

ORIGINAL ARTICLE

Activating Peroxisome Proliferator-Activated Receptors (PPARs): a New Sight for Chrysophanol to Treat Paraquat-Induced Lung Injury

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Abstract—The aim of this study is to evaluate the protective effects of chrysophanol (CH) against paraquat (PQ)-induced pulmonary injury. Fifty BALB/C mice were randomized into five groups: (1) control, (2) PQ, (3) PQ + dexamethasone (Dex, 2 mg/kg), (4) PQ + CH (10 mg/kg), and (5) PQ + CH (20 mg/kg). A single dose of PQ (50 mg/kg, i.p.) was intraperitoneally given to induce acute lung injury. Then mice were treated with CH (10 and 20 mg/kg/day, orally) for 7 days. At the end of the experiment, animals were euthanized and then bronchoalveolar lavage fluid (BALF) and lung tissues were collected for histological observation, biochemical analysis, and Western blot analysis. Malondialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD), interleukin-6 (IL-6), IL-1 β , and tumor necrosis factor- α (TNF- α) levels in BALF were determined. The levels of SOD and MDA in the lung were also detected. The peroxisome proliferator-activated receptor (PPAR)- γ and nuclear factor-kappaB (NF- κ B) pathway proteins in the lung were determined by Western blot. Histological examination indicated that CH attenuated lung inflammation caused by PQ. Biochemical results showed that CH treatment significantly reduced the levels of MDA, MPO, and inflammatory cytokines and increased the level of SOD, compared to those in the PQ group. Meanwhile, Western Blot results revealed that CH increased PPAR- γ expression and inhibited NF- κ B pathway activation after PQ challenge. These findings suggested the potential therapeutic effects of CH which is derived from a natural product on PQ-induced pulmonary injury.

KEY WORDS: chrysophanol; paraquat; lung injury; PPAR- γ ; NF- κ B.

INTRODUCTION

As one of the most widely used herbicides, paraquat (PQ, 1-10-dimethyl-40-bipyridylum dichloride), a quaternary nitrogen herbicide, is a highly toxic compound for both animals and humans [1]. PQ contributes to aggressive tissue damage in the lung, kidney, and liver in serious PQ intoxication. It is noteworthy that the major target organ in PQ poisoning is the lung. PQ-induced lung injury is characterized by edema, hemorrhage, inflammatory cell

infiltration, and alveolar spaces [2]. Meanwhile, it is acknowledged that oxidative stress plays a crucial role in the pathogenesis of PQ-stimulated pulmonary injury [3]. On the other hand, there are also sufficient studies reporting that inflammatory cascade was closely associated with the progression of lung disorders [4].

Peroxisome proliferator-activated receptors (PPARs) belonging to ligand-activated transcription factors possess several biological functions such as regulation of lipid metabolism in acute lung injury [5]. When activated by their ligands, PPARs undergo a conformational change, leading to recruitment of distinct coactivators and corepressors. Then, the PPAR complex combining with *cis*-retinoid X receptor (RXR) would be translocated into the nucleus, where it regulates downstream gene transcription by binding to the peroxisome proliferator response element. In

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cells, there are three PPAR isoforms (PPAR- α , PPAR- β/δ , and PPAR- γ) existed [6]. Previous studies have indicated that PPAR- γ agonists are capable of inhibiting LPS-induced inflammatory responses in airway epithelial cells, neutrophilia, and alveolar macrophages by suppressing the release of pro-inflammatory cytokines and nuclear factor- κ B (NF- κ B) activation [7]. Remarkably, NF- κ B, a major nuclear transcription factor, is a regulator which drives the generation of cytokines in inflammatory processes [8, 9]. Chen *et al.* have reported that NF- κ B pathway plays an important role in the development of pulmonary diseases [10]. Therefore, compounds activating PPAR- γ expression or inhibiting NF- κ B pathway presumably have a protective effect against inflammatory diseases such as lung injury.

During the few past decades, various nature products have been used for the intervention of disease [11, 12]. Chrysophanol (CH), a member of the anthraquinone family, was originally extracted from plants of the *Rheum* genus [13]. At present, CH has been shown to have multiple pharmacological effects, such as anti-diabetic [14], anti-depressive [15], and anti-inflammatory [16] activities. However, no available study has evaluated the effects of CH treatment on PQ-induced lung injury in a mouse model as yet. Herein, we sought to investigate whether CH could protect against pulmonary inflammation caused by PQ stimulation and to explore the potential mechanism in mice.

