

## Effects of hydrogen water on paraquat-induced pulmonary fibrosis in mice

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**Objective:** Chronic paraquat (PQ) intoxication causes pulmonary fibrosis. PQ-induced pulmonary injury is caused by reactive oxygen species (ROS) that are produced during the reduction-oxidation (redox) cycle. In addition, the hydrogen has antioxidative effects. The present study aimed to determine whether or not the intake of hydrogen water (HW) prevents pulmonary injury caused by chronic PQ intoxication.

**Methods:** PQ was administered to 10-week-old male C57BL/6 mice, and the mice were randomly assigned to either the HW group (PQ + HW group) or the tap water group (PQ group). After 3 weeks, the mice were assessed for pneumodynamic and histologic changes.

**Results:** Pneumodynamic analysis revealed that elastance (E) ( $P = 0.010$ ) and hysteresivity ( $\eta$ ) ( $P = 0.048$ ) were significantly lower in the PQ + HW group than that in the PQ group. Although no significant difference was observed in compliance, it tended to be higher in the PQ + HW group. Histologic findings showed that inflammatory cell infiltration and fibrosis were comparatively lower in the PQ + HW group.

**Conclusion:** The results of the present study suggest that HW may prevent the development of PQ-induced pulmonary injury.

**Key words:** paraquat-induced pulmonary fibrosis, hydrogen water

### Introduction

Paraquat (PQ) (1,1'-dimethyl-4,4'-bipyridinium dichloride) has been used as an herbicide for many years in Asian countries, including Japan. However, due to a large number of fatal accidents, the concentration of PQ in various products has been lowered, and it is now mandatory to present a photograph ID to purchase PQ herbicides. Nevertheless, patients are still sometimes admitted to the hospital for attempted suicide by PQ poisoning. The mortality rate for PQ poisoning is as high as 60%, and patients who survive the acute poisoning often die later due to respiratory failure.

When PQ enters the cells, it undergoes repeated reduction-oxidation (redox) cycling, which produces a variety of reactive oxygen species (ROS) leading to irreversible fibrosis of pneumocytes.<sup>1-7</sup> On the other hand, hydrogen water (HW), a colorless, tasteless, and odorless gas, has been widely reported for its antioxidative effects.

Ohsawa et al. have previously reported that the antioxidative effects of HW are produced through the selective reduction of strong oxidizing ROS such as hydroxyl radicals and peroxynitrite.<sup>8</sup> The methods of administration of hydrogen ( $H_2$ ) include inhalation of  $H_2$  gas, drinking HW, and intraperitoneal or intravenous administration of saline solution with dissolved  $H_2$ .  $H_2$  has already been shown to be effective in animal experiments involving cisplatin-related renal damage,<sup>9</sup> atherosclerosis,<sup>10</sup> and metabolic syndrome.<sup>11</sup> Clinical studies on the amelioration of Parkinson's disease are currently being conducted.<sup>12</sup>

Various models of pulmonary injury have been prevented by  $H_2$ .<sup>13-15</sup> Therefore, it is expected that  $H_2$  is effective for PQ-induced pulmonary injury.

We administered PQ to mice and comparatively examined those that were treated with HW and those that received tap water. We then performed pneumodynamic and histologic assessments of the effects of oral

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administration of HW on PQ-induced pulmonary injury.

## Materials and Methods

### *Animals*

Ten-week-old male C57BL/6 mice (CLEA) were used. The mice were placed in a room with the appropriate humidity and temperature and acclimated for 1 week using a 12-hour light/12-hour dark cycle. The mice were randomly assigned to two groups; one group received PQ and HW in their drinking water (PQ + HW group,  $n = 12$ ) and the other group received PQ and tap water (PQ group,  $n = 9$ ). All animal experiments were performed in accordance with the guidelines for animal experiments of the Kitasato University School of Medicine (2014-048).

