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Carvedilol attenuates paraquat-induced lung injury by inhibition of proinflammatory cytokines, chemokine MCP-1, NF-κB activation and oxidative stress mediators

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ABSTRACT

Paraquat is a highly toxic herbicide that selectively accumulates in the lungs and causes pulmonary damage through the oxidative and inflammatory processes. Carvedilol is a nonselective beta and alpha-adrenergic blocking agent that has been shown to possess powerful antioxidant and anti-inflammatory properties. In the present study, we evaluated the protective effects and the underlying mechanisms of carvedilol on paraquat-induced lung injury in a mouse model. Mice were injected with a single dose of paraquat (20 mg/kg, ip), and treated with carvedilol (10 and 20 mg/kg/day, orally) for eight days. At the end of the experiment, lung tissue and blood samples were collected for histological and biochemical analysis. The results showed that carvedilol treatment improved the histopathological changes in the lung tissue of mice exposed to paraquat. Carvedilol significantly decreased the levels of malondialdehyde (MDA), carbonyl protein, myeloperoxidase (MPO), and nitric oxide (NO), while increased the levels of glutathione (GSH), superoxide dismutase (SOD), catalase and glutathione reductase compared with paraquat group. Carvedilol treatment also significantly reduced the levels of proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, transforming growth factor (TGF)-β1 and monocyte chemoattractant protein (MCP)-1 in the lung tissue. Treatment of mice with carvedilol decreased paraquat-induced expression of nuclear factor kappa B (NF-κB). In addition the plasma levels of matrix metalloproteinase (MMP)-9 and the lung hydroxyproline content significantly reduced by carvedilol treatment. Taken together, these results indicate that carvedilol is able to decrease the severity of paraquat-induced lung injury through inhibition of inflammation and oxidative stress.

1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridinium chloride) is one of the most widely used herbicides and is highly toxic to animals and humans. Because of its high toxicity, paraquat has been banned in the European Union since 2007. However, it is a major suicide agent in many countries. Paraquat poisoning causes dysfunction of multiple organs, mostly the lung. Paraquat selectively accumulates in the lungs, resulting in pulmonary edema, hemorrhage, destruction of alveolar epithelial cells and ultimately fibrosis. The most common cause of death in acute paraquat poisoning is pulmonary fibrosis and progressive respiratory failure [1].

Oxidative stress and inflammation are two key mechanisms suggested to be involved in the pathogenesis of paraquat-induced lung injury. Paraquat undergoes redox cycling process and generates a reactive oxygen species (ROS) and depletes cellular NADPH. Reactive oxygen species are thought to be responsible for the direct cell injury and induce intracellular transcription factors, proinflammatory cytokines, inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX) all of which cause inflammation and destruction of the lung tissue. It has also shown that paraquat intoxication leads to activation and proliferation of fibroblasts and deposition of collagen and other extracellular matrix proteins in the alveolar walls. There is no effective therapy for paraquat poisoning, though several anti-inflammatory or antioxidant agents have been investigated against paraquat induced lung injury [2,3].

Carvedilol is a nonselective beta and alpha-adrenergic blocking agent clinically approved for the treatment of congestive heart failure and to reduce high blood pressure. Carvedilol has also been

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found to exert an antioxidant and anti-inflammatory effects. It has been suggested that the potent antioxidant properties of carvedilol result from the tricyclic carbazole moiety in its structure. Carvedilol exerts antioxidant properties independent of adrenoceptor blockade. It was reported that the antioxidant activity of carvedilol and its metabolites is much greater than that of vitamin E [4,5]. Several studies demonstrated that carvedilol is able to inhibit proinflammatory and fibrogenic cytokines, leukocyte accumulation, lipid peroxidation, and matrix metalloproteinases, all of which promote tissue destruction and fibrosis [6,7]. The present study was conducted to assess the possible protective effect of carvedilol against paraquat-induced lung injury in a mouse model. In this study, we examined the changes in oxidative stress and inflammatory markers and histopathological features in lung tissues.

2. Material and methods

2.1. Animal

Experiments were performed on Swiss albino mice weighing 25–30 g. Mice were kept in our animal house under controlled conditions and allowed free access to tap water and a standard diet. All animal procedures were performed in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1996).

2.2. Experimental design

The animals were randomly divided into five groups (n = 8), as follows: (1) control group, mice received saline solution. (2) car group, mice received carvedilol (20 mg/kg/day, orally). (3) PQ group, mice received paraquat (20 mg/kg, ip). (4) PQ + Car 10 group, mice received paraquat (20 mg/kg, ip) and carvedilol (10 mg/kg/day, orally). (5) PQ + Car 20 group, mice received paraquat (20 mg/kg, ip) and carvedilol (20 mg/kg/day, orally). Paraquat was dissolved in saline solution and injected intraperitoneally in a single toxic dose of 20 mg/kg of body weight. The dosage of paraquat was based on our previous experiments [2]. Carvedilol was suspended in 0.9% sodium chloride and administered by gavage once daily for 8 consecutive days. Carvedilol was administered one hour before the injection of paraquat. At the end of the experiment, all animals from each group were anesthetized with ketamine and xylazine and their thoracic cavities were opened. Blood was drawn from the heart, and the plasma fraction was collected by centrifugation. Lung tissues were immediately removed and washed in normal saline solution. Then, one part of each lung tissue sample was fixed in formalin for histological examination, and another part was frozen in liquid nitrogen and kept at −80 °C until analysis.

2.3. Preparation of lung tissue homogenate

Frozen tissue samples were homogenized in ice-cold Tris–HCl buffer (pH 7.4, containing protease inhibitor cocktail) using a homogenizer (Heidolph, Germany). All homogenized samples were centrifuged at 20,000*g* for 20 min in a refrigerated centrifuge at 4 °C. The supernatants were collected and stored at −80 °C for biochemical analysis.

2.4. Histopathological examination of lung tissue

The fixed tissue samples were embedded in paraffin and sections of 4 μm thickness were prepared and stained with hematoxylin and eosin (H&E). The slides were evaluated by an expert pathologist using a light microscope. The severity of lung damage was scored according to the following histological parameters: Alveolar wall thickness, the amount of cellular infiltration and hemorrhage. The histological parameters were graded on a scale of 0–3 (0 = none, 1 = mild, 2 = moderate, 3 = severe).

2.5. Measurement of malondialdehyde (MDA)

The level of MDA in the lung tissues was determined using the thiobarbituric acid (TBA) method [8]. Briefly, 100 μl of the supernatant was added to a reaction mixture containing 100 μl of 20% (w/v) trichloroacetic acid and 200 μl of TBA (0.1 M). The mixture was incubated at 90 °C for 60 min to complete the reaction. After cooling, the samples were centrifuged at 10,000*g* for 5 min at 4 °C and the supernatants were collected. The absorbance of the supernatants was measured at 532 nm using a 96-well microplate reader (BioTek, USA).

