



Research Article

# The Ameliorative Effects of Allopurinol on Paraquat-Induced Pulmonary Fibrosis in Rats

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## ABSTRACT

**Background:** Pulmonary fibrosis is described as a chronic idiopathic inflammatory disease of the interstitial lungs. It is associated with a potentially fatal prognosis, and patients show insignificant response to treatment. To treat paraquat (PQ)-induced pulmonary injury and fibrosis, multiple approaches have been used. We aimed to determine the effects of allopurinol (Allo), a xanthine oxidase inhibitor, on PQ-induced pulmonary fibrosis in rats.

**Methods:** A total of 30 female Sprague-Dawley rats were divided randomly into five groups (200±20 g). Group 1 (control) and group 2 (PQ group) were intraperitoneally administered PQ (20 mg/kg) once on day seven without any treatment, while groups 3–5 orally received 50, 100, and 200 mg/kg of Allo seven days before and three weeks following the administration of PQ, respectively. The animals were sacrificed three weeks after PQ administration. For the histopathological analysis and assessment of serum malondialdehyde (MDA) and hydroxyproline (HP) contents, the animals' blood and lungs were collected.

**Results:** The PQ group showed significantly higher lung HP, serum MDA, and lung index in comparison with the control. Treatment with Allo, especially at 100 and 200 mg/kg, decreased HP, MDA, and lung index significantly, compared to the PQ group. Allo could prevent inflammatory cell infiltration, presence of fibroblasts, and PQ-related alveolar thickening.

**Conclusion:** The results revealed that Allo has potential protective effects on PQ-related pulmonary fibrosis, and the role of xanthine oxidase in the exacerbation of PQ-induced pulmonary fibrosis was confirmed.

## Introduction

Pulmonary fibrosis is described as a chronic idiopathic inflammatory disease of the interstitial lungs. It is associated with a potentially fatal prognosis, and patients often show insignificant response to treatment.<sup>1,2</sup> Nevertheless, the pathophysiology of this disease remains undetermined. The lower-airway accumulation of activated inflammatory cells is assumed to produce dangerous quantities of reactive oxygen species (ROS), leading to lung damage.

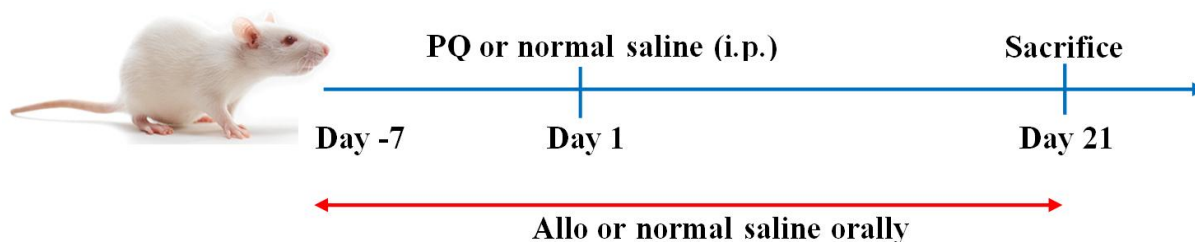
The content of the extracellular matrix is increased by activated fibroblasts, which in turn destroys the normal structure of the lungs and decreases vital gas exchange.<sup>3,4</sup> A studied model of fibrogenesis is experimental paraquat (PQ)-induced lung fibrosis, which has been endorsed by many studies.<sup>5-8</sup> This pulmonary fibrosis model is similar to the model in humans, assessing the potential effects of therapeutic agents. PQ, as a quaternary nitrogen

herbicide, is used for controlling broadleaf weed worldwide. The molecular mechanism of PQ toxicity is not completely understood, making it difficult to treat toxicities of the central nervous system, kidneys, heart, and liver. However, pulmonary fibrosis and lung damage are recognized as the most common causes of mortality and injury.<sup>9-13</sup>

In the mid 1950's, allopurinol (Allo) was synthesized for the production of new antineoplastic agents. Nevertheless, its inhibitory effects on xanthine oxidase (XO) were reported, reducing both serum uric acid and urinary contents.<sup>14</sup> In 1966, the Food and Drug Administration approved Allo for gout treatment. Administration of this drug remains the most effective approach for primary and secondary hyperuricemia.<sup>15</sup> Allo is recognized as a competitive inhibitor and a substrate for XO enzymes at low concentrations, while it is a noncompetitive inhibitor at higher concentrations.

