyperbaric oxygen therapy (HBOT)

Rats were placed in a hyperbaric chamber 3 days after paraquat administration and received 1 hour of hyperbaric oxygen (HBO2) at 2.0 ATA in 100% O_2 each day for five consecutive days [12]. Compression air was performed at a rate of 1 kg/cm^2/min to 2.0 ATA/100% oxygen and maintained for 60 minutes. The chamber was flushed with 100% oxygen at a rate of 5 L/min to avoid carbondioxide accumulation. Decompression was performed at 0.2 kg/cm^2/min. Chamber temperature was maintained between 22°C and 25°C. To minimize the effects of diurnal variation, all HBO2 exposures were started at around 10:00 AM.

Tissue preparation and blood sampling

After the final HBOT exposure, rats were fasted overnight before blood sampling was performed. Animals were anesthetized with 50 mg/kg ketamine and xylazine (10 mg/kg) (ip) and blood sample was collected from the aorta. Blood samples were centrifuged at 3000 rpm for 5 min, and the plasma was then collected for iNOS analysis and stored at -80°C until analysis. Lung samples were then collected in liquid nitrogen for analysis of oxidant, antioxidant enzymes and molecular biologic studies, and stored at -80°C until analysis. Histopatological evaluation was also performed.

Biochemical analyses

Plasma concentration of iNOS was analyzed in ELISA device, in accordance with the rat-specific kit protocols (Cusabio, China).

Total antioxidant (TAS) and total oxidant (TOS) levels, which are among the oxidative stress parameters, were measured using kits (Rell Assay, Gaziantep, Turkey) that work with spectrophotometric methods [13, 14]. In Beckman Coulter AU680 analyzer (Beckman Coulter, Miami, FL, USA). Oxidative stress index (OSI= [(TOS/TAS)×100]) was calculated by using the obtained TAS and TOS data. The results were calculated dividing the concentrations of the parameters analyzed in the lung tissue by the most recently obtained total protein levels. The

Table 1. Histopathologic lung damage criteria according to modified scoring system [15]

Scor	Edema	Hemorrhage	Leukocyte infiltration	Alveolar septal thickening
0	No edema	No hemorrhage	Less than 10 cells	No thickened alveolar walls
1	Edema in one quadrant	Hemorrhage in one quadrant	At least 10 cells	Thickened walls in one quadrant
2	Edema in two quadrant	Hemorrhage in two quadrant	At least 25 cells	Thickened walls in two quadrant
3	Edema in three quadrant	Hemorrhage in three quadrant	At least 50 cells	Thickened walls in three quadrant
4	Edema in four quadrant	Hemorrhage in four quadrant	Over 75 cells	Thickened walls in four quadrant

Table 2. The levels of Total Antioxidant Status (TAS), Total Oxidant Status (TOS), Oxidative Stress Index (OSI) in Control (C), Paraquat (PQ) and Paraquat + Hyperbaric Oxygen Treatment (PQ + HBOT) groups

Groups	C (n=7)	PQ (n=7)	PQ + HBOT (n=7)	p
TAS	22.6 ± 0.83	19.9 ± 0.21*,†	26.1 ± 0.75*	0.001
TOS	0.62 ± 0.05	0.66 ± 0.02	0.63 ± 0.01	0.560
OSI	2.78 ± 0.29	3.31 ± 0.14†	2.44 ± 0.09	0.007

Data are expressed as mean± standard error. p: Shows the differences between all groups. *$P \leq 0.05$ vs C group, †$P \leq 0.05$ versus PQ + HBOT group.
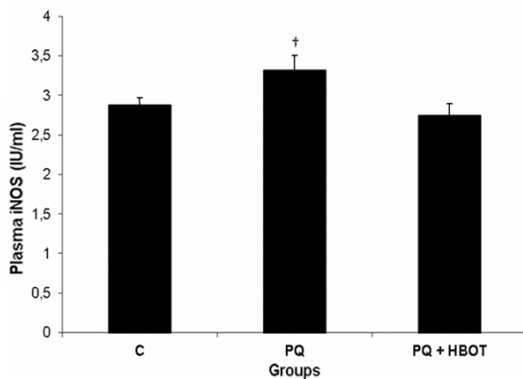
Figure 1. Plasma iNOS concentrations in Control (C), Paraquat (PQ), Paraquat + Hyperbaric Oxygen Treatment (PQ + HBOT) groups. Data are expressed as mean± standard error. †$P \leq 0.05$ versus PQ + HBOT group. iNOS, inducible nitric oxide synthase.

results were shown as concentration per gram protein or concentration milligram protein.

