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Chloroquine attenuates paraquat-induced lung injury in mice by altering inflammation, oxidative stress and fibrosis

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ABSTRACT

Paraquat is one of the most extensively used herbicides and has high toxicity for humans and animals. However, there is no effective treatment for paraquat poisoning. The aim of the present study was to evaluate the effects of chloroquine on paraquat-induced lung injury in mice. Mice received a single intraperitoneal injection of paraquat and a daily intraperitoneal injection of the indicated dosages of chloroquine or dexamethasone. The histological changes, inflammation and oxidative stress in the lungs were examined at day 3, and the degree of pulmonary fibrosis was examined at day 28. H&E staining showed that chloroquine markedly attenuated lung injury induced by paraquat. In addition, the inflammatory responses induced by paraquat were inhibited after treatment with chloroquine, as indicated by the decreased number of leukocytes, the reduced levels of TNF-α, IL-1β and IL-6 in the bronchoalveolar lavage fluid, the reduced NO content, and downregulation of iNOS expression in lung tissues. No different effect was found between high-dose chloroquine and dexamethasone. Additionally, the treatment with chloroquine increased the activity of SOD and decreased the level of MDA in the lung tissues. The expressions of the anti-oxidative proteins, Nrf2, HO-1 and NQO1, were also upregulated by chloroquine treatment. The high-dose chloroquine was more effective than dexamethasone in its anti-oxidation ability. Finally, the results of Masson's staining illustrated that chloroquine markedly attenuated fibrosis in the paraquat-exposed lungs. Immunohistochemistry staining showed that the expressions of the pro-fibrotic proteins TGF-β and α-SMA were downregulated after treatment with chloroquine. In conclusion, chloroquine effectively attenuated paraquat-induced lung injury in mice.

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1. Introduction

Paraquat (PQ) is one of the most extensively used nonselective contact herbicides around the world, particularly in developing countries [1]. However, it is highly toxic to humans and animals and is a primary cause of death from intentional and accidental ingestion [2,3]. Thus, PQ poisoning is a great public health problem in these countries [4,5]. The predominant cause of PQ-induced death is respiratory failure because the lung is the major target organ of PQ after it is ingested [6]. Acute lung injury induced by PQ is characterized by hemorrhage, edema and pulmonary fibrosis. Mechanically, inflammation and oxidative stress are considered to play a vital effect in the pathological processes of these changes. At the early stage of PQ-induced acute injury, inflammatory cells are activated and infiltrate into the alveolar walls and the flood of neutrophils and macrophages increases the proinflammatory cytokine levels [7,8]. Additionally, the production of superoxide radicals is dramatically increased and directly or indirectly damages alveolar epithelial cells [9–11]. After several weeks following exposure to PQ, myofibroblast infiltration, collagen accumulation and the differentiation of fibroblasts are found in the alveolar septum [11,12]. Little is known about the mechanisms of PQ-caused pulmonary fibrosis. Inflammation and oxidative stress may be involved in the pathogenesis of pulmonary fibrosis [13]. In addition, this process may be promoted by some pro-fibrotic factors, such as transforming growth factor-β (TGF-β), collagens, α-smooth muscle actin (α-SMA) and matrix metalloproteinase (MMP).

Currently, PQ poisoning is usually treated by gastric lavage and purgation, followed by the administration of glucocorticoids and immunosuppressors to attenuate lung injury. However, these drugs are not specific for PQ poisoning and have severe adverse effects. The lack of effective therapeutic strategy against PQ intoxication results in high mortality, so it is urgent to develop efficient antidotes. A compound that targets inflammation, oxidative stress and pro-fibrotic factors would be beneficial for the treatment of PQ intoxication.

Chloroquine (CQ) is a lysosomotropic weak base that has been used for malaria treatment for several decades. Data in previous literature indicate that CQ has multiple bioactivities against inflammation, oxidation and fibrosis properties. In the 1990s, CQ was reported to have an anti-oxidative effect on *Plasmodium yoelii nigeriensis*-infected mice [14,15]. Later, CQ was found to attenuate inflammatory responses and inhibit inflammatory cytokine secretion in various models [16,17]. Moreover, CQ

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could improve liver fibrosis by downregulating the expressions of TGF-β and α-SMA [18]. In addition, CQ has lung protective effects in acute hemorrhagic necrotizing pancreatitis [19,20]. Importantly, CQ could rescue alveolar type II cells (A549 cells) from PQ-induced death [21]. Based on the above findings, we hypothesize that CQ could attenuate PQ-induced lung injury. The aim of the present study was to evaluate the protective effects of CQ against PQ-induced inflammation, oxidative stress and fibrosis in the lung.

2. Materials and methods

2.1. Animals

A total of 118 male C57BL/6 mice (18–22 g, 8 weeks old, Laboratory Animal Centre of China Medical University, Shenyang, China) were maintained in standard cages at controlled conditions of temperature (22 ± 2 °C) with relative humidity of 50% ± 10%, 12 h light/dark cycle and free access to water and food. All the protocols were conducted according to the ethical standards and approved by the Ethics Committee of China Medical University.

