The dose of cyclophosphamide for treating paraquat-induced rat lung injury

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Background/Aims: Cyclophosphamide (CP) is a promising treatment for severe cases of paraquat (PQ) poisoning. We investigated the effective dose of CP for mitigating PQ-induced lung injury.

Methods: Adult male Sprague-Dawley rats were allocated into five groups: control, PQ (35 mg/kg, intraperitoneal injection), and PQ + CP (1.5, 15, or 30 mg/kg). The dimensions of lung lesions were determined using X-ray microtomography (micro-CT), and histological changes and cytokine levels were recorded.

Results: The micro-CT results showed that 15 mg/kg CP was more effective than 1.5 mg/kg CP for treating PQ-induced lung injury. At a dose of 1.5 mg/kg, CP alleviated the histological evidence of inflammation and altered superoxide dismutase activity. Using 15 mg/kg CP reduced the elevated catalase activity and serum transforming growth factor (TGF)-β1 level.

Conclusions: A CP dose of > 15 mg/kg is effective for reducing the severity of PQ-induced lung injury as determined by histological and micro-CT tissue examination, possibly by modulating antioxidant enzyme and TGF-β1 levels.

Keywords: Paraquat; Cyclophosphamide; Reactive oxygen species; X-ray microtomography
DNA strands at guanine N-7 positions (called interstrand and intrastrand cross-linkages) and consequently suppresses the immune system. Although CP has the potential to be a clinically effective treatment for patients with PQ intoxication, its dose and effectiveness remain controversial. In the 1980s, a CP dose of 5 mg/kg was used, whereas a dose of 15 mg/kg is currently recommended for PQ intoxication. The use of CP as an antidote must be managed carefully because inappropriate administration of CP to rats has been shown to result in fatal lung fibrosis [13,14].

In this study, we investigated the effectiveness of various doses of CP for suppressing the size of lung lesions assessed via X-ray microtomography (micro-CT) and histological and biochemical tests in PQ-intoxicated rats.

METHODS

Animals
Adult male Sprague-Dawley rats (body weight, 280 to 300 g), maintained on a standard laboratory diet and water, were used. The rats were divided into five groups. Control rats received intraperitoneal injections of 1 mL saline (n = 5). PQ rats received intraperitoneal injections of 35 mg/kg PQ (Sigma Chemical, St. Louis, MO, USA) in 1 mL saline (n = 10). PQ + CP rats received intraperitoneal injections of CP (at 1.5, 15, or 30 mg/kg, n = 10 for each dose; Sigma Chemical) immediately after the PQ injections. In a preliminary study of ours, all rats (n = 10) died after an intraperitoneal injection of 40 mg/kg PQ and all rats survived after an intraperitoneal injection of 20 mg/kg PQ. An intraperitoneal injection of 35 mg/kg PQ led to 60% mortality. Therefore, we used this dose as the lethal dose of PQ in this study. After 72 hours, the rats were anesthetized with a mixture of Zoletil (anesthetic) and Rompun (muscle relaxant) and exsanguinated via the abdominal aorta. Then the chest wall was opened and blood samples were collected and centrifuged, and serum was stored at -70°C.

Image acquisition using micro-CT
After exsanguination, 4 to 5 mL air was instilled via the trachea, and then the right main bronchus was tied to prevent air leakage. The right lung was removed and scanned using micro-CT (desktop Micro-CT SkyScan 1172, SkyScan, Aartselaar, Belgium). The specimens were attached to a stage that rotated 360°, with images acquired every 0.7°. CT was performed at settings of 100 kVp and 100 μA. The scanned data were reconstructed using 1,000 x 1,000 matrices (26.5 μm pixel size) and 26.5 μm sections. Peripheral patchy consolidation and ground-glass opacities represented lung injury. In the axial images, the area of lung injury was measured in the right lower lobe (RLL) of the lung at three levels: 1) the bifurcation of the first branch of the bronchus, 2) the middle portion of the RLL, and 3) the peripheral portion of the RLL. The area of lung injury expressed as the percentage of the total axial lung area was calculated using CT analyzer version 1.11.10.0+ (SkyScan).

