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Anti-inflammatory effect of thalidomide in paraquat-induced pulmonary injury in mice

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ABSTRACT

Thalidomide has been used in inflammatory and autoimmune disorders due to its anti-inflammatory activity. Paraquat (PQ) poisoning causes severe lung injury. PQ-induced pulmonary inflammation and fibrosis are due to its ability to induce oxidative stress, inflammatory and fibrotic reactions. This study was designed to evaluate the anti-inflammatory and anti-fibrotic effect of thalidomide on PQ-induced lung damage in a mouse model. Mice were injected with a single dose of PQ (20 mg/kg, i.p.), and treated with thalidomide (25 and 50 mg/kg/day, i.p.) for six days. Lung tissues were dissected six days after PQ injection. The results showed that thalidomide ameliorated the biochemical and histological lung alterations induced by PQ. Thalidomide decreased production of inflammatory and fibrogenic cytokine tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and transforming growth factor (TGF)-β1. In addition thalidomide reduced myeloperoxidase (MPO), nitric oxide (NO), and hydroxyproline content in lung tissue. Taken together, the results of this study suggest that thalidomide might be a valuable therapeutic drug in preventing the progression of PQ-induced pulmonary injury.

1. Introduction

Paraquat (1,1′-dimethyl-4,4′-bipyridinium chloride) is a widely used herbicide that can cause severe lung injury in humans. Exposure to PQ leads to accumulation of PQ in the lungs resulting in pulmonary edema, alveolar destruction, proliferation of bronchial epithelial cells and eventually fibrosis. Pulmonary fibrosis is a major hallmark and a leading cause of death in PQ poisoning [1,2].

Paraquat redox cycling and subsequent generation of reactive oxygen species (ROS), hydroxyl free radical ($HO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) is the primary mechanism for initiating lung damage by PQ. These mediators induce intracellular transcription factors and then many proinflammatory agents including inducible nitric oxide synthase (iNOS), inflammatory cytokines, and cyclooxygenase (COX) all of which exaggerate the inflammatory process [3,4]. From the molecular aspects, nitric oxide (NO) and proinflammatory cytokines, particularly TNF-α, IL-6, IL-1β, and TGF-β1 may be the core of the pathogenesis of PQ-induced lung injury and fibrosis [4–6]. Although there is no effective therapy for PQ poisoning, anti-inflammatory drugs have been used in the clinical treatment of PQ-poisoned patients [7,8]. Generally, corticosteroids and immunosuppressive drugs are the mainstay of treatment for PQ-induced lung injury.

As mentioned above, it may be hypothesized that an effective treatment against PQ-induced lung injury and fibrosis should have considerable anti-inflammatory and anti-fibrotic effects. It has been reported that thalidomide as an anti-inflammatory agent is effective in the prevention of pulmonary inflammation and fibrosis in the experimental models [9–11]. Thalidomide (α-N-phthalimido glutarimide) is a glutamic acid derivative that was initially introduced as a sedative drug but was withdrawn from the market for its teratogenic effects. Thalidomide has various pharmacological properties, including immunomodulation, anti-inflammation and anti-angiogenesis. Clinical and experimental studies have demonstrated the efficacy of thalidomide or its analogs in the treatment of a variety of disorders including erythema nodosum leprosum, multiple myeloma, rheumatoid arthritis, Crohn's disease, prostate cancer and lupus erythematosus [12]. It has also been shown that thalidomide exhibits anti-inflammatory and anti-fibrotic activity by suppressing the production of proinflammatory cytokines, growth factors and nitric oxide which play an important role in the tissue destruction and fibrosis in chronic inflammatory situations [11,13–15].

In the present study, we examined whether thalidomide had protective effects on PQ-induced lung injury in mice. Furthermore, we investigated the mechanism underlying the therapeutic effect of thalidomide on pulmonary inflammation and fibrosis.