Real-time polymerase chain reaction (RT-PCR)

Total RNA isolation was extracted from lung tissue samples by the GeneJET RNA Purification Kit (Thermo, Cat No: # K0732) according to the manufacturer's protocol. Total mRNA concentrations were measured at 260 nm using a Maestro Nano Micro-Volume spectrophotometer (Maestrogen Inc., Las Vegas, NV). Samples were stored at -80°C until further analysis.

Complementary DNAs (cDNA) were synthesized by EasyScript™ cDNA Synthesis Kit (abm). cDNAs were stored at -20°C until used in the real-time polymerase chain reaction.

A total of 20 µL of PCR mixture containing cDNA template (equivalent to 20 ng total RNA), PCR-grade H_2O, 10 µM each of forward primer, reverse primer and EvaGreen 2X qPCR Master Mix (abm), was amplified by using an LightCycler 480 Real Time PCR System (Roche Diagnostics, Germany) with an initial melt at 95°C for 10 min followed by 35 cycles at 95°C for 15 s, 58°C for 60 s and 72°C for 20 s. The following gene-specific primers were used: *TNF-α* (forward: 5'-CCA CCA CGC TCT TCT GTC TAC-3'; reverse:5'-GCT ACG GGC TTG TCA CTC G-3'; 148 bp expected); *IL-6* (forward: 5'-CTT CCA GCC AGT TGC CTT CTT G-3'; reverse: 5'-TGG TCT GTT GTG GGT GGT ATC C-3'; 109 bp expected); *bFGF* (forward: 5'-GGC TAT GAA GGA AGA TGG AC-3'; reverse: 5'-GTT CGT TTC AGT GCC ACA-3'; 141 bp expected); *TGF-β1* (forward: 5'-GCT AAT GGT GGA CCG CAA CAA C-3'; reverse: 5'-CAG CAG CCG GTT ACC AAG-3'; 229 bp expected); *β-actin* (forward: 5'-CTA TCG GCA ATG AGC GGT TCC-3' reverse: 5'-TGT GTT GGC ATA GAG GTC TTT ACG-3'; 147 bp expected). The number of amplification steps required to reach an arbitrary intensity threshold (C_t) was computed. The relative gene expression was represented as 2 $(-\Delta C_t)$, where ΔC_t= Cttarget-Ctβ-actin. Fold change for the treatment was calculated as $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$= ΔCttreatment-ΔCtvehicle.