Surgical procedures and tissue processing for structural analysis
After micro-CT, the right lung was inflated using a fixative (4% [v/v] buffered formaldehyde; in situ fixation). The lungs were dissected free and submitted for routine histological procedures for qualitative structural analysis. Briefly, cubes of lung were fixed by immersion for 24 hours, and then dehydrated with graded ethanol and embedded in paraffin. The serial sections were mounted on silane-coated slides. Inflammation, alveolar thickness, and hemorrhage in hematoxylin and eosin (H&E)-stained sections were evaluated by a pathologist according to the modified methodology described by Szapiel et al. [15]. Alveolar wall thickness was graded using the following criteria: 0, no alveolitis; 1+, mild thickening of the alveolar septum, involving less than 20% of the lung and accompanying good preservation of the alveolar architecture; 2, moderate thickening of the alveolar septum, involving 20% to 50% of the lung; and 3, severe thickening of the alveolar septum, affecting more than 50% of the lung. The amount of cellular infiltration in the damaged lungs was graded using the following criteria: 0, no infiltration; 1, mild inflammatory cell infiltration, involving less than 20% of the lung; 2, moderate infiltration, involving 20% to 50% of the lung space; and 3, severe infiltration, involving more than 50% of the lung. Hemorrhage was graded using the following cri-
criteria: 0, no hemorrhage; 1, mild hemorrhage, involving less than 20% of the lung; 2, moderate hemorrhage, involving 20% to 50% of the lung; and 3, severe hemorrhage, involving more than 50% of the lung. The mean of the grades measured at three levels in horizontal images of the lower lung was determined.

**Cytokine measurement**

After the pulmonary circulation was flushed with 10 to 20 mL phosphate-buffered saline injected into the right heart chamber, the left lung was placed in a tube, frozen rapidly in enzyme-linked immunosorbent assay (ELISA) buffer, and stored at -70°C.

Lung tissue was placed in homogenizing buffer (50 mM Tris-HCl at pH 7.5, containing 1 methylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, and 2.5 mM N-ethylmaleimide) at a ratio of 1 g lung tissue to 9 mL homogenizing buffer. Then the lung tissue was homogenized on ice using a Polytron (Brinkman Instruments, Westbury, NY, USA). The lung homogenates were spun for 5 minutes at 300 x g to sediment the tissue debris. The fluorometric method of Ohkawa et al. [16] (excitation at 532 nm; emission at 551 nm) was used to determine the tissue superoxide dismutase (SOD) and catalase activities. Transforming growth factor (TGF)-β1, TNF-α, and interleukin (IL)-6 were measured in serum samples using commercial ELISA kits (Quantikine, R&D Systems, Minneapolis, MN, USA).

**Statistical analysis**

The results are presented as the mean ± SD. The control, PQ-treated, and PQ + CP groups were compared using the Kruskal-Wallis test. Pairs of groups were compared using the Mann-Whitney U test. The statistical analyses were performed using SPSS version 14.0 (SPSS Inc., Chicago, IL, USA). A p < 0.05 was considered statistically significant.

**RESULTS**

**Mortality**

Of the 10 PQ-treated rats (35 mg/kg), six died before sacrifice. The timing and number of deaths in the PQ group were as follows: 0 at < 24 hours, 2 at 24 to 48 hours, and 4 at 48 to 72 hours. All of the deaths in the CP-treated groups were observed at 48 to 72 hours. The numbers of dead rats are shown in Table 1.

**Light microscopy and micro-CT**

Fig. 1 illustrates the structural changes in lungs and palliative effects of CP on the severity of PQ-induced damage. Micro-CT showed that CP reduced the severity of lung lesions (Fig. 2). CP at a dose of 15 mg/kg was more effective at reducing PQ-induced lung injury than 1.5 mg/kg CP (Fig. 3). The sizes of the lesions were not significantly different between the CP doses of 30 and 15 mg/kg.