MATERIALS AND METHODS

Reagents

CH was purchased from the National Institutes for Food and Drug Control (Beijing, China). Dexamethasone (Dex) was purchased from Xiansheng Drug Store (Nanjing, China). PQ was provided by Sigma. Tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were supplied by BioLegend (San Diego, CA, USA). The levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were evaluated using commercially available kits subscribed from Jiancheng Institute of Biotechnology (Nanjing, China). All antibodies were purchased from Cell Signaling Technology.

Animals

A total of 50 female BALB/c mice (18–22 g), acquired from the Jiangning Qinglongshan Animal

Cultivation Farm (Nanjing, China), were maintained in an animal facility under standard laboratory conditions for 7 days prior to experiments with water and standard chow *ad libitum*. All experimental procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental Design

The animals were randomly divided into five groups with 10 mice in each group as follows: (1) normal control group; (2) PQ group, mice received PQ (50 mg/kg, i.p.); (3) PQ + Dex group, mice received PQ (50 mg/kg, i.p.) and Dex (2 mg/kg/day); (4) PQ + CH (10 mg/kg) group, mice received PQ (50 mg/kg, i.p.) and CH (10 mg/kg/day); and (5) PQ + CH (20 mg/kg) group, mice received PQ (50 mg/kg, i.p.) and CH (20 mg/kg/day). PQ was dissolved in saline solution (NaCl 0.9 %) and intraperitoneally given to mice at a single toxic dose of 50 mg/kg. CH was also dissolved in saline (NaCl 0.9 %) and administered intragastrically. Two hours after PQ stimulation, CH was intragastrically treated at the dose of 10 and 20 mg/kg/day for 7 days. Dex was intragastrically treated as a positive control.

Bronchoalveolar Lavage

Two days after PQ challenge, the animals were sacrificed and bronchoalveolar lavage was collected three times through a tracheal cannula with 0.5 ml of autoclaved PBS to obtain the bronchoalveolar lavage fluid (BALF). The total leukocyte count was determined with a hemocytometer. BALF samples were centrifuged at 3000 rpm for 10 min at 4 °C, and then the cell-free supernatants were stored in –80 °C for the detection of cytokine concentrations, SOD activity, and MDA content. The pellets were resuspended in 100 μ l of saline, centrifuged onto slides, and stained with Wright–Giemsa staining (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for neutrophil counting.

Lung Wet-to-Dry Weight Ratios

The right lungs were excised at the end of the experiment, and the wet weight was determined. Subsequently, the lungs were placed at 60 °C for 48 h to remove all moisture and then the dry lungs were weighted and the lung wet-to-dry (W/D) ratio was calculated.

Cytokine in BALF

The levels of TNF- α , IL-1 β , and IL-6 in BALF were determined using ELISA kits according to the instructions recommended by the manufacturers. The optical density (OD) of each well was determined at 450 nm by a microplate spectrophotometer.

Measurement of SOD and MDA in the Tissue

Lung samples were prepared to make 1:10 (*w/v*) homogenates followed by centrifugation at 12,000 $\times g$ (4 °C) for 20 min to obtain supernatants for the next determination of SOD and MDA. Thereafter, SOD activity in the lung was conducted in the light of manufacturer's recommendation (Jiangcheng Institute of Biotechnology, Nanjing, China). And MDA level was determined with the thiobarbituric acid-reactive substance (TBARS) method using a commercial detection kit (Jiangcheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions.

Pulmonary Histopathology

The lungs were removed at the end of the experiment. Afterward, the samples were fixed in 4 % neutral-buffered formalin for 48 h, embedded in paraffin, and cut into 3- μ m sections. Then, hematoxylin-eosin staining was performed using the standard protocol. After that, pulmonary pathological changes were observed under a light microscope.

Western Blot Analysis

Lung tissues were harvested and frozen in liquid nitrogen immediately until homogenization. Proteins were extracted with lysis buffer (RIPA with protease and phosphatase inhibitor) for 15 min on ice. The total protein concentrations were determined by a BCA protein assay kit. Equal amounts of protein were mixed with five times loading dye (Laemmli buffer) and 2-mercaptoethanol followed by heating for 5 min at 95 °C. The samples were loaded per well on a 10 % sodium dodecyl sulfate polyacrylamide gel and transferred to PVDF membranes. The blots were blocked with 5 % bovine serum albumin (BSA) (5 g BSA was dissolved in 100 ml TBST) for 2 h at room temperature and incubated with primary antibody overnight at 4 °C. After washing, the bound antibodies were incubated with peroxidase-conjugated secondary anti-rabbit antibodies. The proteins were visualized by a ECL Key-GEN system (KeyGEN Biotechnology, Nanjing, China) and scanned

with a Clix ChemiScope chemiluminescence imaging system (Gel Catcher 2850, China). GAPDH was detected as an internal control of protein loading.