Histopathologic examinations

Lung tissue samples were fixed in 10% formalin, embedded in paraffin, sectioned (thickness of 4 µm), and slides were stained with hematoxylin and eosin (H&E). Slides were examined under light microscope (Olympus BX51, Tokyo,
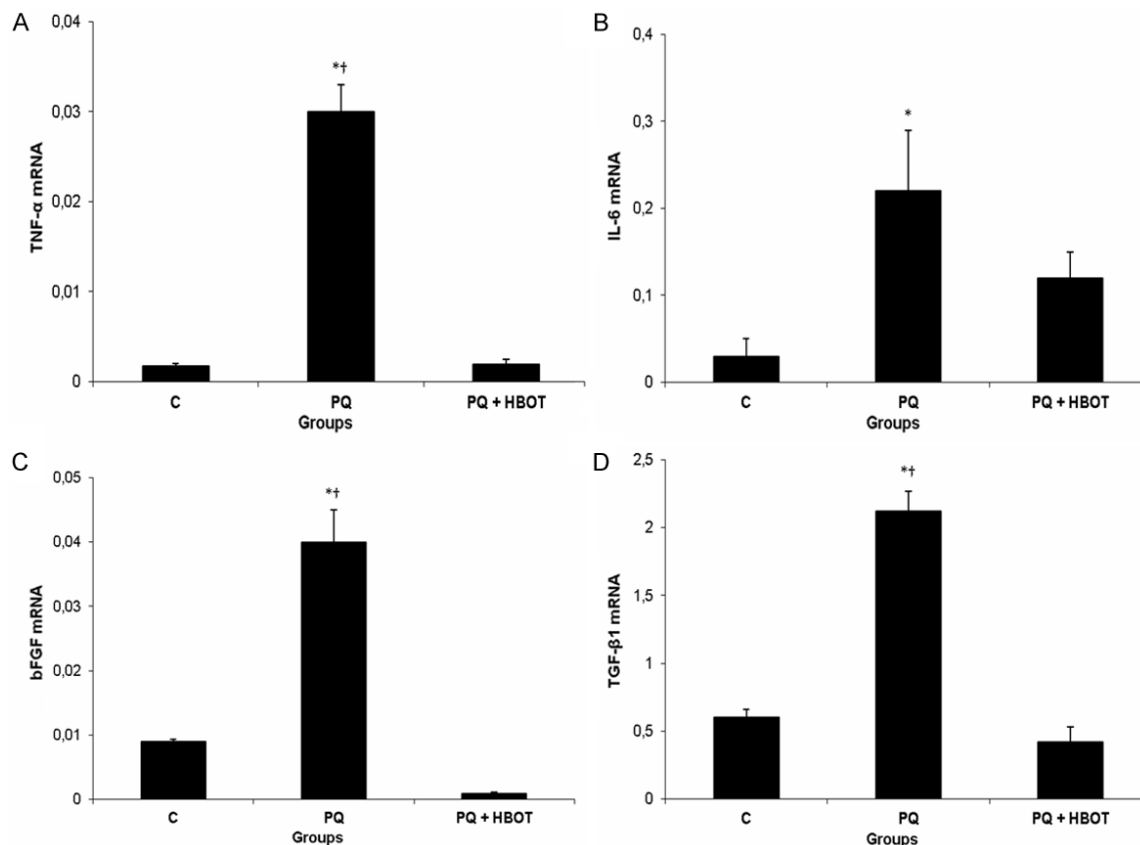
Figure 2. Effect of HBOT on mRNA expression of *TNF-α, IL-6, bFGF and TGF-β1* in rats with paraquat-induced acute lung injury. A. The *TNF-α* mRNA expression for each group. B. *The IL-6* mRNA expression for each group. C. The *bFGF* mRNA expression for each group. D. The *TGF-β1* mRNA expression for each group. Groups: Control (C; n=7), Paraquat (PQ; n=7), Paraquat + Hyperbaric Oxygen Treatment (PQ + HBOT; n=7). Data were expressed as mean ± standard error. *P ≤ 0.05 vs control group, †P ≤ 0.05 vs PQ + HBOT group. TNF-α, Tumor necrosis factor-alpha; IL-6, interleukin 6; bFGF, basic fibroblast growth factor; TGF-β1, transforming growth factor-beta1.

Japan) by a pathologist who was blinded to the treatment each animal had received. The pathologic slides were graded histopathologically according to the severity of lung damage by using a modified scoring system (**Table 1**) [15]. In this scoring system, four lung damage indices; edema (score: 0-4), hemorrhage (score: 0-4), leukocyte infiltration (score: 0-4), and alveolar septal thickening (score: 0-4) are graded for a total score of 0-16.

Statistical analysis

Statistical analysis was done with SPSS (Statistical Package for Social Sciences, Chicago, IL, USA) 16.0 pocket program. All results were given as means ± standard error (SE). For analysis of concentration effect and time course, a multiple range of anova and Bonferroni post-hoc tests were used. Values smaller than $P ≤ 0.05$ were accepted as statistically significant.

Results

Effect of HBOT on tissue TAS, TOS and OSI levels

There were statistically significant differences in tissue TAS levels among the groups of C, PQ, and PQ + HBOT (*P*=0.001). Compared with the C and PQ + HBOT groups, tissue TAS level was decreased significantly in PQ group (*P*=0.017 and *P*=0.001). Tissue TAS level significantly increased in the PQ + HBOT group compared with the C and PQ groups (*P*=0.038, *P*=0.001) (**Table 2**).