Histological changes were assessed using H&E-stained sections. The mean semiquantitative results of the microscopic observation are summarized in Table 2. In summary, the severity of inflammation was reduced by CP doses > 1.5 mg/kg.

**PQ-induced oxidative damage and circulating inflammatory cytokines**

SOD and catalase activities decreased significantly (p < 0.05) in PQ-treated rats compared to the control group (Table 3). SOD activity returned to control levels at CP doses > 1.5 mg/kg. Catalase activity returned to control levels at CP doses > 15 mg/kg.

**Table 1. Mortality in each group 72 hours after no treatment, paraquat injection, and paraquat and cyclophosphamide injections**

|                | Control | PQ (35 mg/kg) | PQ + CP (1.5 mg/kg) | PQ + CP (15 mg/kg) | PQ + CP (30 mg/kg) |
|----------------|---------|---------------|---------------------|--------------------|-------------------|
| Starting no.   | 5       | 10            | 10                  | 10                 | 10                |
| No. of deaths (%) | 0 (0)   | 6 (60)        | 1 (10)              | 1 (10)             | 2 (20)            |
| Final no.      | 5       | 4             | 9                   | 9                  | 8                 |

PQ, paraquat; CP, cyclophosphamide.
To investigate the effect of PQ on circulating inflammatory cytokines, specifically TGF-β1, IL-6, and TNF-α, we measured their levels in blood. TGF-β1 levels were significantly higher in PQ-treated rats than in the control group \((p < 0.05)\). CP at doses > 5 mg/kg reduced the elevated levels of TGF-β1. IL-6 and TNF-α levels did not differ between PQ-treated rats and the control group, and were higher in the CP-treated groups than in the PQ-treated group.

**DISCUSSION**

PQ is a pesticide, which when ingested, is highly toxic to humans. PQ accumulates in the lungs via the alveolar cells, inducing the production of intracellular reactive oxygen species (ROS) and the development of lung inflammation and fibrosis. Early therapies have concentrated on reducing PQ absorption from the gastrointestinal tract and increasing its elimination. Unfortunately, there is no substantiated clinical evidence that either reducing PQ absorption (using Fuller’s earth, bentonite, or activated charcoal) or increasing PQ elimination (using forced diuresis, hemodialysis, or hemofiltration) increases survival \([1,3,17]\). Interestingly, however, many clinical studies have shown that combined treatment with methylprednisolone and CP pulse therapy improves the survival rates of severely

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**Figure 1.** Photomicrographs of lung sections stained with H&E (original magnification \(\times 100\)). (A) Healthy control with a normal lung structure and no evidence of increased alveolar wall thickness, hemorrhage, or cellular infiltration. (B) Paraquat injection (35 mg/kg) only, with numerous inflammatory cells infiltrating the alveolar septum and spaces together with hemorrhage and congestion. Paraquat plus (C) 1.5, (D) 15, and (E) 30 mg/kg cyclophosphamide. There is a decrease in inflammatory cell infiltration and alveolar wall thickness from panel (C) to (E).
PQ-intoxicated patients [7-10,18].

CP has a wide range of immunomodulatory effects, which influence virtually all components of the cellular and humoral immune response and reduce the severity of inflammation. In early studies, high doses of CP produced leukopenia in 1 to 2 weeks and reduced the severity of PQ-related inflammation in poisoned patients, suggesting that immunomodulatory agents are useful tools for treating severe PQ intoxication [19,20]. It is also interesting that high doses of CP (200 mg/kg) have the potential to cause fatal lung injuries [13,14]. However, this finding has generated controversy, and the appropriate dose of CP and its anti-inflammatory mechanism in the treatment of PQ intoxication remain unknown. Clinical studies have recommended a CP dose of 15 mg/kg, although some studies have shown that a lower dose (5 mg/kg) can reduce the clinical severity of PQ intoxication [8,18,19]. No studies have compared the