2. Material and methods

2.1. Animal and chemicals

Male Swiss albino mice, weighing 25–30 g, were housed in a room with a 12-h light/dark cycle. Animals were allowed free access to tap water and ad libitum food. All animal procedures were performed in

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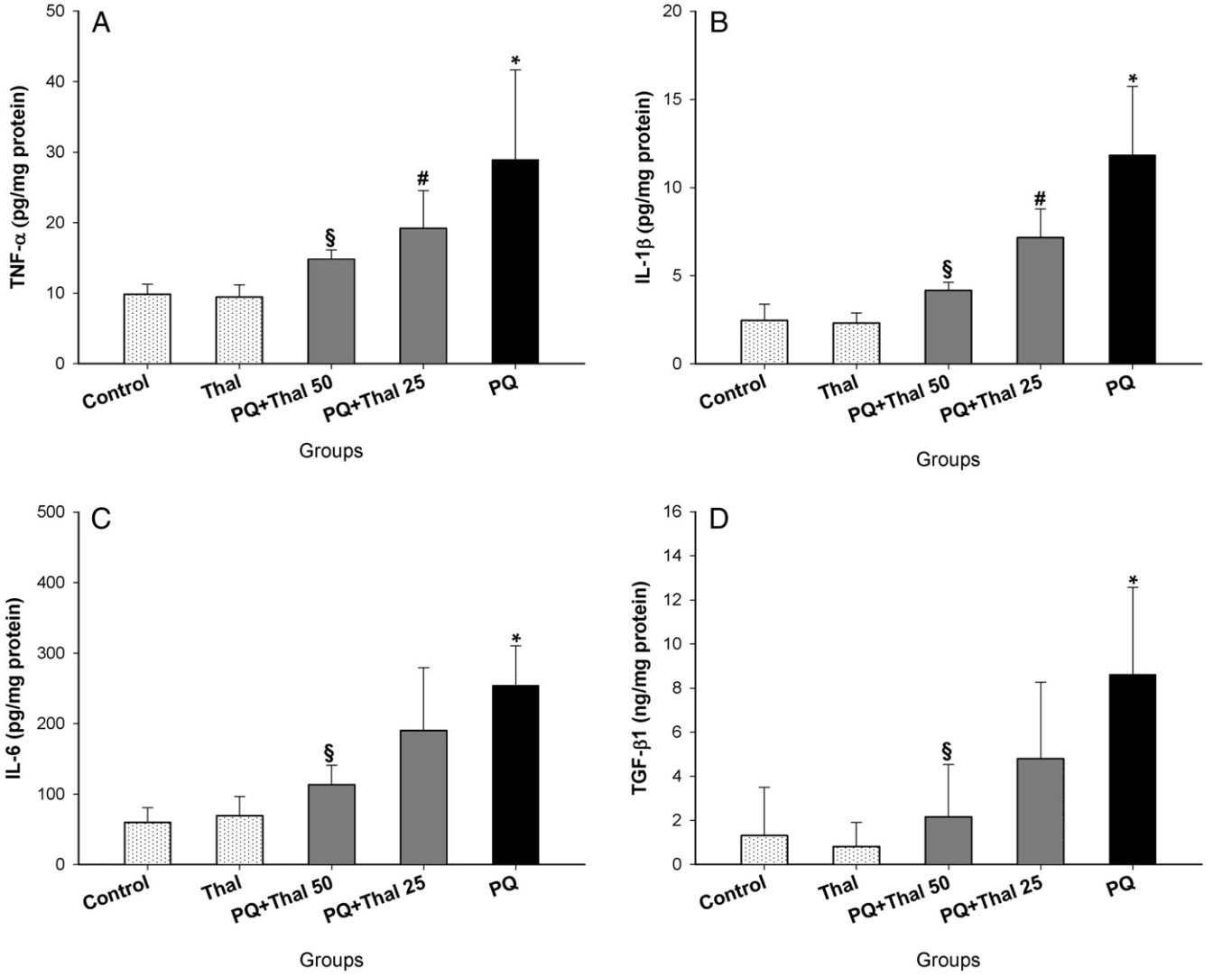