Statistical Analysis

The results are expressed as means \pm SDs and analyzed with one-way analysis of variance (ANOVA) with the Tukey multiple comparison test. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

Effects of CH on PQ-Induced Lung Wet-to-Dry Ratio

The index of lung edema was measured by calculating the W/D ratio of lung tissue. As revealed in Fig. 1, the lung W/D ratios in PQ-stimulated mice were evidently higher *versus* those of control mice. On the contrary, the W/D ratios in CH (10 and 20 mg/kg) groups and the Dex (2 mg/kg) group significantly decreased compared to those in the PQ group. The data clearly indicated the obvious reduction of pulmonary edema content with the treatment of CH.

Effects of CH on Inflammatory Cells in BALF and Myeloperoxidase Activity in Lung Tissues

The myeloperoxidase (MPO) activity was measured to elucidate the neutrophil accumulation in pulmonary tissues. Meanwhile, the number of total cells and neutrophils was analyzed to reveal the migration and infiltration of pulmonary cells (Fig. 2). As illustrated in Fig. 1, the number of total cells, neutrophils, and MPO activity in the PQ group significantly increased as compared to those in the control group. Treatment with CH (10 and 20 mg/kg) and Dex (2 mg/kg) effectively decreased the number of total cells, neutrophils, and MPO level compared to those in the PQ group. Our results suggested that CH exhibited an inhibitory effect on cell infiltration.

Effects of CH on Cytokines in BALF

Cytokines participate in the initiation and amplification of inflammatory cascade of acute lung injury (ALI). As shown in Fig. 3, it was proved that the levels of TNF- α , IL-1 β , and IL-6 in BALF were significantly increased in the PQ group compared to those in the control group. The CH (10 and 20 mg/kg) and Dex (2 mg/kg) treatment remarkably decreased the generations of TNF- α , IL-1 β ,