2.6. Measurement of carbonyl proteins

The levels of carbonyl proteins in the lung tissue were determined by a colorimetric method [9]. 50 μl of the sample was added to 100 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl and incubated at room temperature for 1 h, with vortexing every 15 min. Then, 100 μl of 20% Trichloroacetic acid was added and centrifuged at 15,000*g* for 5 min. The supernatant was discarded and the pellet was washed with 1 ml ethanol-ethyl acetate (1:1 v/v) to remove excess DNPH. The final pellet was redissolved in 150 μl of 6 M guanidine hydrochloride in 20 mM potassium phosphate (pH 2.3). The absorbance was measured at 370 nm and carbonyl content was calculated using the molar extinction coefficient of 22 mM^{-1} cm^{-1}. The results were expressed as nmol carbonyl per mg tissue.

2.7. Measurement of LDH

The plasma levels of lactate dehydrogenase (LDH) were measured using a commercial kit (Pars Azmun, Iran) and a spectrophotometer (Shimadzu UV-1800, Japan). The results were expressed as units per liter (U/L).

2.8. Measurement of reduced glutathione (GSH)

To determine GSH, 50 μl of the supernatant was added to 50 μl of 10% trichloroacetic acid. After shaking, the mixture was centrifuged at 10,000*g* for 5 min at 4 °C and the supernatant was separated from the pellet. An aliquot of the resulting supernatant (50 μl) was added to 150 μl of phosphate buffer (0.2 M, pH 7.6, 1 mM EDTA) and 1 mM DTNB (5,5′-Dithiobis-2-nitrobenzoic acid). The absorbance of the yellow color was measured at 412 nm using a microplate reader.

2.9. Measurement of superoxide dismutase (SOD) and glutathione reductase (GR)

The activity of SOD and glutathione reductase enzymes in the lung tissue was measured using a SOD assay kit (BioVision) and Glutathione Reductase Assay Kit (Cayman Chemical) according to the manufacturer's protocol.

2.10. Measurement of catalase (CAT)

CAT activity was measured according to the method described previously [10]. Briefly, a 10 μL aliquot of the supernatant was added to a cuvette containing 0.5 ml of phosphate buffer (50 mM, pH 7). The reaction was started by the addition of

0.5 ml hydrogen peroxide (30 mM) and the change in absorbance was measured at 240 nm by using a spectrophotometer (Shimadzu UV-1800, Japan). One unit of enzyme activity was defined as the amount of enzyme decomposing 1 mM hydrogen peroxide per minute.

2.11. Measurement of nitric oxide (NO)

The concentrations of nitrite and nitrate in the lung tissue were evaluated as an index of NO production. Total levels of nitrate and nitrite were measured using a Nitric Oxide Assay Kit (Enzo Life Sciences). Results were expressed as nmol/mg tissue.

2.12. Measurement of myeloperoxidase (MPO)

The activity of MPO in the lung tissue was determined by measuring the H_2O_2–dependent oxidation of tetramethylbenzidine (TMB). Briefly, 50 µl of the sample was added to 50 µl of TMB solution (15 mM) and 100 µL of H_2O_2 (25 mM) diluted in phosphate buffer (50 mM, pH 5.4). The assay was performed in a microplate and the rate of change in absorbance was measured at 370 nm using a microplate reader. One unit of MPO activity was defined as the quantity of enzyme degrading 1 µmol of H_2O_2 per min at 25 °C. The results were expressed as mU MPO/mg tissue

2.13. Measurement of pro-inflammatory cytokines

The levels of cytokines TNF-α, IL-1β, IL-6, TGF-β1 and chemokine MCP-1 in the lung tissue were measured by using mouse ELISA kits from eBioscience according to the manufacturer's instructions.

2.14. RT-PCR analysis of NF-κB in lung tissue

Total RNA was extracted from lung tissue samples using TRIzol Reagent (Roche) and treated with DNase I. Synthesis of the cDNA was performed using a First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The mRNA expression level of NF-κB was measured by semi-quantitative RT-PCR. The sequences of primers were as follows: NF-κB forward, ACCTTTGCTGGAAACACACC and reverse, ATGGCCTCGGAAGTTTCTTT; GAPDH forward, CAAGGTCATCCATGACAACTTTG and reverse, GTCCACCACCCTGTTGCTGTAG.

2.15. Measurement of Hydroxyproline

The concentration of hydroxyproline in the lung tissue was measured according to the method described in our previous study [2]. Briefly, all samples were hydrolyzed in 6 N HCl for 12 h at 120 °C. 50 µl of the samples were added to a 96-well plate and kept at 60 °C until the HCl was completely evaporated. Hydroxyproline oxidation was initiated by addition of 100 µl of chloramine T reagent (0.05 M). After shaking, the plate was incubated for 20 min at room temperature. To develop the color 100 µl of p-dimethylaminobenzaldehyde solution (1 M) was added to each well and the plate was incubated for 20 min at 60 °C. After cooling, the absorbance was read at 570 nm using a microplate reader. The hydroxyproline concentrations were calculated using a standard curve of known concentrations of hydroxyproline. The results were expressed as microgram of hydroxyproline per mg tissue.

2.16. Measurement of MMP-9

The plasma levels of matrix metalloproteinase-9 (MMP-9) were measured using an ELISA kit (Mouse Total MMP-9 Quantikine ELISA Kit, R&D Systems) according to the manufacturer's instructions.

2.17. Statistical analysis

Data were expressed as mean ± SD. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. $P < 0.05$ was considered significant.

3. Results

3.1. Effects of carvedilol and paraquat on body weight

We examined the body weight of the mice from day 1 to day 8. Administration of paraquat (20 mg/kg, ip, single dose) caused a significant weight loss in mice as compared to the control group (Fig. 1). During the whole experimental period, body weight loss was significantly attenuated in the mice treated with PQ + carvedilol (20 mg/kg/day) as compared to that in the PQ group. Carvedilol treatment at dose of 10 mg/kg/day also slightly increased the body weight of mice but the difference between this group and the PQ group was not significant (except the second day).

The difference of body weight between the control and carvedilol-only treated group was not significant over the whole experimental period.

3.2. Effects of carvedilol and paraquat on the ratio of lung weight to body weight (LW/BW)

At the end of the experiment, pulmonary edema was evaluated using the ratio of wet lung weight to body weight. Paraquat administration caused an increase in the ratio of LW/BW, however treatment with carvedilol (20 mg/kg/day) significantly decreased the LW/BW ratio ($P = 0.016$) (Fig. 2). No significant change in the LW/BW ratio was observed in PQ + low dose carvedilol (10 mg/kg/day) compared with PQ group ($P = 0.056$).