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**Figure 1.** The experimental protocol of the study. Female rats were pretreated with 50, 100, and 200 mg/kg of Allo or normal saline orally seven days before and three weeks after the administration of PQ. PQ or normal saline was administered on day one at a single dose intraperitoneally (i.p.).

Its formation, besides its long persistence in tissues, largely accounts for its pharmacological activity. Furthermore, it has been reported that XO inhibition exerts antioxidant properties.<sup>16</sup> The XO enzyme forms superoxide ( $O_2^{\bullet-}$ ) and hydrogen peroxide ( $H_2O_2$ ), which is majorly involved in chronic heart failure, different inflammatory diseases, and vascular and tissue damage.<sup>17-20</sup> In animal models, as well as small-scale clinical trials in humans, Allo has exhibited positive therapeutic effects. XO-derived ROS has been introduced as a mediator of proinflammatory gene expression and inflammatory signal transduction pathways.<sup>16</sup> Therefore, this study examined the effects of Allo on the fibrogenic activity of PQ in a pulmonary fibrosis model.

## Materials and Methods

### Chemicals

Jalinous Pharmaceutical Company (Tehran, Iran) provided Allo in this study. Sigma–Aldrich Co. (St. Louis, MO, USA) supplied PQ (methyl viologen), thiobarbituric acid (TBA), chloramine T, trichloro acetic acid (TCA), L-hydroxyproline (HP), dimethyl benzaldehyde, and tetraethoxypropane (TEP). All other chemicals were of an analytical grade.

### Animals

The animal house of Ahvaz Jundishapur University of Medical Sciences (AJUMS) provided 30 female eight-week-old Sprague–Dawley rats ( $200 \pm 20$  g), which were kept in a 12:12 h light-dark cycle inside polypropylene cages with free access to standard rat chow and drinking water under controlled temperature ( $20 \pm 2^\circ C$ ). In our study, the procedures for animals were in line with the guidelines of AJUMS Animal Ethics Committee.

### Experimental design

After randomly dividing the animals into five experimental groups (six per group), they were treated as follows: group 1 (control); group 2 (PQ group), PQ (20 mg/kg/5 ml in normal saline, i.p.); and groups 3–5, oral administration of 50, 100, and 200 mg/kg of Allo, respectively seven days before and three weeks after PQ administration.<sup>2</sup> (Figure 1)

### Sample Collection

Xylazine (10 mg/kg), as well as ketamine (90 mg/kg), was used to anesthetize the rats at the end of the experiment

(day 28). Blood samples from the jugular vein were collected. After isolating, weighing, and washing the lungs with saline rapidly, centrifugation was carried out to separate the serum at 3000 rpm for 10 minutes; then, it was stored at  $-80^\circ C$  until further analysis.

### MDA assay

MDA reaction with TBA was measured to determine lipid peroxidation, as explained by Esterbauer and Cheeseman.<sup>21</sup> Briefly, after mixing the serum sample (0.5 mL) with TCA (2.5 mL; 10% w/v) and centrifuging for 10 minutes at 3000 rpm, 2 mL of the supernatant was added to the test tube of TBA solution (1 mL; 0.67% w/v). A solution with a pink color emerged after keeping the mixture for 10 minutes in boiling water. The mixture was left to cool down, and then, a spectrophotometer (UV-1650 PC, Shimadzu, Japan) was used to read absorbance at 532 nm. In this study, serum MDA is presented as  $\mu\text{mol/L}$ , and tetraethoxypropane (TEP) is considered as the standard.

### HP assay

A colorimetric assay was used to quantify the left lung total collagen content and HP content of the lungs.<sup>22,23</sup> For this purpose, after homogenizing the minced lobes of the left lung in 6 M HCl and hydrolyzed at  $130^\circ C$  for five hours, NaOH was used to adjust pH to 6.5–7.0, and distilled water was used to adjust the sample volume to 30 mL. After mixing chloramine T solution (1.0 ml; 0.05 mol/L) with the sample solution (1.0 ml) and incubation for 20 minutes at room temperature, 1.0 ml of 20% dimethyl benzaldehyde solution was added and then incubated for 20 minutes at  $60^\circ C$ . Absorbance was measured at 550 nm. According to HP standards, the values were presented as mg HP per g wet lung weight.