Fig. 1. Effect of thalidomide on PQ-induced production of TNF-α (A), IL-1β (B), IL-6 (C) and TGF-β1(D) in mouse lung tissues. Thalidomide treatment (50 mg/kg/day, i.p.) significantly reduced PQ-induced production of proinflammatory cytokines TNF-α, IL-1β, IL-6 and TGF-β1. Data are means ± SD (two replicates in each assay), n = 8. *$P < 0.001$ compared with normal group; §$P < 0.001$, #$P < 0.05$ compared with PQ group.

compliance with the "Guide for the Care and Use of Laboratory Animals" (National Academies Press, Washington, DC, USA, 1996). Thalidomide, tetramethylbenzidine, Tris–HCl buffer, dimethyl sulfoxide (DMSO), hydrogen peroxide (H_2O_2), hydroxyproline, chloramine T and p-dimethylaminobenzaldehyde were purchased from Sigma-Aldrich. Paraquat was purchased from Afrashimi Co. (Iran).

2.2. Experimental design

The animals were randomly divided into five groups (8 mice in each group), as follows: (1) Normal control group, mice received saline solution. (2) Thal group, mice received Thal (50 mg/kg/day, ip). (3) PQ group, mice received paraquat (20 mg/kg, i.p.). (4) PQ + Thal 25 group, mice received paraquat (20 mg/kg, i.p.) and thalidomide (25 mg/kg/day, i.p.). (5) PQ + Thal 50 group, mice received paraquat (20 mg/kg, i.p.) and thalidomide (50 mg/kg/day, i.p.). Paraquat was dissolved in saline solution (NaCl 0.9%) and injected intraperitoneally in a single toxic dose of 20 mg/kg of body weight. The dosage of PQ was based on our preliminary experiments showing induction of lung injury with lowest mortality. Thalidomide was dissolved in 0.5% carboxymethylcellulose and administered intraperitoneally for six consecutive days.

2.3. Tissue collection

At the end of the experiment, mice were anesthetized with ketamine and xylazine and their thoracic cavities were opened. One part of the right lung was fixed in formalin for histological examination, and the remaining lung tissues were immediately removed and washed in normal saline solution and frozen in liquid nitrogen.

2.4. Histological examination

Lung samples were fixed with 10% formalin embedded in paraffin and sections were stained with hematoxylin and eosin (H&E). The severity of lung damage was scored using the criteria as follows. Score 0 = no injury. Score 1 = alveolar inflammation without thickening of the alveolar septum. Score 2 = extensive alveolar inflammation and thickening of the alveolar septum. Score 3 = destruction of the alveolar spaces or extensive thickening of the airway/vessel wall [16].

2.5. Measurement of TNF-α, IL-1β, IL-6, and TGF-β1levels

The samples were homogenized in Tris–HCl buffer (pH = 7.4) containing protease inhibitors (trypsin and other serine and cysteine

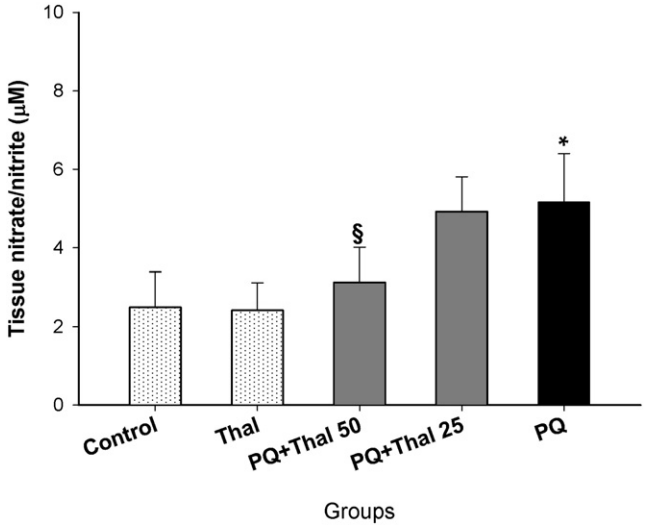

Fig. 2. Effect of thalidomide on PQ-induced production of nitrate/nitrite in mouse lung tissues. Thalidomide treatment (50 mg/kg/day, i.p.) decreased the effect of PQ on the levels of nitrate/nitrite as a marker of NO production. Data are means ± SD (two replicates in each assay), n = 8. *$P < 0.001$ compared with normal group; §$P = 0.001$ compared with PQ group.