### *PQ ingestion*

An anesthesia system (SN-487-OT air + O<sub>2</sub>; Shinano, Tokyo) designed for laboratory animals was used for the administration of isoflurane (Forane; Abott Japan, Tokyo) via inhalation. An anterior cervical T-shaped incision was then created to fix the trachea onto the surface of the body. An intratracheal aerosolizer (MicroSprayer, IA-1C-M; PennCentury, Pennsylvania, USA) was inserted into the oral cavity and directed into the trachea. Upon a visual confirmation of the presence of the aerosolizer in the trachea, a 20- $\mu$ l dose of a 0.25 mg/ml PQ solution (Tokyo Kasei Kogyo, Tokyo) was intratracheally administered. After decannulation, the wound was closed using nylon sutures.

### *HW*

HW (Blue Mercury, Tokyo) containing supersaturated molecular H<sub>2</sub> at a pressure of 0.4 MPa was used. HW was transferred from an aluminum bag to a closed glass vessel connected to a double ball-bearing water pipe and silicon cap, allowing the mice to drink water *ad libitum*. The bottles were changed once a day and residual H<sub>2</sub> gas was monitored using a dissolved H<sub>2</sub> analyzer (HMD-X1; Hikari Bellcom, Kanagawa). Ohsawa et al. used a similar method for the administration of HW, and an H<sub>2</sub> concentration of  $\geq 0.4$  mM was maintained after the first day using an H<sub>2</sub> electrode.<sup>10</sup> Tap water was provided by an automatic water feeder, allowing the mice to drink water *ad libitum*.

### *Measurement of lung function*

Three weeks after intratracheal administration of PQ, pentobarbital was intraperitoneally administered and a tracheal incision was made under anesthesia. An 18 G

hypodermic needle was inserted into the 4-mm incision, and flexiVent (SCIREQ Scientific Respiratory Equipment, Montreal, Canada), a high-performance respiratory function analyzing computer system, was loaded. An incision was made on the diaphragm to stop spontaneous respiration and was ventilated at a rate of 150 beats/minute and at a volume of 10 ml/kg. To prevent lung collapse, PEEP (positive end-expiratory pressure) at 2–3 cm H<sub>2</sub>O was applied.

First, for total lung capacity (TLC), the lungs were inflated to 30 cm H<sub>2</sub>O for 3 seconds to stabilize the lungs. Snapshot measurements were then taken to calculate resistance (R), compliance (C), and elastance (E) of the respiratory system as a single compartment model, which includes the trachea, lungs, and thoracic wall. The pistons of the ventilator were spun multiple times at a fixed pace, and the resulting volume changes in quasi-sinusoidal and airway pressures measured at this time were used to calculate R, C, and E.

Forced oscillation technique (FOT) measured respiration that occurred at random frequencies (0.25–20 Hz), and Newtonian resistance (R<sub>n</sub>), tissue damping (G), tissue elastance (H), and eta (hysteresivity) were measured for each frequency. A comparison of these parameters in the central and peripheral airways was then performed.

TLC, Snap Shot (SCIREQ Scientific Respiratory Equipment), and FOT were continuously measured and conducted in triplicate. The data were analyzed using the flexiVent software.

### *Lung histopathology*

After performing flexiVent measurements, the mice were euthanized using an overdose of pentobarbital, an anesthetic agent. For histological assessment, the lungs were isolated, fixed with 4% paraformaldehyde, and then embedded in paraffin. Lung sections of 3- $\mu$ m thickness were then prepared and subjected to hematoxylin and eosin (H&E) and Heidenhain's Azan staining.