Significant differences were not observed in the levels of tissue TOS among the groups of C, PQ, and PQ + HBOT (*P*=0.560). It was observed that augmentation in tissue TOS level of the PQ group compared with the C and PQ + HBOT groups was not significant (*P*=0.902 and *P*=0.535) (**Table 2**).
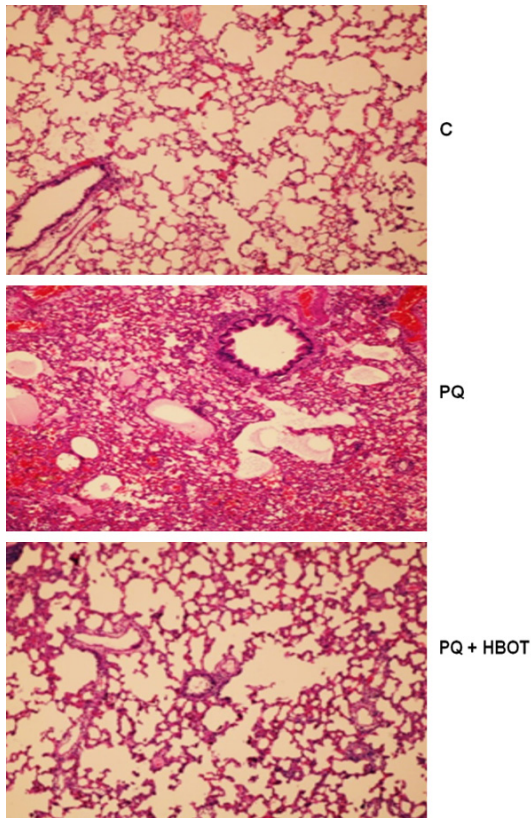
Figure 3. Effect of HBOT on Histopathologic Examinations. Control group demonstrated normal alveolar structure in lung. PQ group demonstrated severe edema, hemorrhage, leukocyte infiltration, and alveolar septal thickening. PQ + HBOT group demonstrated minimal hemorrhage, leukocyte infiltration, and alveolar septal thickening, H&E ×100. PQ, paraquat; HBOT, hyperbaric oxygen treatment.

OSI levels differed a significantly among the groups of C, PQ, and PQ + HBOT (*P*=0.007). The high levels of OSI were found in PQ group compared with PQ + HBOT group, *P*=0.001 (**Table 2**).

Effect of HBOT on plasma iNOS

Plasma iNOS levels were observed to be statistically significant different among C (2.88 ± 0.02 IU/ml), PQ (3.32 ± 0.19 IU/ml), and PQ + HBOT (2.75 ± 0.15 IU/ml) (*P*=0.041). Compared with the PQ + HBOT group, the plasma iNOS levels were increased significantly in PQ group (*P*=0.05) (**Figure 1**).

Effect of HBOT on mRNA Expression of TNF-α, IL-6, bFGF and TGF-β1

There were significant differences in *TNF-α, IL-6, bFGF* and *TGF-β1* gene mRNA expression

levels in the lung tissue among C, PQ, PQ + HBOT groups, (*P* ≤ 0.05). Rat lung expression of *TNF-α, IL-6, bFGF and TGF-β1* mRNA was increased in the PQ group compared with the C group (*P* ≤ 0.05). HBOT produced a significant decrease in rat lung expression of *TNF-α, bFGF and TGF-β1* mRNA (*P* ≤ 0.05) in the PQ + HBOT group. But, low levels of *IL-6* gene mRNA expression were not significant in the PQ + HBOT group compared with PQ group (**Figure 2**).

Effect of HBOT on histopathologic examinations

Histopathologic examinations revealed normal lung structure in the control group Severe lung injury was seen in PQ group. In the PQ + HBOT group, minimal hemorrhage, leukocyte infiltration, and alveolar septal thickening were observed (**Figure 3**). Detailed damage scores and comparisons between groups were shown in **Table 3**.

Discussion

PQ is a highly toxic compound for humans and animals causes fatal injury to organs upon high dose ingestion. The major cause of death in paraquat poisoning is respiratory failure. PQ accumulates in the lungs via the alveolar cells, inducing the production of intracellular reactive oxygen species (ROS) and the development of lung inflammation and fibrosis [16]. Direct exposure to PQ causes severe irritation to the eyes and skin, and ingestion of concentrated products may result in fatal injury to lungs because of edema, hemorrhage, and subsequent fibrosis as well as damage to other organs [17]. Small amounts of paraquat can cause severe and irreversible systemic damage refractory to any known treatment [18]. The mechanism of paraquat toxicity has not been fully understood.