### Histopathological examination

After dissecting a section of the lung (5  $\mu\text{m}$ ) and its fixation in 10% phosphate-buffered formaldehyde, it was stained with hematoxylin and eosin (H&E staining). Connective tissues, recruitment of inflammatory cells, and collagen deposition were evaluated in the lungs.

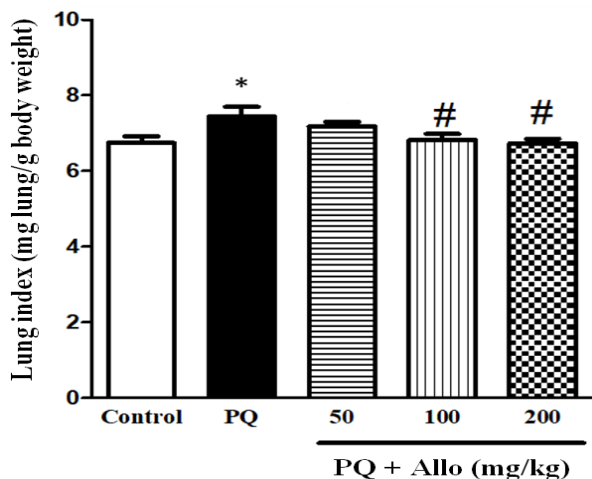
### Statistical analysis

Values are presented as mean  $\pm$  SD. For comparisons, one-way ANOVA and Tukey's test were applied in this study. The significance level was 0.05.

## Results

### Lung index

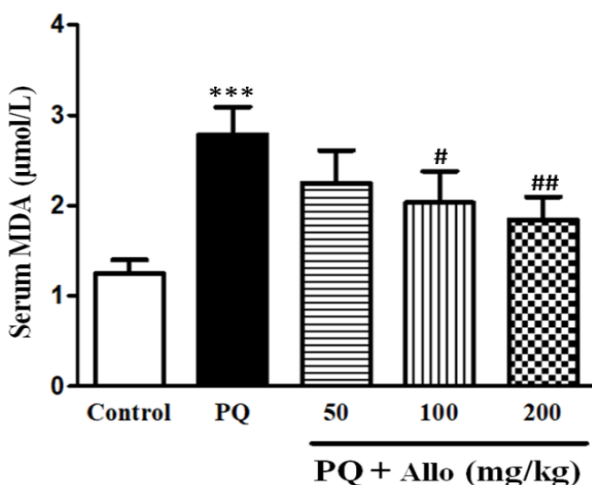
Body weight was calculated every week during the experiment. The lung index was measured after sacrificing the animals as the wet lung weight ratio to body weight (mg/g). The lung indices were  $6.74 \pm 0.17$  and  $7.44 \pm 0.25$  mg/g, respectively in the control and PQ groups. The Allo (100 and 200 mg/kg) and control groups showed no significant differences (Figure 2).



**Figure 2.** Effects of Allo pretreatment at 50, 100, and 200 mg/kg on the lung index in a model of PQ-induced pulmonary fibrosis. Values are presented as mean ± SD (n = 6). \*P < 0.05 and #P < 0.05, significant differences vs. the control and PQ groups, respectively.

### MDA levels

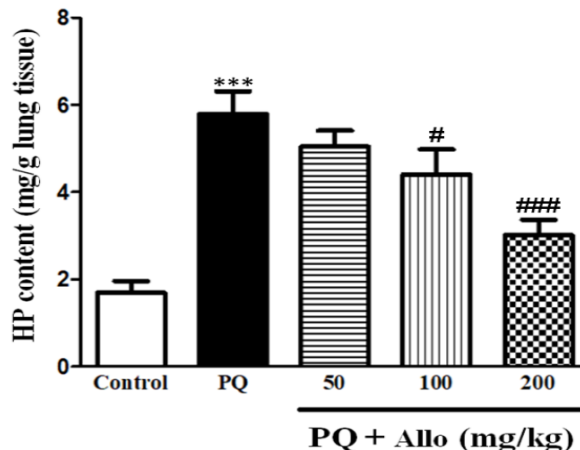
The serum MDA content (lipid peroxidation index) increased in rats exposed to PQ in comparison with the controls (P < 0.001). The serum MDA levels were  $1.24 \pm 0.15$  and  $2.78 \pm 0.31$  μmol/lit in the control and PQ groups, respectively; Allo-pretreated rats showed a reduction in MDA level (Figure 3).