2.2. Experimental procedure

The mice were randomly divided into 6 groups: (1) control (n = 18), (2) CQ 50 mg/kg (n = 18), (3) PQ (n = 22), (4) PQ + CQ 25 mg/kg (n = 20), (5) PQ + CQ 50 mg/kg (n = 20), and (6) PQ + dexamethasone (PQ + DEX, 5 mg/kg) (n = 20). The mice in the PQ, PQ + CQ and PQ + DEX groups received a single intraperitoneal injection of 30 mg/kg PQ [22,23] and the mice in the other groups received an equivalent volume of saline. The mice in the CQ and PQ + DEX groups received an indicated dose of CQ or DEX 1 h after the PQ injection and then once a day. The mice in the control and PQ groups were given an equivalent volume of saline. Three mice in the PQ group, 1 mouse in the PQ + CQ 25 mg/kg group, 2 mice in the PQ + CQ 50 mg/kg group and 1 mouse in the PQ + DEX group died during the experiment. For short-term experiments, the mice were sacrificed at day 3. Six mice in each group were used for histological examinations and 6 mice were used for the bronchoalveolar lavage fluid (BALF) collection, biochemical and molecular biological analyses. Six mice in each group were used for long-term lung fibrosis examination and were sacrificed at day 28. All the mice were randomly selected and the remaining 3 mice were used for preliminary experiments.

2.3. BALF collection, protein content determination and cell count

Three days after PQ injection, the mice were anaesthetized and 2 ml of BALF were collected by lung perfusion with saline via tracheal cannulation. The protein content in the BALF was determined using a Bradford Protein Quantification Kit (Beyotime Institute of Biotechnology, Haimen, China). For cell counting, the BALF was centrifuged at 250 ×*g* for 5 min at 4 °C and the pellets were suspended with 0.5 ml phosphate buffer solution (PBS). Subsequently, 10 μl of the suspension was placed on a glass slide and fixed with methanol for 15 min. Cells in the suspension were stained using Giemsa staining solution according to the manufacturer's instruction (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Total leukocytes, neutrophils and macrophages were counted with a hemocytometer.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Contents of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 in the BALF were determined using commercial ELISA kits according to the manufacturer's protocols (Wuhan Boster Biological Technology, Ltd., Wuhan, China).

2.5. Measurement of bio-markers of oxidative stress and NO production

The malondialdehyde (MDA) content, superoxide dismutase (SOD) activity and NO content in the lung tissues were measured by biochemical kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer's protocols.

2.6. Histological analyses of lung tissues

The lung tissues were fixed in 4% paraformaldehyde and embedded with paraffin. The paraffin blocks were cut into 5-μm-thick sections, which were then dewaxed with xylene and hydrated with graded ethanol. For histological examination, the sections were stained with hematoxylin and eosin or Masson's trichrome. For immunohistological assay, the sections were boiled in 0.1 M sodium citrate buffer in a microwave oven for 10 min to perform the antigen retrieval. After washing with PBS, the sections were incubated with primary anti-α-SMA antibody (1:200, bs-10196R, Bioss, Beijing, China) or anti-TGF-β antibody (1:200, bs-0086R, Bioss) at 4 °C overnight and then incubated with biotinylated goat anti-rabbit IgG (1:200, Beyotime) at 37 °C for 30 min. The immune-staining was visualized by reaction with diaminobenzidine tetrahydrochloride (Beyotime). The specimens were examined under a light microscope (DP73, Olympus, Tokyo, Japan). The H&E staining was used for histological scoring according to an established scoring system [24]. The mean score obtained from three randomly selected sections in each sample was used as the final score of each sample.

2.7. RNA extraction and real-time PCR

Total RNA was extracted from the lung tissues using a total RNA extraction kit (BioTeke Corporation, Beijing, China) according to the manufacturer's protocol. First-strand complementary DNA (cDNA) was synthesized from the total RNA with an oligo-(dT) primer using super M-MLV reverse transcriptase (BioTeke). Then the mRNA levels of iNOS, Nrf2, HO-1 and NQO1 in the lung tissues were determined via quantitative real-time PCR using the 2× Power Taq PCR Master Mix (BioTeke) and SYBR Green (Solarbio Science & Technology, Co., Ltd., Beijing, China) in a 20 μl reaction system on an Exicycler 96 (Bioneer, Daejeon, Korea) with β-actin used as a loading control. Relative gene expression levels were normalized with β-actin, calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold changes compared with the control group. The sequences of primers used in the study are shown as follows: iNOS, forward: 5′-GCAGGGAATCTTGGAGCGAGTTG-3′, reverse: 5′-GTAGGTGAGGGCTTGGCTGAGTG-3′; Nrf2, forward: 5′-CTTCCATTTACGGAGACCC-3′, reverse: 5′-GAGCACTGTGCCCTTGAGC-3′; HO-1, forward: 5′-ACAGATGGCGTCACTTCGT-3′, reverse: 5′-GTGAG GACCCACTGGAGGA-3′; NQO1: forward: 5′-GCTTTAGGGTCGTCTTGGC-3′, reverse: 5′-ACAATCAGGGCTCTTCTCG-3′; β-actin, forward: 5′-CTGTGCCCATCTACGAGGGCTAT-3′, reverse: 5′-TTTGATGTCACGCACG ATTTCC-3′.

2.8. Western blotting analysis

The lung tissues were homogenized in RIPA lysis buffer supplemented with phenylmethanesulfonyl fluoride (Beyotime) on ice. Protein extracts were centrifuged at 12,000 ×*g* for 10 min at 4 °C and the supernatants were collected. The protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime). Equal amounts of proteins from each sample were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% non-fat milk and incubated with anti-iNOS antibody (1:400, BA0362, Bioss), anti-Nrf2 antibody (1:500, bs-1074R, Bioss), anti-HO-1 antibody (1:200, sc-10789, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NQO1 antibody (1:500, bs-2184R, Bioss) and anti-β-actin antibody (1:1000, sc-47778, Santa

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