proteases). All homogenized samples were centrifuged (20,000 ×*g*, 4 °C) in a refrigerated centrifuge for 20 min and the supernatant was taken and frozen at −80 °C. Supernatant samples were thawed and analyzed for murine cytokine TNF-α, IL-1β, IL-6, and TGF-β1levels using mouse-specific ELISA kits (eBioscience). Cytokine concentrations in the samples were expressed as pg cytokine/mg of protein [13].

2.6. Determination of tissue NO levels

An aliquot of the supernatant was used to determine NO levels. Tissue levels of nitrate and nitrite as a marker of total NO concentration were measured by a nitrate/nitrite colorimetric assay kit (Cayman Chemical Company).

2.7. Determination of myeloperoxidase (MPO) activity

MPO activity of lung tissues was determined by the method described by Dinis-Oliveira et al. [7]. Briefly, MPO content was measured by mixing 50 μl of supernatant with 50 μl of tetramethylbenzidine dissolved in dimethyl sulfoxide. MPO activity was started by addition of 50 μl of hydrogen peroxide (H_2O_2) dissolved in phosphate buffer (50 mM, pH 5.4). The rate of change in absorbance was measured spectrophotometrically at 650 nm using a microplate reader. MPO activity was defined as the quantity of enzyme which decomposes 1 μl of H_2O_2 per min at 25 °C. The results were expressed as mU MPO/mg of protein.

2.8. Hydroxyproline (HP) assay

The HP content of the lung tissue was determined by the method described by Murrell et al. [17]. Briefly, 100 mg samples were hydrolyzed in 1 mL of 6 N HCl for 12 h at 120 °C. All samples were kept at 60 °C until the HCl was completely evaporated. After reconstitution in 1 mL H_2O, the pH adjusted to 6–7 with NaOH. One hundred microliters of the samples were added to a 96-well plate. Hydroxyproline oxidation was initiated by addition of 50 μl of chloramine T (0.05 M). After shaking, the plate was incubated for 20 min at room temperature. To develop the color 50 μl of p-dimethylaminobenzaldehyde solution (1 M) was added to each well and the plate was incubated at 60 °C for 20 min. After cooling, the absorbance was read at 550 nm using a spectrophotometer. The HP concentrations were calculated using a standard curve of HP concentrations between 10 and 100 μg/ml.

2.9. Statistical analysis

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

3. Results

3.1. Effect of thalidomide on the levels of TNF-α, IL-1β, IL-6, and TGF-β1 in mouse lung tissues

To evaluate the effect of thalidomide on PQ-induced lung injury, we measured the levels of important proinflammatory and profibrotic cytokines TNF-α, IL-1β, IL-6 and TGF-β1 in lung tissues (Fig. 1). Thalidomide was applied in two doses of 25 and 50 mg/kg. Administration of PQ caused significant elevation of tissue levels of TNF-α, IL-1β, IL-6 and TGF-β1 as compared with those in the control group. Treatment of mice with thalidomide significantly reduced PQ-induced production of TNF-α, IL-1β, IL-6 and TGF-β1 compared to the PQ group in a dose-dependent manner. Although there was a significant reduction in the levels of TNF-α and IL-1β in the group treated with the lower dose of thalidomide (25 mg/kg) (Fig. 1A and B), the same effect was not found in the levels of IL-6 and TGF-β1 (Fig. 1C and D). These results suggest that thalidomide at a dose of 50 mg/kg may be more effective on PQ-induced inflammatory cytokine production.