Oxidative stress induced by free radicals may be the primary cause of tissue injury and may occur in inflammation [19]. An imbalance between ROS and the antioxidant defence system results in oxidative stress, which is closely linked to the pathogenesis of acute and chronic lung injury. Increased levels of ROS can cause direct tissue injury and promote inflammatory responses via the regulation of diverse pro-inflammatory mediators in the lungs [20].

TAS and TOS are the additive measurement parameters of antioxidant effects that enable

Table 3. Comparison of scored histopathological values in lung tissue samples between Control (C), Paraquat (PQ) and Paraquat + hyperbaric oxygen treatment (PQ + HBOT) groups

Scored values	C (n=7)	PQ (n=7)	PQ + HBOT (n=7)	p
Edema	0.28 ± 0.18	2.14 ± 0.40*,†	0.28 ± 0.18	0.000
Hemorrhage	0.85 ± 0.14	3.00 ± 0.21*,†	1.14 ± 0.14	0.000
Leukocyte infiltration	0.14 ± 0.14	3.85 ± 0.14*,†	1.00 ± 0.21*	0.000
Alveolar septal thickening	0.14 ± 0.14	2.57 ± 0.20*,†	1.28 ± 0.18*	0.000
Total damage score	0.36 ± 0.09	2.90 ± 0.17*,†	0.93 ± 0.12	0.001

Data are expressed as mean ± standard error. p: Shows the differences between all groups. *P ≤ 0.05 vs C group, †P ≤ 0.05 versus PQ + HBOT group.

the measurement of all the antioxidant and oxidant parameters by being evaluated together. The TOS/TAS ratio, in other words the OSI, is an indicator of the oxidative stress degree and shows the anti-oxidation and oxidation redox balance [21]. In a study by Rifaioglu et al. [22], it was observed that TAS levels were decreased while TOS values were found to be significantly higher in the PQ (15, 30, 45) groups compared with control group [22]. Kılıç et al. [23] reported that GSH and TAS levels were significantly decreased and the OSI was higher in the bleomycin-induced lung injury rats when compared to controls [23]. In our study we found that serum TAS levels of rats in the PQ group decreased when compared with the other groups. Serum TOS values of the PQ group were higher than the C and PQ + HBOT, but the differences were not statistically significant. In addition, an increase was observed in OSI levels in the PQ group compared with PQ + HBOT group. After HBOT application, we observed significantly increased TAS levels and decreased TOS and OSI levels. There are important studies in the literature about how the HBOT application affects the antioxidant enzymes in various tissues in the organism. Sanchez and colleagues [24] applied HBOT to patients with diabetic foot, and they reported that antioxidant enzyme levels were increased in the HBOT group when compared to the antibiotic treatment group. Koca et al. [25] recently showed that inflammatory cytokines and oxidative stress decreased in HBOT groups. Harabin and colleagues [26] reported an increase in SOD activity in the lung and decrease in GSH-Px activity in brain and lung tissues in HBOT-applied rats. These results seem compatible with our study results. This demonstrates that HBOT may have a preventive effect on lung aganist oxidative injury.

iNOS that is released in any acute event (trauma, stress, acute inflammation, etc.) may have either a protective or detrimental effect on tissue. High NO concentrations produced by iNOS increase the damage [27]. In this study, an increase was observed in plasma iNOS level in the PQ group compared with the PQ + HBOT group. In the study performed by Zocrato [28], it was shown that paraquat poisoning increases endothelial *iNOS* expression. The cytokine TNFα has been shown to play a majör role in driving the expression of *iNOS* in inflammatory states [29]. In our experiments, there was increased mRNA levels of *TNF-α*, which is consistent with a possible role of this cytokine in the PQ group. It has been reported that TNF-α is necessary for *iNOS* expression and iNOS-dependent vascular dysfunction [30]. These results suggest that increasing *TNF-α* mRNA levels can cause a increase in levels of iNOS. These high levels of plasma iNOS and *TNF-α* mRNA expression may be the results of their interaction and activating each other in the PQ group.

HBOT not only increased antioxidant enzyme expression, such as Cu/Zn-superoxide dismutase, catalase, and glutathione peroxidase, but also significantly decreased pro-oxidant enzyme levels, such as iNOS [31]. Huang et al. [32], showed that protein expression of iNOS and the serum NO content were dramatically reduced in the HBOT application group as compared to the spinal cord injury group. In this study, there were significant decreases plasma iNOS in the PQ + HBOT group compared with the PQ group. HBOT may exert significant effects plasma level of iNOS and protect lung tissue from paraquat induced acute lung injury.