3.3. Effects of carvedilol against paraquat-induced pathological changes

Histopathological changes caused by paraquat and carvedilol treatment in the lung tissues of mice were assessed by H&E staining (Fig. 3A). Paraquat administration induced a significant inflammatory response characterized by widespread inflammatory cell infiltration in the alveolar space and septum, thickening of the

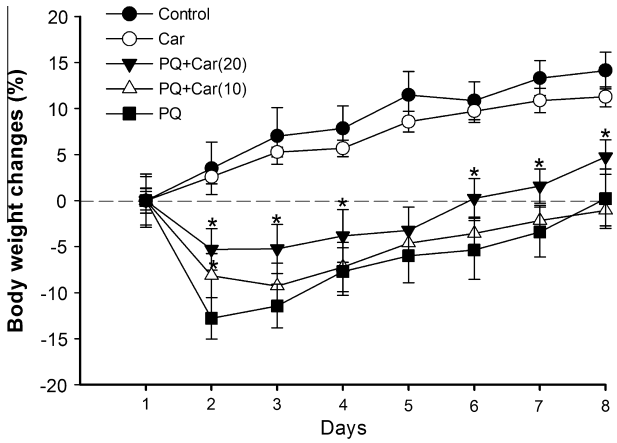

Fig. 1. Effects of paraquat and carvedilol treatment on body weight changes in mice. Carvedilol at a dose of 20 mg/kg/day attenuated paraquat-induced weight loss in mice. Data are means ± SD. *$P < 0.01$ compared with PQ group.

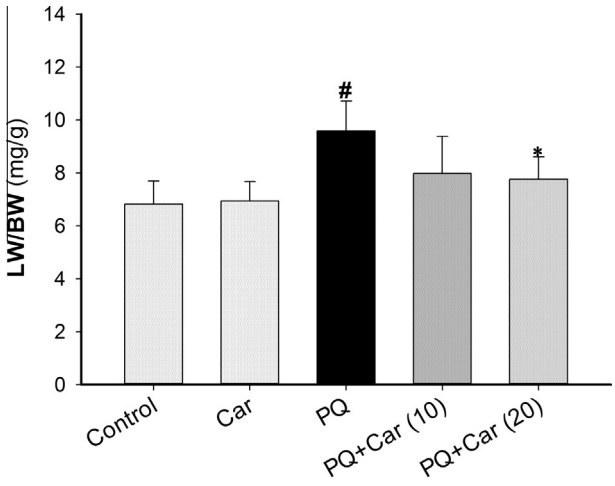

Fig. 2. Effects of paraquat and carvedilol treatment on the ratio of lung weight to body weight (LW/BW). The ratio of LW/BW was determined at the end of the study, on day 8. Carvedilol at the dose of 20 mg/kg/day decreased paraquat-induced increase in the ratio of LW/BW in mice. Data are means ± SD. $^{\#}P < 0.001$ compared with control group; $^{*}P < 0.05$ compared with PQ group.

alveolar septum, and alveolar collapse and hemorrhage. These histopathological changes were markedly improved by the treatment with carvedilol at the dose of 20 mg/kg/day. No histopathological alteration was observed in the lung of carvedilol only-treated mice. As shown in Fig. 3B, the total histopathological score was significantly elevated in the paraquat treated mice compared with the control mice ($P < 0.001$). Treatment with carvedilol (20 mg/kg/day) showed a significant decrease ($P = 0.016$) in the histopathological score when compared with the PQ group. However, the histopathological score was not significantly lower in the mice treated with the low dose of carvedilol (10 mg/kg/day) as compared to the PQ group.

3.4. Effects of carvedilol and paraquat on the levels of MDA, protein carbonyl and LDH

Oxidative stress induced by paraquat was examined by measuring the level of MDA, a marker of lipid peroxidation, and protein carbonyl content as a marker of protein oxidation (Table 1). The results showed that paraquat administration significantly increased the level of MDA and protein carbonyl content in the lung tissue. Treatment with carvedilol (20 mg/kg/day) significantly prevented paraquat induced increase of MDA and protein carbonyl formation ($P < 0.01$). Treatment of mice with the low dose of carvedilol (10 mg/kg/day) also significantly decreased the levels of protein carbonyl ($P < 0.01$). Furthermore, in the present study the plasma levels of LDH as a marker of cell membrane damage were measured (Table 1). The plasma levels of LDH in mice treated with paraquat were significantly increased in comparison to normal