**Figure 3.** Effects of Allo pretreatment at 50, 100, and 200 mg/kg on serum MDA content in PQ-induced pulmonary fibrosis. Values are presented as mean ± SD (n = 6). \*\*\*P < 0.001, significant difference vs. control group; #P < 0.05 and ##P < 0.01, significant difference vs. PQ group.

### HP Content

The lung HP content as an index of collagen accumulation was  $1.69 \pm 0.26$  and  $5.79 \pm 0.53$  mg/g tissue in the control and PQ groups, respectively (Figure 4). Treatment with Allo in doses 100 and 200 mg/kg significantly decreased lung HP in comparison with PQ group.



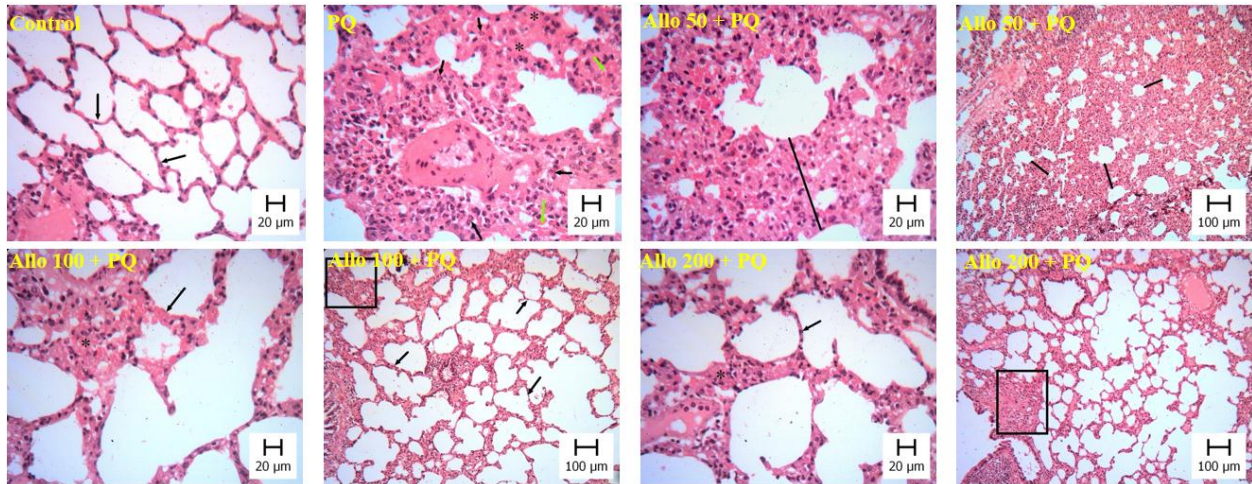
**Figure 4.** Effects of Allo pretreatment (50, 100, and 200 mg/kg) on lung HP content in a PQ-induced pulmonary fibrosis model. Data are presented as mean ± SD (n = 6). \*\*\*P < 0.001, significant difference vs. control group; #P < 0.05 and ###P < 0.001, significant difference vs. PQ group.

### Histological Changes

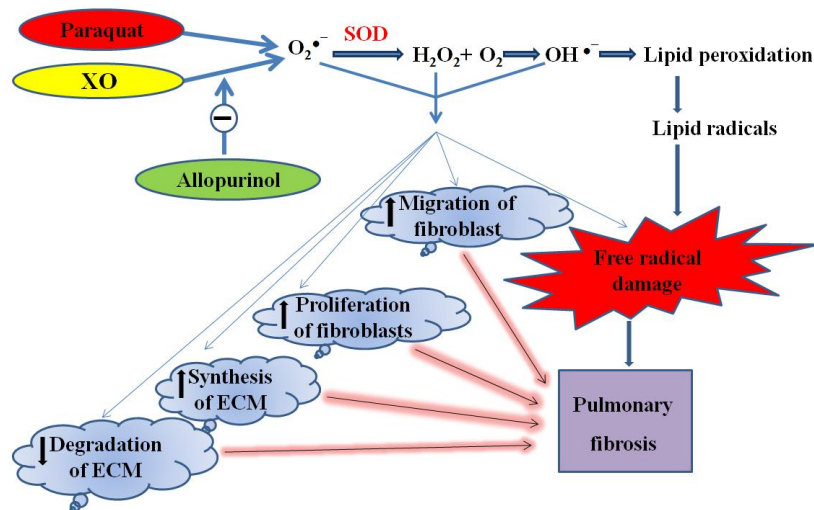
According to the Photomicrographic analysis, grade 0 and grade 8 were more prominent in the control and PQ groups, respectively, based on the infiltration of fibroblasts, inflammatory cells, and extracellular matrix. The pretreated rats indicated grades 6-7 at 50 mg/kg, while grades 4-5 were more prevalent in the photomicrographs at 100 and 200 mg/kg (Figure 5).