3.2. Effects of thalidomide and PQ on tissue NO levels

We measured the lung tissue levels of nitrate/nitrite as an indicator of NO generation. As shown in Fig. 2, lung tissue levels of nitrate/nitrite were increased in PQ-treated mice ($P < 0.001$). Treatment of mice with thalidomide (50 mg/kg) significantly decreased PQ-induced production of nitrate/nitrite ($P = 0.001$).

3.3. Effects of thalidomide and PQ on MPO activity

We measured MPO activity as a sensitive marker of neutrophil infiltration or inflammation in lung tissue. Administration of PQ significantly increased level of MPO, a marker of neutrophil infiltration into the inflamed lung tissue, in comparison to control group ($P < 0.001$) (Fig. 3). Treatment with thalidomide (50 mg/kg) significantly reduced elevated MPO activity compared to that in the PQ group ($P = 0.019$).

3.4. Histological studies

Histological examination of lung tissues from each study group was performed to evaluate the severity of PQ-induced lung damage. PQ administration induced marked interstitial edema, widespread inflammatory cell infiltration in the alveolar space and septum, diffuse alveoli collapse and thickening of the alveolar septum (Fig. 4A). Histopathological changes in mice treated with PQ plus thalidomide (50 mg/kg) were significantly lower than those in the PQ group. Furthermore, as shown in Fig. 4B, in PQ-treated mice, pathological score was significantly higher than that of the control group. Treatment with thalidomide significantly decreased the pathological score compared with the PQ group ($P < 0.05$), indicating that thalidomide ameliorated PQ-induced lung injury.

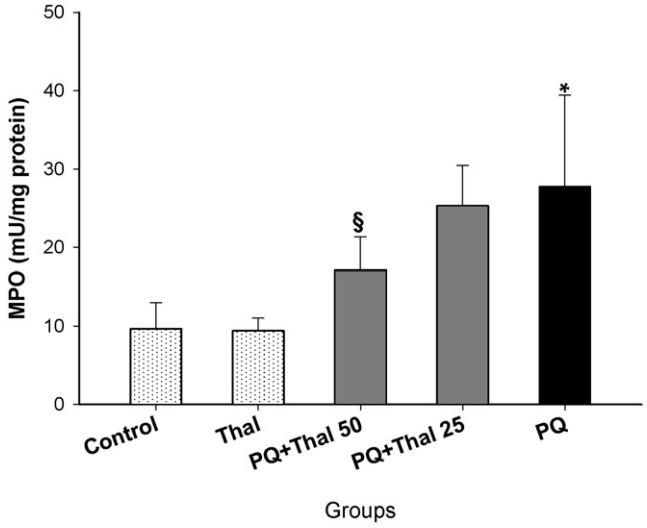

Fig. 3. Administration of thalidomide (50 mg/kg/day, i.p.) decreased MPO activity induced by PQ (20 mg/kg, i.p.) in lung tissues. Data are means ± SD (two replicates in each assay), n = 8. *$P < 0.001$ compared with normal group; §$P < 0.05$ compared with PQ group.

3.5. Effects of thalidomide and PQ on hydroxyproline content of lung tissue

Hydroxyproline is the main component of collagen protein in the body. We measured the hydroxyproline content as a marker of fibrosis in the lung tissue. As shown in Fig. 5, the hydroxyproline content in lung of the PQ-treated mice significantly increased compared with the control group. Administration of thalidomide (50 mg/kg) significantly reduced the content of hydroxyproline in lung tissue ($P = 0.034$). These findings were consistent with the histological results.