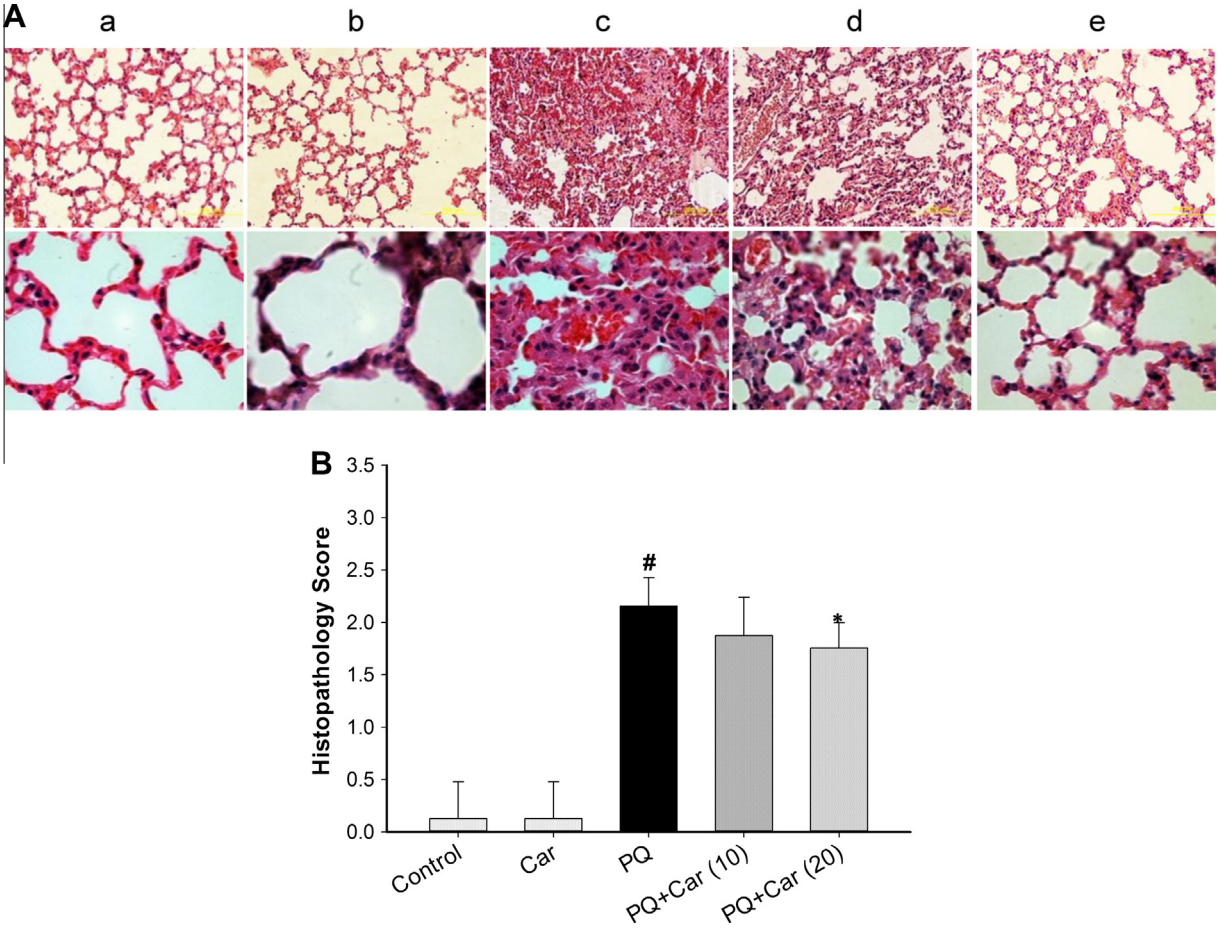

Fig. 3. (A) Histopathological appearance of lung tissues in (a) normal control, (b) carvedilol only (c) PQ, (d) PQ + carvedilol (10 mg/kg/day) and (e) PQ + carvedilol (20 mg/kg/day) groups. Widespread thickening of alveolar septum, infiltration of inflammatory cells, and hemorrhage were observed in PQ group. Treatment with carvedilol reduced the histopathological alterations induced by paraquat. The lung tissue sections were analyzed by H&E staining (magnification is ×400 and ×600). (B) Total histopathological score of lung tissue in each group. Histopathology score of lung was significantly lower in the PQ + carvedilol (20 mg/kg/day) group than in the PQ group. The results are presented as the mean of the total score ± SD. $^{\#}P < 0.001$ compared with control group; $^{*}P < 0.05$ compared with PQ group.

Table 1
Effects of carvedilol on the levels of MDA, protein carbonyl and LDH in each group.

Groups	MDA (nM/mg tissue)	Protein carbonyl (nM/mg tissue)	LDH (U/L plasma)
Control	8.8 ± 5.16	0.26 ± 0.07	138.9 ± 51.3
Carvedilol	9.57 ± 5.6	0.26 ± 0.04	119.7 ± 73.4
PQ	39.9 ± 5.6$^{\#}$	1.17 ± 0.31$^{\#}$	322.3 ± 73.7$^{\#}$
PQ + Carvedilol (10)	32.9 ± 9.8	0.71 ± 0.13**	275.6 ± 65.8
PQ + Carvedilol (20)	19.46 ± 12**	0.5 ± 0.29**	189.2 ± 71.1*

Data are means ± SD.
$^{\#}$ $P < 0.01$ compared with control group.
* $P < 0.05$.
** $P < 0.01$ compared with PQ group.

control mice. Administration of carvedilol (20 mg/kg/day) significantly reduced paraquat induced increase of LDH ($P = 0.02$).

3.5. Effects of carvedilol and paraquat on the levels of GSH, SOD, CAT and GR

Sustained oxidative stress caused by paraquat leads to the depletion of GSH along with the antioxidant enzymes SOD, CAT and GR in the lung tissue when compared with those in the control group (Fig. 4A–D). Treatment with carvedilol (20 mg/kg/day), significantly restored the levels of GSH ($P < 0.001$), SOD ($P = 0.032$), CAT ($P = 0.014$) and GR ($P = 0.032$) activity in the lung tissue as compared with those in the PQ group. The level of GSH was also significantly increased at the low dose of carvedilol (10 mg/kg/day, $p < 0.01$).

3.6. Effects of carvedilol and paraquat on the levels of MPO and NO

A single injection of paraquat caused significant elevation in lung tissue levels of MPO and NO as compared to the control group (Fig. 5A and B). Treatment with carvedilol (20 mg/kg/day), significantly reduced the activity of MPO ($P = 0.033$) and the levels of NO ($P < 0.001$) in lung tissue as compared to those in the PQ group.

3.7. Effects of carvedilol and paraquat on the levels of pro-inflammatory cytokines

The levels of proinflammatory cytokines TNF-α, IL-1β, IL-6, TGF-β1 and chemokine MCP-1 in the lung tissue were significantly elevated in the paraquat treated group as compared to the normal control group ($P < 0.001$) (Fig. 6A–E). Treatment of mice with carvedilol (20 mg/kg/day) significantly decreased the production of TNF-α ($P = 0.024$), IL-1β ($P = 0.004$), IL-6 ($P = 0.001$), TGF-β1 ($P < 0.001$) and MCP-1($P = 0.001$) in the lung tissue as compared to the mice treated with paraquat. Treatment of mice with the low dose of carvedilol (10 mg/kg/day) also significantly decreased the levels of IL-6 ($P = 0.027$) and TGF-β1 ($P < 0.001$).

3.8. Effects of carvedilol and paraquat on the mRNA expression level of NF-kB

As shown in Fig. 7, the mRNA expression level of NF-κB in the lungs of mice treated with paraquat significantly increased when compared with control mice ($P < 0.01$). Treatment with carvedilol