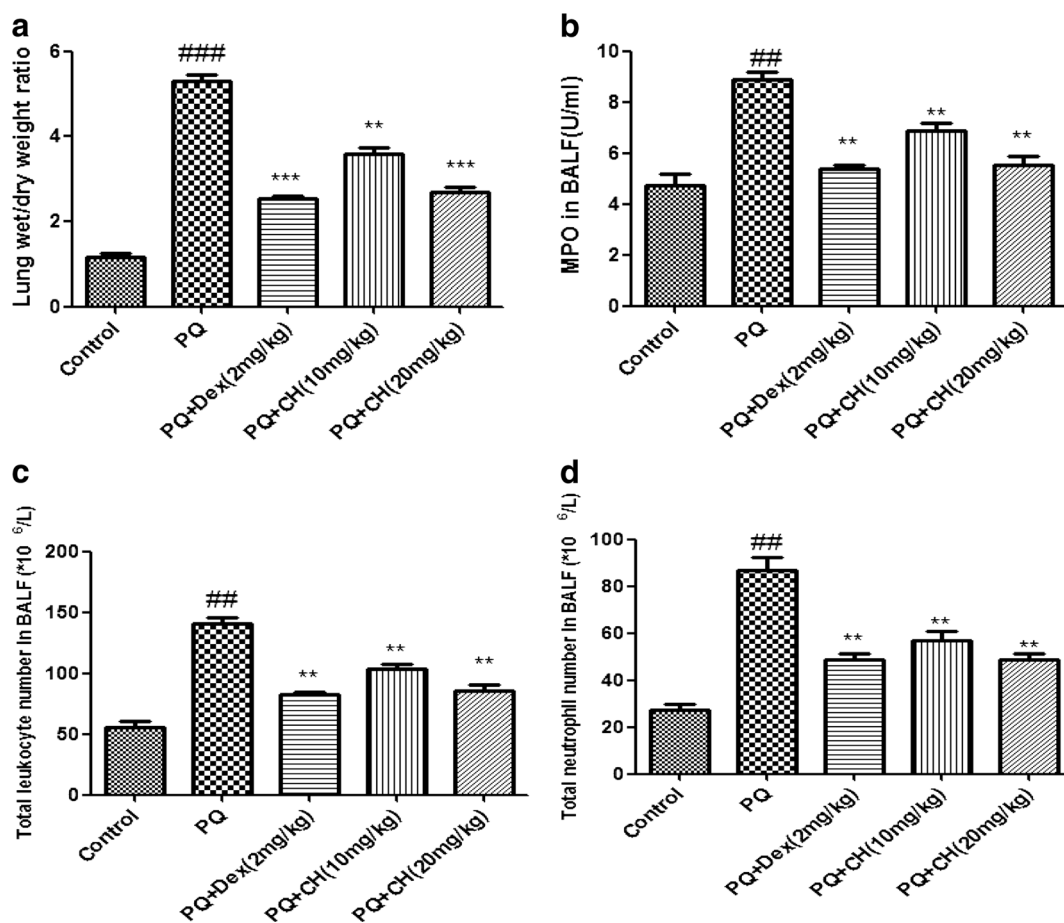


Fig. 1. The effects of CH on PQ-induced acute lung injury. **a** Lung W/D ratio. **b** MPO activity. **c** The number of total cells in BALF. **d** The number of neutrophils in BALF. Values are expressed as mean \pm SD. [#] $P < 0.05$; ^{##} $P < 0.01$; ^{###} $P < 0.001$, compared to the control group. ^{*} $P < 0.05$; ^{**} $P < 0.01$; ^{***} $P < 0.001$, compared to the PQ group.

and IL-6. CH reduced the synthesis and release of inflammatory cytokines in PQ-induced ALI.

Effects of CH on Oxidative Stress

Lipid peroxidation in BALF as well as lung tissues was determined by assaying the generations of MDA and SOD. As revealed in Fig. 2, the trend of MDA and SOD levels in the BALF between groups is consistent with that of MDA and SOD levels in the tissue. However, the levels of MDA and SOD in the BALF exhibited more significant differences among groups than those in the tissue. PQ stimulation significantly declined the SOD content, while CH administration effectively restored the level of SOD. Meanwhile, exposure to PQ displayed a strikingly high

MDA level whereas the treatment with CH remarkably ameliorated this condition.

Effect of CH on PQ-Induced Pathological Changes of the Lung

Hematoxylin and eosin (H&E) staining was performed to evaluate the protective effects of CH on physiological impairment. Scarce obvious histological alteration was observed in the lung specimen. By contrast, in the PQ group, histological evaluation of the lungs by light microscopy demonstrated alveolar wall hyperemia and excessive neutrophil infiltration around the pulmonary vessel after the simulation of PQ. CH treatment groups obviously attenuated the severity of lung injury. These findings suggested that CH