The inflammatory reaction plays an important role in acute lung injury (ALI) in patients following paraquat poisoning. Clinically, the expression of inflammatory cytokines such as IL-6, TNF-α in the blood is significantly elevated in patients with paraquat poisoning. Numerous basic and clinical studies have demonstrated that overexpression of TNF-α can induce acute lung injury [33]. Liu et al. [34] have demonsrat-

ed that paraquat caused a significant acute systemic inflammatory response as demonstrated by the increased serum concentrations of the pro-inflammatory mediators TNF-α, IL-1β, and IL-6. Lee et al. [35] reported that IL-6 plasma levels were not elevated at 6 and 12 hours in a PQ-intoxicated rat model (50 mg/kg), although a different study showed that IL-6 levels in lung tissues were higher 1 day after PQ injection in a rat model (18 mg/kg) [36]. In this study, *TNF-α* and *IL-6* mRNA expression in the lung tissue were significantly increased in the PQ group and were reduced with HBOT application. But, low levels of *IL-6* gene mRNA expression were not significant in the PQ + HBOT group compared with PQ group. Recent experimental reports have demonstrated that HBOT exposure significantly reduced the lung injury and proinflammatory cytokines such as TNF-α, IL-1β, IL-6 [37]. These data show that HBOT alleviates PQ-induced lung injury and inhibits *TNF-α* and *IL-6* mRNA levels. Reduced *TNF-α* and *IL-6* mRNA levels may, at least in part, contribute to the beneficial effect of HBOT.

bFGF is involved in many physiological and pathophysiological processes like growth, wound and bone healing, cell differentiation and proliferation, but also tumour progression and metastasis, due to its ability to stimulate the activity of fibroblasts, endothelial cells, smooth muscle cells and neurons, [38, 39]. In our study, we found that lung *bFGF* mRNA levels of rats in the PQ + HBOT group decreased when compared with the PQ group. There has not been any study in the literature between bFGF and PQ that can be compared with the results of this study. Kang et al. [40], demonstrated that it was increased secretion of bFGF in cells after initial HBOT exposure. In another study by Jung et al. [41], it was showed that HBOT was not verified on serum concentrations of bFGF [41]. In this study and previous studies on this subject, the duration and type of administration of HBOT differ leading to the differences in the results.

TGF-β1 is a polypeptide cytokine that promotes inflammatory responses by releasing related inflammatory cells [42]. TGF-β1 plays a critical role in tissue injury development of multiple organs, including lung [43]. Additionally, TGF-β1 has been reported to play an important role in ALI induced with PQ and hyperoxia [44]. It was discovered that the rat serum TGF-β1 levels in the paraquat groups were significant higher than that in the control group and the rat pulmonary *TGF-β1* mRNA expression levels were also higher than that in the control group [45]. In our study, the TGF-β1 level increased 3 days after PQ intoxication and was reduced with HBOT application. Romero-Valdovinos et al. [46] showed that growth of fibroblasts and in the expression of *TGF-β* mRNA were decreased in keloid and nonkeloid fibroblast after chronic exposition to hyperbaric oxygenation compared with normal oxygen partial pressure [46]. This suggests that HBOT modulates the TGF-β1 level, thereby reducing PQ-induced lung injury.

In conclusion, results from the study showed that oxidative stress, reduction of antioxidant capacity, and inflammation play a crucial role in the PQ-induced lung injury. HBOT markedly inhibited the secretion of inflammatory mediators, decreased OSI and iNOS levels, and significantly increased TAS activity. In addition, it reduced lung oedema, haemorrhage, leukocyte infiltration and alveolar septal thickening on histopathologic examination. The histopathological findings support the biochemical findings. The data suggest that HBOT is a highly promising agent in protecting early lung tissue damage induced by paraquat exposure.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Raziye Akcilar, Department of Physiology, Faculty of Medicine, University of Dumlupınar, Kütahya, Turkey. Tel: +90-507 953 94 74; Fax: +90-274-265 22 85; E-mail: raziyeakcilar@gmail.com

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