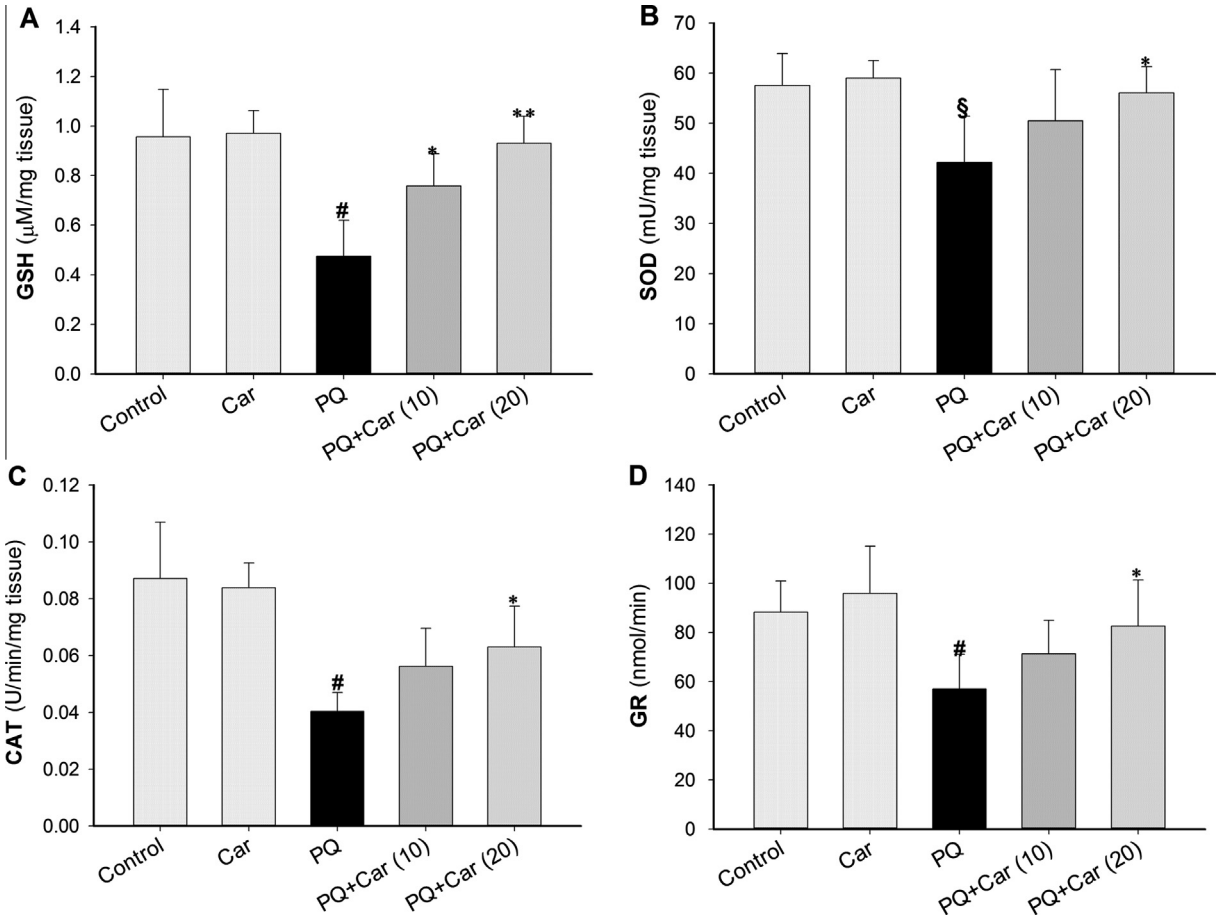

Fig. 4. Effect of carvedilol treatment on paraquat-induced changes in the levels of (A) GSH, (B) SOD, (C) CAT and (D) GR. Data are means ± SD. $^{\S}P < 0.01$, $^{\#}P < 0.001$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.001$ compared with PQ group.

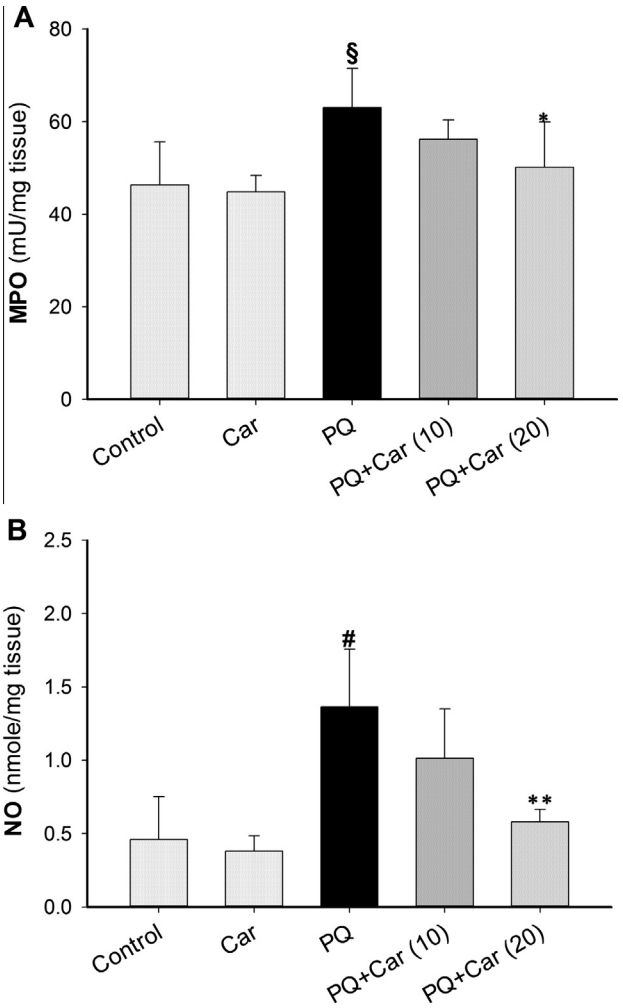

Fig. 5. Effects of carvedilol and paraquat on the levels of (A) MPO and (B) NO in the lung tissue. Treatment with carvedilol (20 mg/kg/day) remarkably reduced the levels of MPO and NO in the lung tissue compared with the PQ group. Data are means ± SD. $^{\S}P < 0.01$, $^{\#}P < 0.001$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.001$ compared with PQ group.

(20 mg/kg/day) significantly decreased the levels of NF-κB mRNA in the lungs of mice exposed to paraquat ($P < 0.01$).

3.9. Effects of carvedilol and paraquat on the hydroxyproline content

Lung fibrosis was assessed by the collagen deposition in the lung tissue. Hydroxyproline is the main component of collagen protein and we measured the hydroxyproline content as a marker of fibrosis in the lung tissue (Fig. 8). The lung hydroxyproline content was significantly increased in mice treated with paraquat compared to normal control mice. However treatment with carvedilol at both doses (10 and 20 mg/kg/day) significantly reduced the lung hydroxyproline content ($p = 0.025$ and $P < 0.001$).

3.10. Effects of carvedilol and paraquat on the levels of MMP-9

We measured total plasma levels of MMP-9 in order to evaluate lung inflammation and fibrosis induced by paraquat. As shown in Fig. 9, Paraquat exposure significantly increased the plasma levels of MMP-9 compared to control group ($P < 0.001$). The levels of MMP-9 were significantly reduced at both doses of carvedilol (10 and 20 mg/kg/day, $P < 0.001$).

4. Discussion

Lung injury is the most serious and fatal complication of paraquat poisoning. The present study revealed that carvedilol treatment ameliorated the severity of paraquat-induced lung injury. To study the possible mechanisms responsible for the protective effect of carvedilol on paraquat-induced lung injury, we determined the oxidative and inflammatory biomarkers in lung tissue.