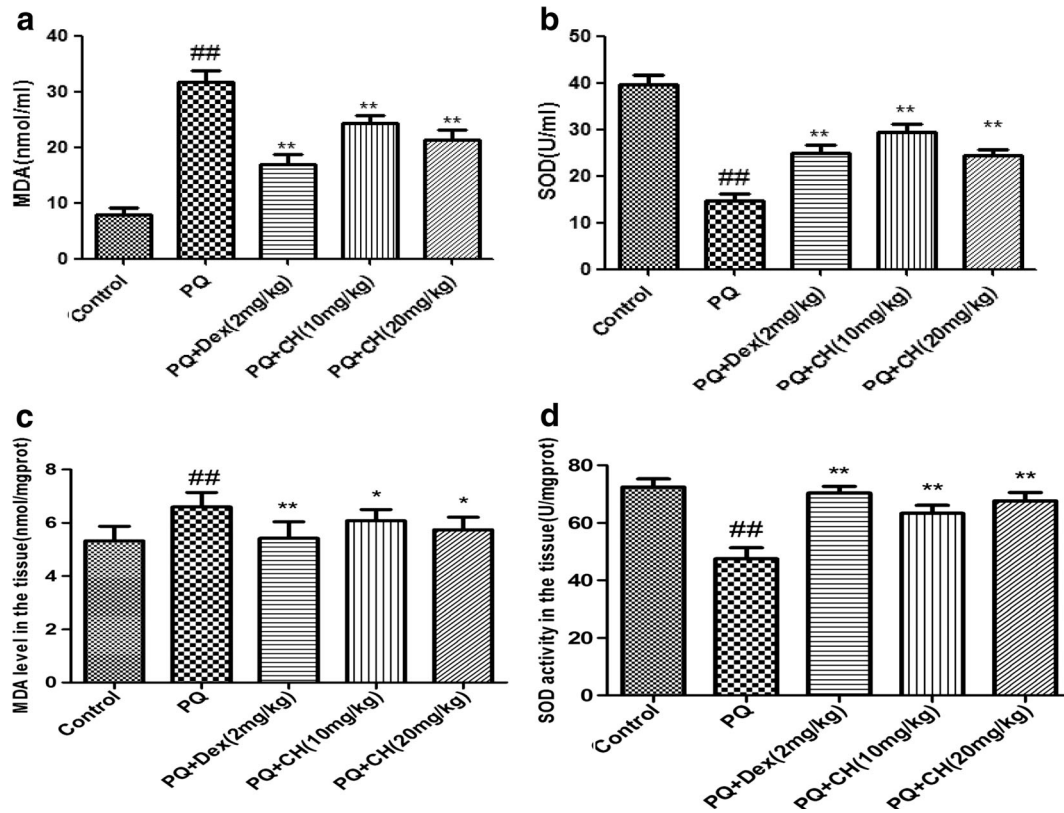


Fig. 2. The effects of CH on the oxidative stress of PQ-induced mice. **a** MDA in the BALF. **b** SOD in the BALF. **c** MDA in the tissue. **d** SOD in the tissue. Values are expressed as mean \pm SD. # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$, compared to the control group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$, compared to the PQ group.

significantly attenuated the histopathology conditions in PQ-induced ALI (Fig. 4).

Effect of CH on PQ-Induced PPAR- γ

We detected the expression of PPAR- γ by Western blot analysis. As shown in Fig. 5, PQ challenge respectively induced an obvious decrease in the expression of PPAR- γ . However, it was proved that the treatment with CH significantly activated PPAR- γ .

Effect of CH on PQ-Induced NF- κ B Pathway

NF- κ B pathway plays a key role in the regulation of inflammatory mediator production. We found that the expressions of p-NF- κ BP65, P-I κ B α , P-IKK α , and P-IKK β were significantly upregulated in lung tissues after PQ stimulation. By contrast, treatment with CH (10 and 20 mg/kg) and Dex (2 mg/kg) obviously ameliorated these situations (Fig. 6).

DISCUSSION

Evidence has emerged indicating that CH exerted the anti-inflammatory and anti-oxidative properties [17]. In this study, our results showed that CH could attenuate PQ-induced pulmonary and lung injury in mice. The ability of CH to reduce PQ-induced toxicity was due to its anti-inflammatory property in lung tissues.

Paraquat is the widely used herbicide around the world. Since its first application on agriculture in 1962, thousands of people died yearly from the intentional or accidental ingestion of PQ. As to our knowledge, the lung tissue is the major target organ as PQ is actively taken up by the alveolar epithelium [18]. Therefore, the injection of PQ was usually used as an inducer for acute lung injury in scientific studies [19].

ALI is a severe clinical syndrome characterized by noncardiogenic pulmonary edema, severe hypoxemia, accumulation of pulmonary cells, and overproduction of