4. Discussion

The aim of the present study was to assess the effect of thalidomide on paraquat-induced pulmonary injury in mice. PQ, a quaternary ammonium herbicide, is a very toxic substance for humans and animals. Intoxication with PQ causes severe lung damage in the chronic stages in humans. Although the mechanism underlying the development of lung injury by PQ is not obvious, previous studies showed that oxidative and inflammatory mediators induced by PQ could result in tissue inflammation and injury. Respiratory distress arises as a result of alveolar epithelial cell disruption, hemorrhage, infiltration of inflammatory cells into the interstitial and alveolar spaces, edema, fibroblast proliferation, collagen deposition and progressive fibrosis [4]. Several cytokines, chemokines, and growth factors have been identified to be involved in the molecular pathogenesis of PQ poisoning. It has been shown that inflammatory and profibrogenic cytokines TNF-α, IL-1β, IL-6 and TGF-β1 play an important role in PQ-induced pulmonary inflammatory and fibrosis [3,5,18]. PQ-induced reactive oxygen species can activate intracellular transcription factors such as nuclear factor (NF)-κB, thereby enhancing TNF-α and IL-1β expression. TNF-α is one of the early cytokines that are released from alveolar macrophages in the early inflammatory and late fibrotic phases of lung injury. TNF-α activates the proliferation of fibroblasts, expression of cellular matrix metalloproteinases (MMPs) and the release of other proinflammatory cytokines. IL-6 is significantly elevates in PQ-induced lung injury and promotes fibrogenesis either alone or concomitant with TNF-α [3]. Increased production of TGF-β1 from lung injury caused by PQ has been associated with inflammation and fibrosis of lung tissue [5,19]. TGF-β1 as an important growth factor promotes tissue fibrosis. In experimental models of pulmonary fibrosis, TGF-β1 has been shown to be an important fibrogenic mediator which stimulates fibroblast proliferation, matrix protein production and collagen synthesis [20]. Moreover, it is known to induce IL-6 production [10]. PQ exposure induces the expression of nitric oxide synthase (NOS) through generation of reactive oxygen species. Although, the role of NO in the pathogenesis of PQ toxicity has been controversial, this finding suggests that NO has a critical role in PQ-induced cytotoxicity [4,6,21]. It has also been reported that NOS inhibitors have protective effects against PQ-induced lung injury [22].

Thalidomide and its analogs are immunomodulatory drugs that exhibit anti-inflammatory, anti-proliferative and anti-angiogenic activities. These effects are responsible for the clinical effectiveness of thalidomide in erythema nodosum leprosum, lupus erythematosus, graft versus host disease, multiple myeloma, rheumatoid arthritis and Crohn's disease [12]. In addition there are several studies that showed thalidomide has therapeutic effects in various fibrotic and inflammatory disorders such as bleomycin-induced lung fibrosis [11], peritonitis [14], pancreatitis [23] and experimental diabetes [13].

Pulmonary fibrosis is a serious consequence of PQ poisoning. We suggested that thalidomide may be an appropriate candidate to inhibit PQ-induced pulmonary inflammation and fibrosis. Most recently, the effect of thalidomide in mouse model of PQ toxicity has been investigated by a Chinese group [24]. Our study has been done independently of the Chinese study. In our study, thalidomide treatment reduced PQ-induced lung injury in mice. We showed that PQ-induced proinflammatory cytokines, including TNF-α, IL-1β and IL-6 in the lungs of mice were reduced by thalidomide treatment in a dose-dependent manner. As mentioned above, these mediators have a critical role in the progression of lung inflammation and fibrosis by PQ. Furthermore, we found that chronic thalidomide treatment had the ability to reduce the levels of growth factor TGF-β1 in the lungs of mice. Indeed, blocking the production of the proinflammatory mediators by thalidomide decreases pulmonary inflammation and suppresses specific pathways that lead to alveolar epithelial cell disruption and lung fibrosis.

Our previous study showed that thalidomide has the ability to suppress production of NO during inflammatory processes [13]. It is reported that thalidomide exhibits NOS-inhibitory activity [14,25]. In our study pulmonary levels of NO in the PQ group were significantly higher than in other groups. Thalidomide administration significantly decreased the elevation of NO production in the PQ-treated group. This reduction in the formation of NO by thalidomide may contribute to the inflammation, lipid peroxidation and fibrosis in the lung tissues in PQ-treated animals.