Previous studies showed that oxidative stress plays an important role in the development and progression of paraquat-induced lung damage. Paraquat is reduced intracellularly by an NADPH-dependent reaction to paraquat radical and oxidized back to paraquat cation in the presence of oxygen. These reactions lead to production of the highly toxic molecules such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl free radical ($HO^{\cdot}$) and peroxynitrite ($ONOO^-$) in the mitochondria and cytosol of cells. Oxygen radicals are highly reactive to cellular macromolecules and cause direct oxidative damage to proteins, membrane lipids and nucleic acids, which may lead to cell death [11,12]. Free radicals can abstract hydrogen atoms from polyunsaturated fatty acids in cell membranes and lipoproteins, causing lipid peroxidation. Oxidation of lipids is considered as an important step in the mechanism of paraquat toxicity in mammalian cells [1]. Reaction of oxygen radicals with unsaturated lipids produces secondary oxidation products such as 4-hydroxynonenal and malondialdehyde (MDA) which has been commonly used as a convenient biomarker of lipid peroxidation process [13]. Intracellular paraquat redox cycling can cause oxidation of amino acids in proteins and lead to the formation of protein carbonyl derivatives. Increased protein carbonylation, as an important biomarker of oxidative stress, has been reported in paraquat induced cytotoxicity [14]. In agreement with other studies, our results showed that paraquat significantly increased MDA level as a marker of lipid peroxidation and carbonylation of proteins in the lung tissue. Treatment with carvedilol significantly prevented lipid peroxidation and protein carbonylation process induced by paraquat. It has been shown that carvedilol is extremely lipophilic and concentrates in lipid membranes and able to protect cell membranes from lipid peroxidation [5]. It has also been demonstrated that administration of carvedilol decreased protein carbonylation as a marker of cellular protein injury [15]. The ability of carvedilol to prevent oxidation of cellular lipids and proteins indicates that carvedilol treatment improves the antioxidant status in the lung tissue of mice exposed to paraquat.

In the present study the plasma levels of LDH as a marker of cell membrane damage were measured. Plasma levels of this intracellular enzyme may also be used to indicate the extent of cell membrane oxidative damage induced by paraquat. Administration of paraquat increased the levels of LDH however treatment with carvedilol reduced the elevation of plasma LDH. This finding is in correlation with histopathological findings and may indicate the protective effect of carvedilol against paraquat induced lung tissue damage.

The cells have endogenous antioxidant defense systems which protect cells against oxygen free radical damage. Reduced glutathione (GSH) as thiol-containing peptide is the major endogenous antioxidant produced by the cells and plays an important role in intracellular protection against reactive oxygen species. During oxidative stress, GSH is converted to its oxidized form, glutathione disulfide (GSSG). GSH is regenerated from GSSG by the enzyme glutathione reductase, using NADPH as an electron donor. It is also involved in a variety of cellular functions such as DNA repair mechanisms and nonenzymatic conjugation with some chemicals to detoxify them [12,16]. The main antioxidant enzymes involved in the elimination of reactive oxygen species include

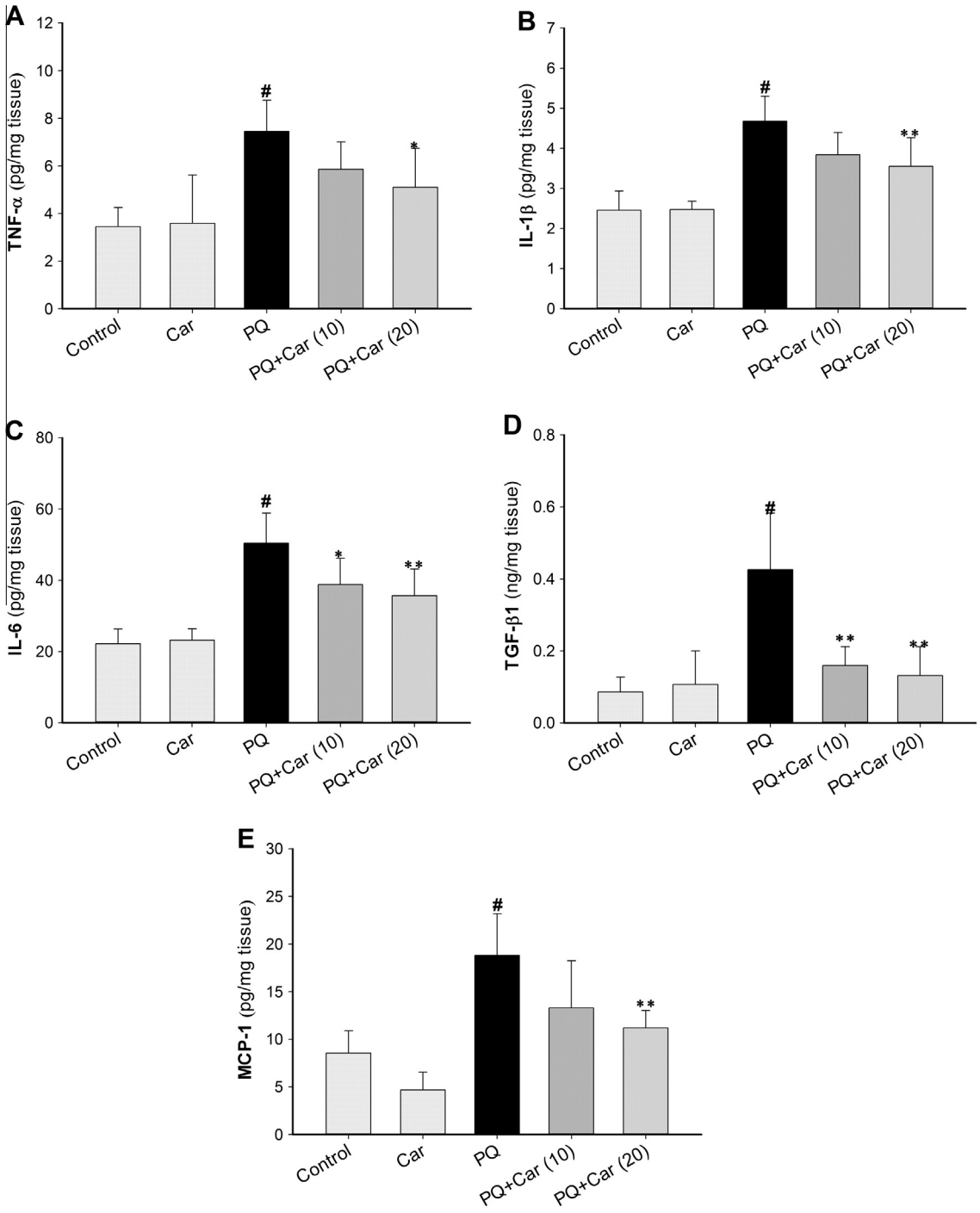

Fig. 6. Effects of carvedilol and paraquat on (A) TNF-α, (B) IL-1β, (C) IL-6, (D) TGF-β1 and (E) MCP-1 production in lung tissue of mice. Administration of paraquat increased the levels of TNF-α, IL-1β, IL-6, TGF-β1 and MCP-1 in the lung tissue, whereas treatment with carvedilol (20 mg/kg/day) prevented the paraquat-induced increase in TNF-α, IL-1β, IL-6, TGF-β1 and MCP-1. Data are means ± SD. $^{\#}P < 0.001$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with PQ group.