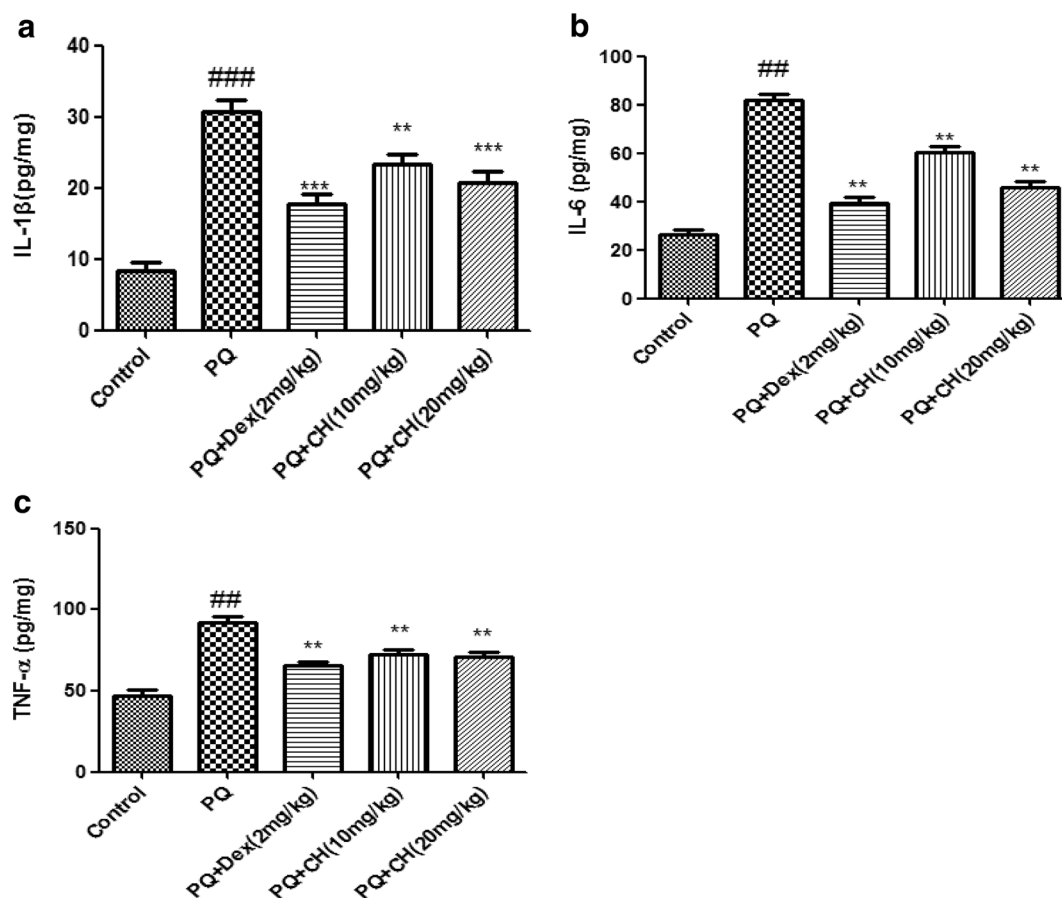


Fig. 3. The effects of CH on the generations of inflammatory cytokines **a** IL-1 β , **b** IL-6, and **c** TNF- α in PQ-induced mice. Values are expressed as mean \pm SD. # P < 0.05; ## P < 0.01; ### P < 0.001, compared to the control group. * P < 0.05; ** P < 0.01; *** P < 0.001, compared to the PQ group.

inflammatory cytokines [20]. Unfortunately, there were few effective drugs to treat ALI as yet [21]. Thus, there is an urgent need to find a safer medicine for the management of acute lung injury. Edema is the pivotal feature of systemic and partial inflammations [22]. The W/D ratio was calculated to determine the water content of lung tissues [23]. As suggested by the evident reduction of W/D ratio, our data confirmed that CH was capable of attenuating the pulmonary edema. The migration and infiltration of neutrophils into the lung is the characteristic symptom of pulmonary disease. Moreover, the suppression of neutrophils participates in the mediation of oxygen species, cytokines, and chemokines and granular enzyme-mediated lung injury [24]. MPO drove superabundant oxidative production which leads to the tissue damage under inflammatory conditions [25]. The experimental results indicated that CH effectively reduced the MPO activity and the inflammatory cell infiltration. Furthermore, the

histopathological observation also confirmed the protective effects of CH on PQ-challenged mice.

Inflammatory cytokines appear in the early stage of the inflammatory response and contribute to the severity of lung dysfunction [26]. IL-1 β conduces to the alveolar epithelial repairment and induces the release of other cytokines [27]. IL-6 is highly related to the acute-phase response of inflammation and the initiation of inflammatory cascade [28]. TNF- α , playing an important role in the motivation of innate immune reaction, is responsible for the pathogenesis of inflammation [29]. As expected, CH was found to effectively downregulate the contents of IL-1 β , IL-6, and TNF- α , which indicated that the protective effects of CH on PQ-induced acute lung injury was partly attributed to the suppression of inflammatory mediators.

Accumulating evidences proposed the common link between acute lung injury and oxidative stress [30]. The excessive oxygen-free radical, which occurs during the