Figure 2. Microtomography (micro-CT) images at the level of the segmental bronchus of the right lower lobe showing patchy peripheral consolidations. (A) A micro-CT image showing patchy peripheral consolidations due to manipulative injury during extraction in a control rat. (B) Approximately 80.7% of the lung was injured in a rat injected with paraquat at 35 mg/kg on the micro-CT image. In rats injected with 30 mg/kg paraquat and then with (C) 1.5, (D) 15, or (E) 30 mg/kg cyclophosphamide, the area of lung injury was 26.2%, 6.5%, and 7.5%, respectively.

Figure 3. Area of lung injury on microcomputed tomography images. Cyclophosphamide (CP) suppressed the area of injury compared to that in the paraquat (PQ) group.

*p < 0.05 compared to the paraquat group.
effects of various CP doses on the severity of PQ intoxication.

We measured SOD and catalase activities in the entire lung because we believe that the levels of ROS-related enzymes reflect the extent of PQ-induced lung injury. In our study, 1.5 mg/kg CP increased SOD activity compared to the PQ-treated group. The SOD activity increased more with 15 mg/kg CP than with 1.5 mg/kg CP, while there was no difference between the animals receiving 15 and 30 mg/kg CP. Catalase activity was alleviated using CP doses > 15 mg/kg. This supports the idea that an anti-inflammatory agent can suppress ROS-induced inflammation and increase antioxidant enzyme levels. This finding is also consistent with previous studies [21,22].

The growth factor TGF-β1 initiates tissue repair; its sustained production can underlie the development of tissue fibrosis [23], and it is an important upstream effector of collagen gene expression [24]. In our study, the TGF-β1 level increased 72 hours after PQ intoxication and was reduced by administering CP doses > 15 mg/kg. This suggests that CP modulates the TGF-β1 level, thereby reducing ROS-induced lung injury.

Interestingly, IL-6 and TNF-α levels did not increase in the PQ group, but increased in the CP group. IL-6 is both a proinflammatory and anti-inflammatory cytokine. Lee et al. [21] showed that IL-6 plasma levels were not elevated at 6 and 12 hours in a PQ-intoxicated rat model (50 mg/kg), although a different study showed that IL-6 levels in lung tissues were higher 1 day after PQ injection in a rat model (18 mg/kg) [25]. Although some studies have suggested that TNF-α is involved in

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Table 2. Morphological evidence of lung injury after no treatment, paraquat injection, and paraquat and cyclophosphamide injections

|                      | Control | PQ (35 mg/kg) | PQ + CP (1.5 mg/kg) | PQ + CP (15 mg/kg) | PQ + CP (30 mg/kg) | p value<sup>a</sup> |
|----------------------|---------|---------------|---------------------|--------------------|--------------------|---------------------|
| Hemorrhage           | 0 ± 0   | 0.2 ± 0.45    | 0 ± 0              | 0.1 ± 0.2          | 0 ± 0              | 0.366               |
| Cellular infiltration| 0.3 ± 0.57 | 1.8 ± 0.84    | 1.0 ± 0.23<sup>b</sup> | 1.0 ± 0.20<sup>b</sup> | 0.6 ± 0.19<sup>b,c</sup> | 0.015               |
| Alveolar septum thickness | 0 ± 0   | 1.6 ± 0.89    | 0.2 ± 0.21<sup>b</sup> | 0.2 ± 0.42<sup>b</sup> | 0.1 ± 0<sup>b</sup> | 0.001               |

Values are presented as mean ± SD.
PQ, paraquat; CP, cyclophosphamide.
<sup>a</sup>Kruskal-Wallis test.
<sup>b</sup>p < 0.05 compared to the PQ group.
<sup>c</sup>p < 0.05 compared to the PQ + CP 1.5 group.