superoxide dismutase (SOD), catalase and glutathione reductase. SOD is a metalloenzyme, which catalyzes the dismutation of superoxide anions into hydrogen peroxide and molecular oxygen. Hydrogen peroxide is a highly reactive oxidizing agent and can be broken down into water and oxygen by the enzyme catalase [17]. In the present study we showed that paraquat-induced lung injury is associated with a significant decrease in SOD, catalase and glutathione reductase enzymes and GSH content of the lung tissue. Depletion of GSH in mice treated with paraquat can be caused by GSH consumption due to the interaction of GSH with reactive oxygen species and toxic products. Reduction of GSH results in accumulation of toxic radicals and enhancement of lipid peroxidation in the lung tissue. We also suggest that, the reduction in SOD, catalase and glutathione reductase enzymes activity can be attributed to the enzyme consumption because of neutralization of excessive superoxide anions and hydrogen peroxide in the lung tissue. It has been reported that pre-treatment with glutathione reductase has a protective effect against harmful paraquat poisoning, probably due to glutathione recycling [16]. Carvedilol treatment inhibited the depletion of GSH and restored SOD, catalase and glutathione reductase activity in the lung tissue of mice exposed to paraquat. The ability to increase the levels of

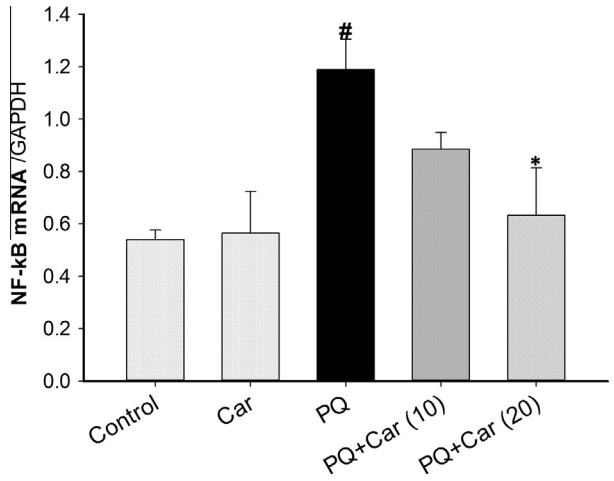

Fig. 7. Effect of carvedilol treatment on paraquat-induced changes in NF-κB mRNA expression in lung tissue. The mRNA levels were normalized by the expression of GAPDH. Data are means ± SD of three experiments. $^{\#}P < 0.01$ compared with control group; $^{*}P < 0.01$ compared with PQ group.

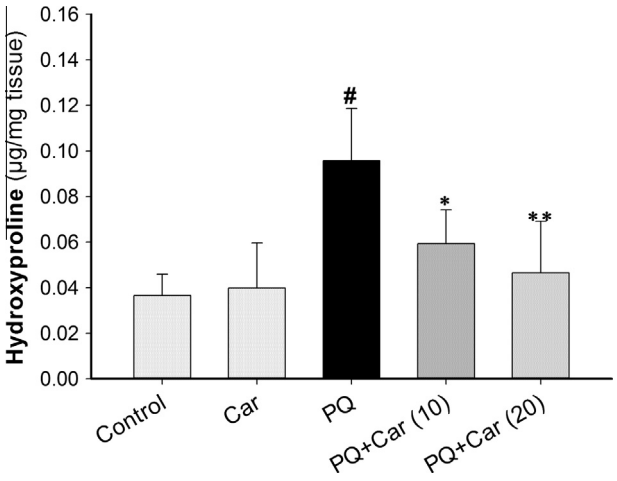

Fig. 8. The lung hydroxyproline content in each group. Paraquat administration increased hydroxyproline levels and treatment with carvedilol inhibited the increase of lung hydroxyproline content. Data are means ± SD. $^{\#}P < 0.001$ compared with control group; $^{*}P < 0.05$, $^{**}P < 0.001$ compared with PQ group.

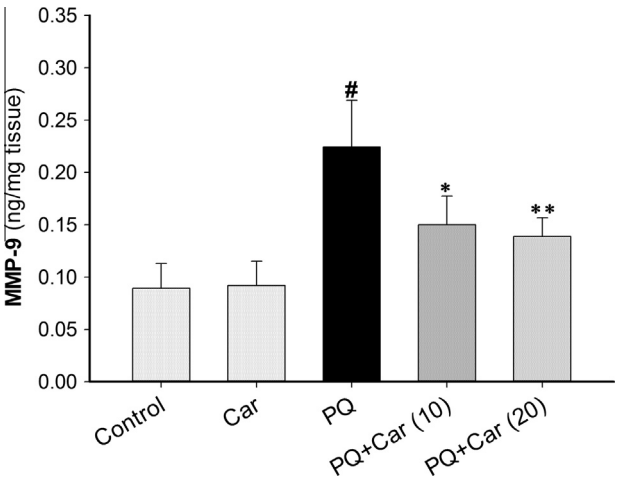

Fig. 9. Effects of carvedilol treatment on paraquat-induced changes in the plasma levels of MMP-9. Treatment with carvedilol inhibited the paraquat-induced increase in MMP-9 levels. Data are means ± SD. $^{\#}P < 0.001$ compared with control group; $^{*}P < 0.01$, $^{**}P < 0.001$ compared with PQ group.

antioxidant enzymes and GSH confirms the antioxidant activity of carvedilol and may be one of the mechanisms of action of carvedilol in paraquat-induced lung injury. Therefore, in the present study we showed that carvedilol treatment altered the oxidant-antioxidant balance in favor of antioxidants and improved lung damage induced by paraquat.

Nitric oxide (NO) has been suggested as another important mediator of paraquat toxicity, even though its role in the pathogenesis of paraquat induced lung injury has been controversial. It has been shown that paraquat may induce nitric oxide synthase (NOS), the enzyme producing NO from L-arginine, through various mechanisms, such as activation of nuclear factor kappa B (NF-κB), proinflammatory cytokines and reactive molecules. NO as a free radical easily reacts with superoxide anion (O_2^-) derived from the redox cycling of paraquat, to form peroxynitrite ($ONOO^-$). Peroxynitrite is a highly toxic oxidant that causes oxidation of proteins, lipids and nucleic acids leading to tissue damage in a process known as nitrosative stress [18,19]. In the present study, our findings showed that paraquat exposure results in an increase in the levels of NO in the lung tissue. Carvedilol administration significantly decreased the excessive production of NO in the lung tissue of mice treated with paraquat. This finding suggests that carvedilol may prevent paraquat induced lung damage by inhibiting NO-mediated cytotoxicity.