### Discussion

There are two phases in pulmonary toxicity with PQ. The first involves injury and destruction of alveolar epithelial cells, resulting in hemorrhage and edema, while the second one involves the infiltration of inflammatory cells into the alveolar space and septa, as well as alveolar cell differentiation into fibroblasts associated with collagen production.<sup>9,10,24,25</sup> PQ can reach the lung through the circulation after ingestion, and accumulates in alveoli. This herbicide can produce a large amount of ROS through its interaction with lung and other organs. ROS can oxidize surrounding lipids and induce lipid peroxidation. Excessive ROS consumes reducing molecules such as glutathione, which can lead to more damage to the lungs and other organs. The amount of oxygen present in the alveoli can induce the production of PQ+ from PQ++ (PQ) by reductases (e.g. NADPH), and reduced form of PQ (PQ+) lead to the generation of superoxide anions (O<sub>2</sub><sup>•-</sup>). Superoxide anion may be finally transformed into hydrogen peroxide and hydroxyl radicals with other pulmonary reductases and ferrous ion (Fe<sup>2+</sup>).<sup>13,26-31</sup>



**Figure 5.** These figures are representative of the role of Allo in lung damage induced by PQ. After collecting lung tissues 21 days after PQ administration, they were stained with H&E. The groups included: control, PQ, PQ pretreated with Allo 50 mg/kg (Allo 50+PQ), PQ pretreated with Allo 100 mg/kg (Allo 100+PQ), and PQ pretreated with Allo 200 mg/kg (Allo 200+PQ). Lung parenchyma is intact and preserved in the control group. Group PQ shows extensive interstitial infiltration and fibrosis. PQ-induced histological changes were markedly prevented in the Allo-pretreated groups at 100 and 200 mg/kg.



**Figure 6.** Graphical abstract of the possible protective effects of allopurinol (Allo) against paraquat (PQ)-induced pulmonary fibrosis. SOD: Superoxide dismutase; ECM: Extracellular matrix; XO: Xanthine oxidase.

These oxidative species can readily obtain hydrogen atoms from alveolar lipids so as to result in alveolar cell injury.<sup>27</sup> It seems that XO increases superoxide anions production mediated by PQ.<sup>32</sup>

PQ increased lung HP, serum MDA and lung index. These effects confirm that PQ induce oxidative stress by elevation in MDA levels, inflammation by infiltration of inflammatory cells in lung alveoli and fibrosis by elevation of tissue HP and presence of fibroblasts. Treatment with Allo, especially in receiving groups of 100 and 200 mg/kg decreased HP, MDA and lung index. The fibroblast and inflammatory cell infiltration, as well as alveolar thickening as a result of PQ, could be prevented by Allo. Although XO inhibition is the most accepted mechanism of Allo activity, its antioxidative activity and potential as a free radical scavenger are also known. Allo prevents glutathione oxidation and lipid peroxidation, which is related to exhaustive physical

exercise.<sup>16,33-38</sup> It has been reported that XO inhibitors suppress oxidative stress and inflammation in liver damage induced by carbon tetrachloride and cirrhosis,<sup>39</sup> as well as doxorubicin-induced cardiotoxicity in rats.<sup>40</sup> As shown in Figure 6, PQ induces oxidative stress and consequently oxidative damage which lead to pulmonary fibrosis. Allo prevents PQ fibrotic effects possibly through suppression of superoxide anion generation.

### Conclusion

The present findings showed that PQ administration leads to lung fibrosis in rats by increasing oxidative stress. Allo prevents fibrosis dose-dependently through its antioxidant properties.

### Acknowledgments

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**Conflict of interests**

The authors claim that there is no conflict of interest.

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