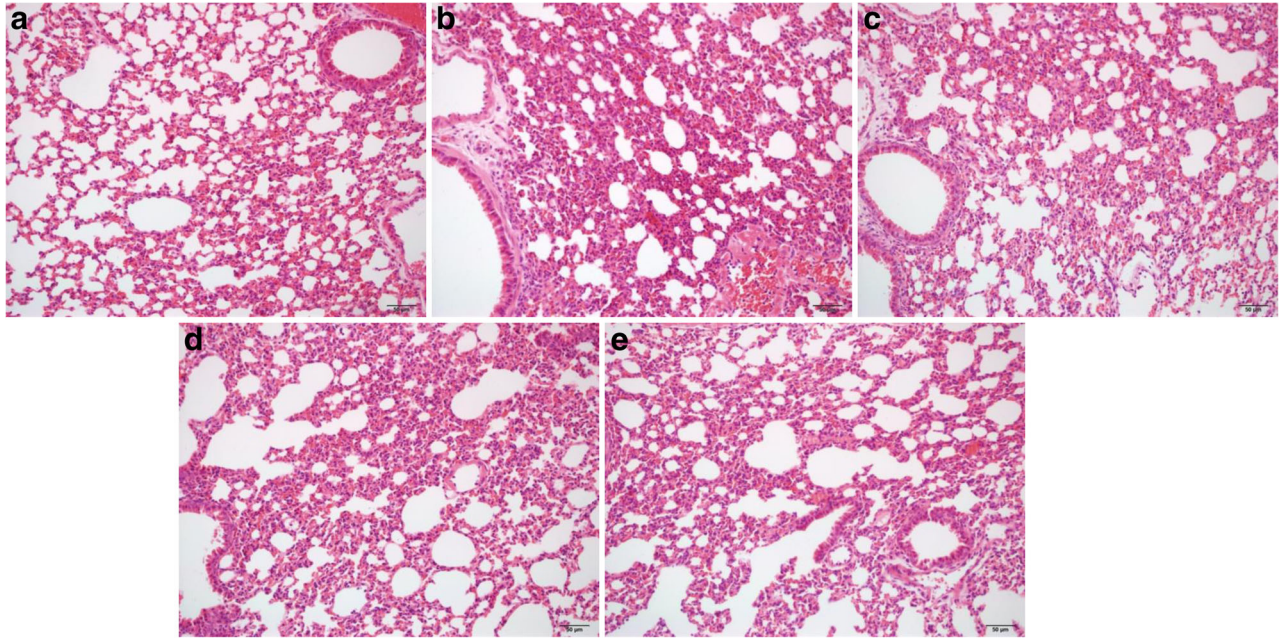


Fig. 4. The effects of CH on PQ-stimulated lung histopathologic changes in lung tissues. **a** The lung section from the control mice; **b** the lung section from the PQ mice; **c** the lung section from the mice administered with PQ and dexamethasone (2 mg/kg); **d** the lung section from the mice administered with PQ and CH (10 mg/kg); **e** the lung section from the mice administered with PQ and CH (20 mg/kg).

pathogenesis of pulmonary lesion, also contributes to biological membrane lipid peroxidation and severe cell damage [31]. Thus, the attenuation of oxidative stress might be beneficial to the treatment of pulmonary disease. MDA is the end product of polyunsaturated fatty acid and usually used as an indicator for the lipid peroxidation [32]. SOD

exhibits various physiological activities including anti-inflammatory and anti-oxidative effects [33]. As the analytical results shown, CH evidently decreased the content of MDA and restored SOD activity in both BALF and lung tissues. Our results suggested that the therapeutic effects of CH might result from the anti-oxidative activity.

It was indicated that PPAR- γ exhibits a cytoprotective effect against cellular stress and inflammatory insults. Above all, our study firstly demonstrated that CH increases the expression/activity and nuclear translocation of PPAR- γ in the lungs of ALI. Next, PQ challenge brings about the activation of NF- κ B through the phosphorylation and degradation of the I κ B α [34]. Substantial researches also elicited that NF- κ B pathway was implicated in the lipid peroxidation and inflammatory process [35]. Kim *et al.* displayed that CH exhibited the anti-inflammatory effects through the NF- κ B signaling [36]. Our Western blotting data ascertained that CH, exerting an ameliorative effect on ALI, significantly increased the expression of PPAR- γ and inhibited the NF- κ B pathway in response to PQ challenge, which demonstrated our hypothesis before.

In conclusion, the results of the present study revealed that CH could effectively attenuate PQ-induced ALI. The potential mechanism of CH might be involved in the amelioration of inflammatory cytokines and oxidative

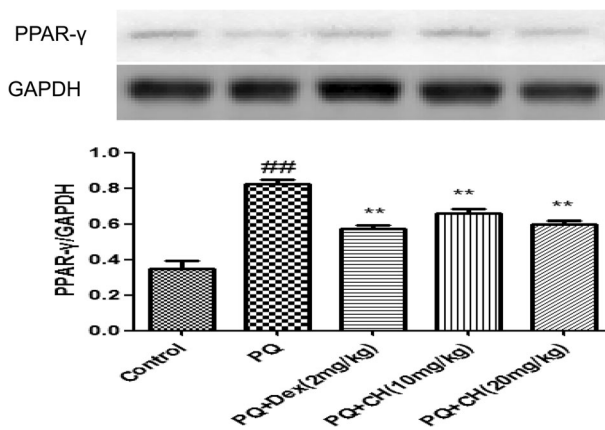


Fig. 5. The effects of CH on the expressions of PPAR- γ in the lung tissues of PQ-induced mice. Values are expressed as mean \pm SD. [#] $P < 0.05$; ^{##} $P < 0.01$; ^{###} $P < 0.001$, compared to the control group. ^{*} $P < 0.05$; ^{**} $P < 0.01$; ^{***} $P < 0.001$, compared to the PQ group.