It has been demonstrated that inflammatory response induced by paraquat is involved in the development of pulmonary injury. During inflammation of the lung, activated neutrophils migrate from pulmonary vasculature into the interstitial and alveolar spaces, where they release proinflammatory mediators and reactive oxygen radicals [20]. In the present study we showed that the activity of myeloperoxidase (MPO) as a biochemical marker of neutrophil infiltration was significantly increased in the lung tissue of mice treated with paraquat. MPO is a peroxidase enzyme found in the azurophilic granules of neutrophils and is released following neutrophil activation by inflammatory stimuli. MPO catalyzes the formation of hypochlorous acid (HOCl), a potent oxidative agent, from chloride and hydrogen peroxide and contributes to tissue damage during oxidative stress and inflammation. Administration of carvedilol caused a significant reduction of the inflammatory cell infiltration and MPO activity in the lung tissue of mice receiving paraquat. In agreement with this finding, it has been reported that carvedilol is able to reduce MPO activity in inflammatory disorders [7,21]. The protective effect of carvedilol against paraquat-induced pulmonary injury may be partially described by the ability of this drug to reduce neutrophil infiltration into the lungs. Consistent with these findings, our histological analysis showed that paraquat induced a significant infiltration of inflammatory cells, thickening of the alveolar septum and hemorrhage, while treatment with carvedilol reduced the histological changes and pathologic score in the lung tissues.

Previous studies showed that several cytokines, chemokines, and fibrogenic mediators play an important role in the molecular mechanisms of pulmonary inflammation and fibrosis induced by paraquat. Inflammatory cells secrete a variety of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, TGF-β1 and chemokine MCP-1, which intensify inflammatory reactions and lead to lung injury [2,22]. TNF-α is one of the first cytokines produced by alveolar macrophages and has the ability to promote alveolar epithelial dysfunction in paraquat induced lung injury. TNF-α triggers the production of other inflammatory cytokines and chemokines and induces infiltration of neutrophils which exacerbate lung tissue damage. It has also been shown that TNF-α can trigger multiple signaling pathways involved in chronic inflammation and plays a major role in the development of fibrosis in various organs such as liver, kidney and lung [23,24]. IL-1β and IL-6 are potent and pleiotropic cytokines thought to be involved in lung inflammation

induced by paraquat. IL-6 has been shown to be increased in paraquat-induced lung injury and promotes fibrogenesis either alone or in combination with TNF-α [24]. It has been shown that IL-1β is a potent inducer of TGF-β, and at least a part of the profibrotic effects of IL-1β is mediated by this growth factor [25]. Transforming growth factor (TGF)-β1 is secreted by numerous cell types including fibroblasts and macrophages. It is an important regulator of cell growth and differentiation and identified as the most important pro-fibrotic cytokine. TGF-β1 has been implicated as a mediator of chronic inflammation and fibrosis in many tissues including the lung. TGF-β1 stimulates the proliferation and differentiation of alveolar epithelial cells and fibroblasts as well as production of matrix metalloproteinases and collagen [26,27]. The monocyte chemoattractant protein (MCP)-1 is a member of the C-C chemokine family and regulates migration and infiltration of monocytes/macrophages. MCP-1 is produced by several cells such as endothelial cells, alveolar epithelial cells and macrophages. This proinflammatory protein is implicated in the pathogeneses of various inflammatory diseases. It has been reported that MCP-1 plays an important role in the development of lung inflammation and fibrosis [28,29]. In agreement with previous studies, we also found that the administration of paraquat caused a significant increase in the levels of proinflammatory cytokines TNF-α, IL-1β, IL-6, TGF-β1 and chemokine MCP-1 in the lung tissue. As mentioned above, these mediators have an important role in the progression of paraquat induced lung inflammation and fibrosis. Treatment with carvedilol significantly decreased the levels of these proinflammatory mediators and eventually attenuated lung damage. It should be noted that the anti-inflammatory effects of carvedilol have been reported in various experimental models. It has been demonstrated that carvedilol reduced cardiac gene expression and production of TNF-α, IL-6, IL-1β and TGF-β1 in acute myocardial infarction and azithromycin-induced cardiotoxicity [6,30]. It has also been shown that the administration of carvedilol is able to significantly reduce the expression and activity of MCP-1 as well as macrophage infiltration in myocardial tissue [31].

Several studies have demonstrated that free radicals are involved in modulating the activation of NF-κB signaling pathway in paraquat-induced lung injury [32,33]. NF-κB is an important transcription factor that regulates cellular responses to reactive oxygen species and inflammatory mediators. NF-κB induces transcription of proinflammatory and fibrogenic cytokines TNF-α, IL-1β, IL-6, TGF-β1 and chemokine MCP-1 and several other adhesion molecules. Upregulation of redox-sensitive transcription factor NF-κB has also been shown to induce inflammatory target protein MMP-9, which contributes to pulmonary inflammation and fibrosis [34]. It has been shown that paraquat-induced activation of NF-κB may induce the expression of inducible form of NOS, which catalyzes the formation of NO [19,35]. As we mentioned above, NO has been suggested as an important mediator of paraquat toxicity. In the present study, we demonstrated that paraquat exposure induced NF-κB activity and treatment with carvedilol significantly reduced paraquat-induced NF-κB activation in the lung tissue. Indeed, carvedilol shows an inhibitory effect on the activation of redox-sensitive transcription factor NF-κB, which may be related to its potent antioxidant properties.

Consistent with previous studies, we showed that lung hydroxyproline content as a marker of fibrosis was significantly elevated in mice treated with paraquat, and treatment with carvedilol reduced the hydroxyproline content in the lung tissue of mice exposed to paraquat. Administration of carvedilol has been reported to inhibit the formation of hydroxyproline in an experimental model of liver injury and myocardial infarction [36,37]. Various studies suggested that the activation of matrix metalloproteinases (MMPs) is involved in pulmonary fibrosis by different signal transduction pathways. MMP-9 is one of the most important pro-fibrotic members of the metalloproteinase family, and it has been suggested that MMP-9 plays an important role in paraquat induced lung inflammation and fibrosis. In the lung tissue, MMP-9 is produced by inflammatory and airway epithelial cells under the influence of proinflammatory cytokines such as TNF-α, IL-1β and TGF-β, and other toxic molecules such as reactive oxygen species [34,38,39]. In the present study, the plasma levels of MMP-9, as a marker of pulmonary inflammation and fibrosis, were increased in mice treated with paraquat, while treatment with carvedilol significantly inhibited the increase of MMP-9 levels. This observation is supported by previous studies demonstrating that carvedilol reduces the expression and activity of MMP-9 in patients with heart failure and experimental models of atherosclerosis and periodontitis [7,40,41].

In conclusion, the current study is the first to suggest that carvedilol may attenuate the paraquat-induced lung injury through antioxidant and anti-inflammatory mechanisms. Further studies are needed to determine whether carvedilol could be used to protect against paraquat-induced lung toxicity in humans.

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