Table 3. Superoxide dismutase and catalase levels in the lung tissues and transforming growth factor-β1, interleukin-6, and tumor necrosis factor-α in the blood after no treatment, paraquat injection, and paraquat and cyclophosphamide injections

|                      | Control | PQ (35 mg/kg) | PQ + CP (1.5 mg/kg) | PQ + CP (15 mg/kg) | PQ + CP (30 mg/kg) | p value<sup>a</sup> |
|----------------------|---------|---------------|---------------------|--------------------|--------------------|---------------------|
| SOD, U/g protein     | 139.8 ± 32.8 | 35.8 ± 5.9<sup>b</sup> | 163.8 ± 58.1<sup>c</sup> | 185.9 ± 62.3<sup>c</sup> | 177.1 ± 131.9<sup>c</sup> | 0.014               |
| Catalase, U/g protein| 19.9 ± 10.6 | 2.9 ± 1.4<sup>b</sup> | 7.3 ± 4.8<sup>b</sup> | 7.7 ± 3.9<sup>c</sup> | 17.2 ± 8.5<sup>c</sup> | 0.003               |
| TGF-β1, ng/g protein | 6.3 ± 0.5   | 8.8 ± 1.0<sup>b</sup> | 8.6 ± 1.5<sup>b</sup> | 6.8 ± 1.6          | 7.4 ± 0.7          | 0.016               |
| IL-6, pg/g protein   | 13.3 ± 2.2  | 13.2 ± 0.7    | 14.9 ± 1.1<sup>c</sup> | 14.8 ± 2.0         | 14.7 ± 1.2         | 0.154               |
| TNF-α, pg/g protein  | 1.1 ± 0.1   | 1.2 ± 0.1     | 1.3 ± 0.2<sup>c</sup> | 1.3 ± 0.1          | 1.4 ± 0.1<sup>c</sup> | 0.028               |

Values are presented as mean ± SD.
PQ, paraquat; CP, cyclophosphamide.
<sup>a</sup>Kruskal-Wallis test.
<sup>b</sup>p < 0.05 compared to the control group.
<sup>c</sup>p < 0.05 compared to the PQ group.
PQ-induced lung injury [26, 27], the time sequence and peak of TNF-α have yet to be revealed in PQ intoxication. Therefore, it is necessary to investigate time sequence variation in IL-6 and TNF-α in PQ intoxication further. In addition, we measured these cytokines in serum, which might not reflect the cytokine levels in lung tissue.

Because the extent of lung injury is very important for predicting patient mortality in clinical situations [28, 29], it is noteworthy that CP attenuated the extent of PQ-induced lung lesions as determined by micro-CT images in our study. This finding has not been reported previously. The micro-CT findings indicated that a CP dose of 15 mg/kg was optimal for effectively reducing the extent of lung injury, although the histological improvement might be greater with a CP dose of 30 mg/kg.

The amount of CP administered is important because cumulative and high doses of CP have many potential adverse effects, including lung injury and hemorrhagic cystitis. In our study, 30 mg/kg CP was no better than 15 mg/kg CP according to our micro-CT and TGF-β1 data. As such, 15 mg/kg CP appeared to be the optimal dose for reducing PQ-induced lung injury. Although our study suggests an optimal dose of CP, future studies should investigate combination therapy with CP and glucocorticoids.

In conclusion, a CP dose > 15 mg/kg was effective at reducing the severity of PQ-induced lung injury as determined by histological and micro-CT tissue examination, possibly by modulating levels of antioxidant enzymes and TGF-β1.

**KEY MESSAGE**

1. Cyclophosphamide (CP) is effective treatment in paraquat intoxicated rats.
2. A CP dose of > 15 mg/kg is effective for reducing the severity of paraquat-induced lung injury, possibly by modulating antioxidant enzyme and transforming growth factor-β1 levels.

**Conflict of interest**

No potential conflict of interest relevant to this article is reported.

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