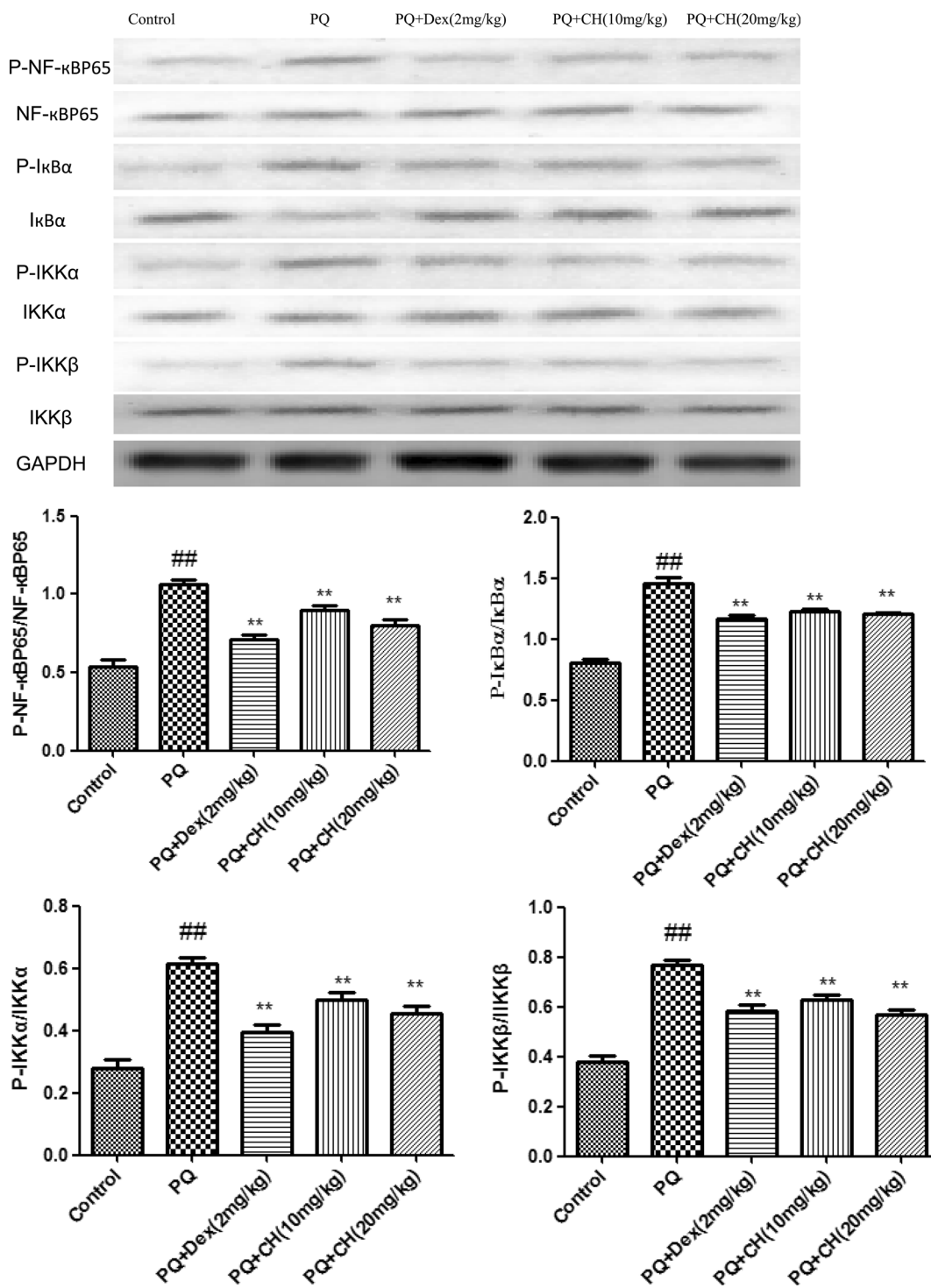


Fig. 6. The effects of CH on the expressions of NF-κB pathway in the lung tissues of PQ-induced mice. Values are expressed as mean ± SD. [#]*P* < 0.05; ^{##}*P* < 0.01; ^{###}*P* < 0.001, compared to the control group. **P* < 0.05; ***P* < 0.01; ****P* < 0.001, compared to the PQ group.

stress through the activated PPAR- γ and inhibited NF- κ B pathway. Despite this, we elaborate the protective effect of CH against paraquat-induced lung injury in the aspect of pharmacodynamics instead of pharmacokinetics in the present study. Adverse effect of CH and its pharmacokinetic situation *in vivo* remain to be investigated. Further studies are warranted to capitalize on the protective effects of CH on ALI caused by PQ in the clinical practice.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest. The authors declare that they have no conflict of interest.

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