In our study we found that the activity of myeloperoxidase (MPO) was significantly increased in lung tissue from mice that had been treated with PQ. MPO is a peroxidase enzyme that is synthesized and secreted by neutrophils and monocytes. MPO catalyzes the formation of hypochlorous acid and other reactive molecular species and contributes to tissue damage during oxidative stress and inflammation [26]. Increased MPO activity represents the extensive infiltration of macrophages and neutrophils in the lungs of PQ exposed mice. Our histological results confirmed the infiltration of polymorphonuclear leukocytes (PMN) into the lungs. Thalidomide administration caused a significant reduction of the interstitial inflammatory cell infiltration and MPO activity in the lung of mice exposed to PQ. In agreement with our study it has been reported that thalidomide was able to reduce MPO activity in various inflammatory conditions [23,27]. This finding indicates that thalidomide reduces lipid peroxidation and improves PQ-induced pulmonary inflammation.

Consistent with previous observations, our study showed that lung hydroxyproline levels increased in PQ-treated mice, and treatment with thalidomide reduced the hydroxyproline content. We found that PQ produced an increase in pathologic score that was decreased by thalidomide. Also, our histological analysis showed

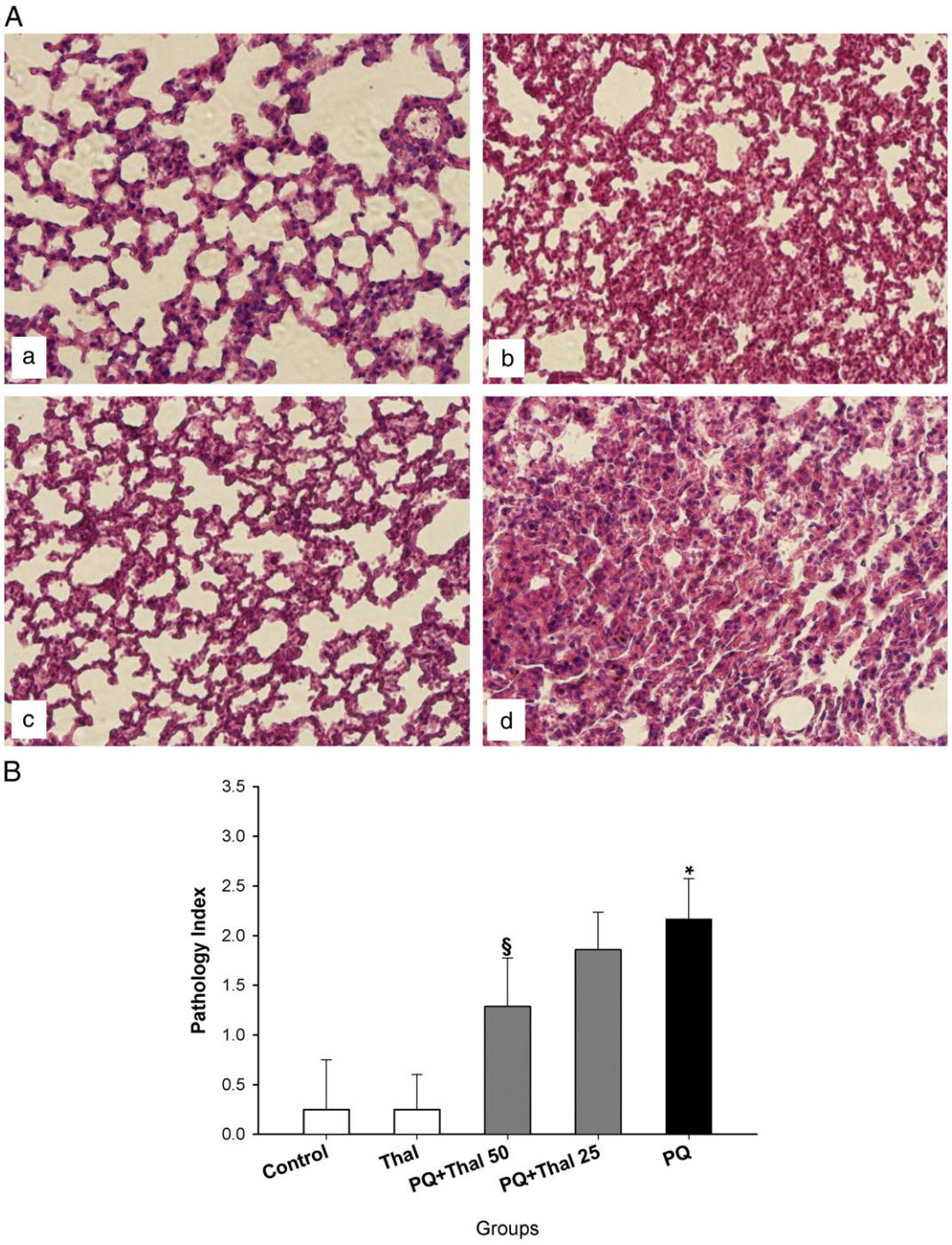

Fig. 4. (A) Histopathological appearance of the lung sections in (a) control, (b) PQ + thalidomide (25 mg/kg/day), (c) PQ + thalidomide (50 mg/kg/day), and (d) PQ groups. Lung injury was induced by a single dose (20 mg/kg, i.p.) of paraquat and mice were treated with thalidomide (25 and 50 mg/kg/day, i.p.). Widespread thickening of alveolar septum, infiltration of inflammatory cells, and cellular proliferation of connective tissues were observed in PQ group. Treatment with thalidomide reduced the histological alterations induced by PQ intoxication. The lung sections were analyzed by hematoxylin and eosin (H&E) staining (magnification is 400×). (B) Pathology score of lung was significantly lower in the PQ + thalidomide (50 mg/kg/day) group than in the PQ group. $^{*}P < 0.001$ compared with normal group; §$P < 0.05$ compared with PQ group.

that PQ-induced lung injury was characterized by a marked thickening of the alveolar septum and infiltration of various inflammatory cells. Histological changes were attenuated by administration of thalidomide in a dose dependent manner. This finding suggests that the beneficial effect of thalidomide on PQ-induced lung inflammation and fibrosis may be associated in part by the inhibition of neutrophil migration into the lungs. The PQ-induced lung damage begins at an early stage of PQ poisoning and the treatment with thalidomide should be applied at an early time point of exposure to PQ.

In conclusion, according to the data presented in this study it seems that thalidomide treatment has a protective effect against PQ-induced pulmonary injury in mice through a number of distinct mechanisms. Nevertheless, further studies are needed to support these findings.

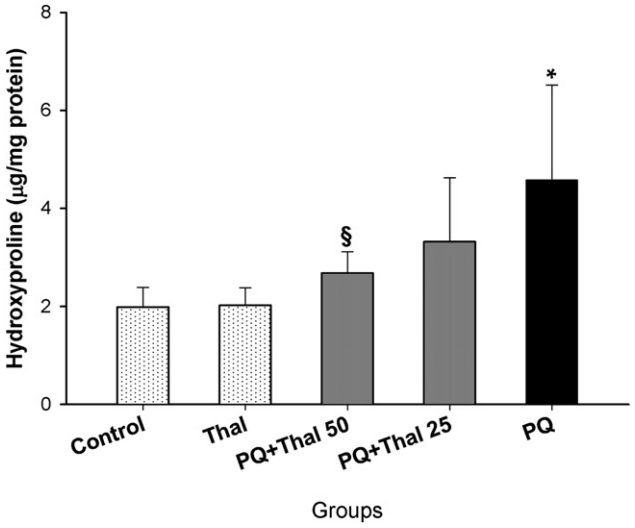

Fig. 5. Hydroxyproline content of lung tissue in experimental groups. PQ exposure increased hydroxyproline levels and treatment with thalidomide (50 mg/kg/day) inhibited the increase of lung hydroxyproline content. Data are means ± SD (two replicates in each assay), n = 8. $^{*}P = 0.001$ compared with normal group; §$P < 0.05$ compared with PQ group.

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