### *Statistical analysis*

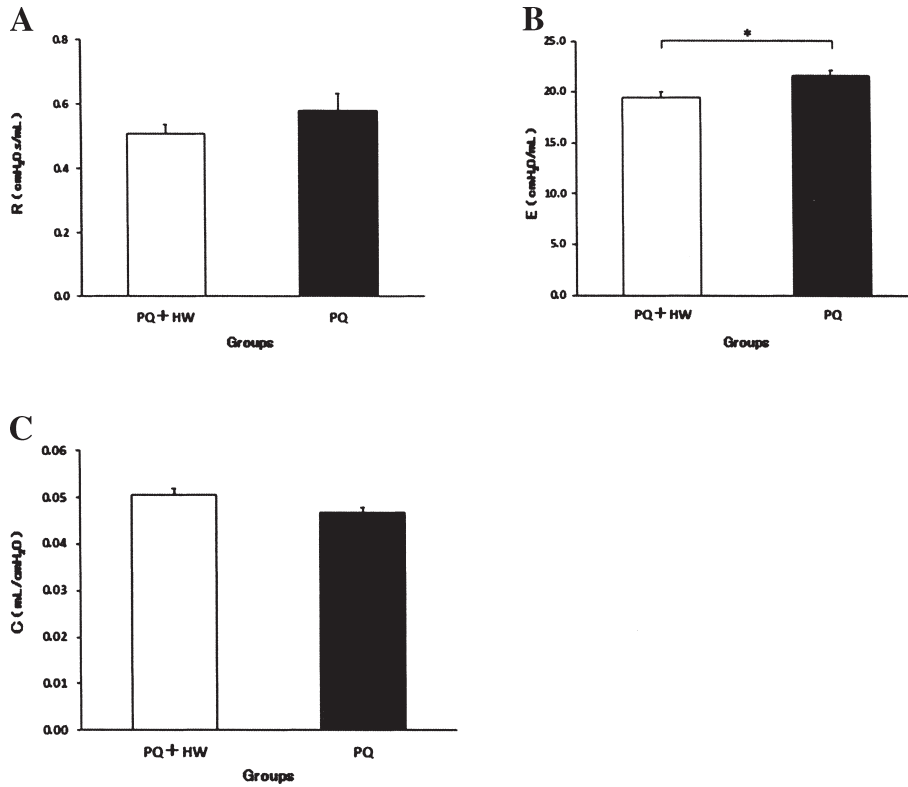
All data were expressed as the mean  $\pm$  standard error of the mean. Both groups were compared using the unpaired student's *t*-test. A P-value of  $<0.05$  was considered to indicate statistical significance.

## Results

### *Lung function*

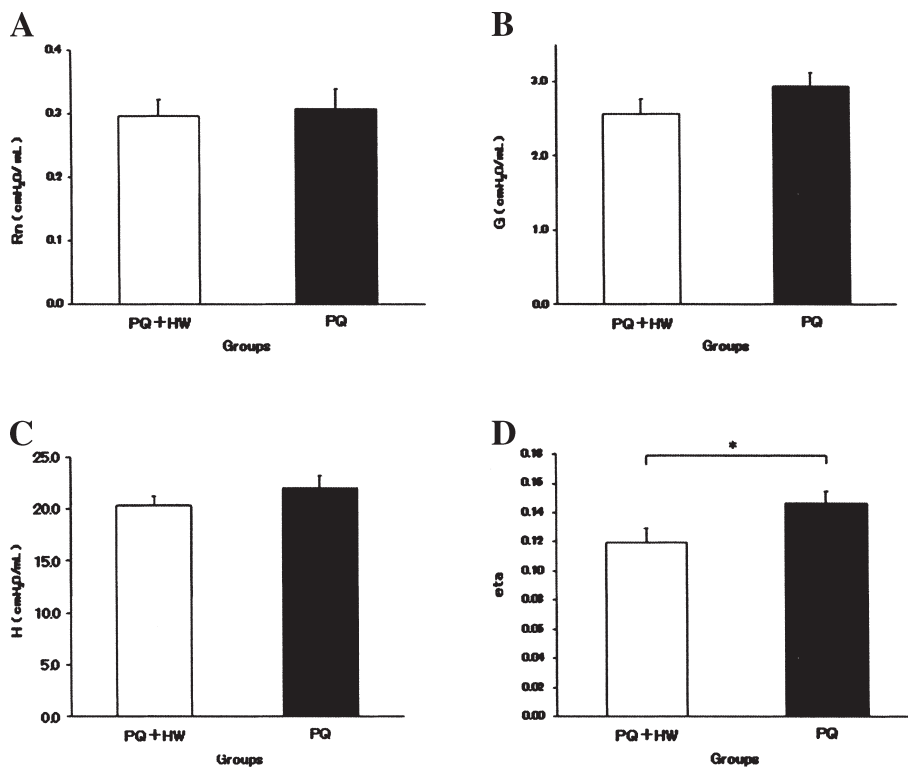
We comparatively examined the PQ + HW and PQ groups to ascertain whether or not HW could reduce PQ-induced

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**Figure 1.** Snap Shot (SCIREQ Scientific Respiratory Equipment)

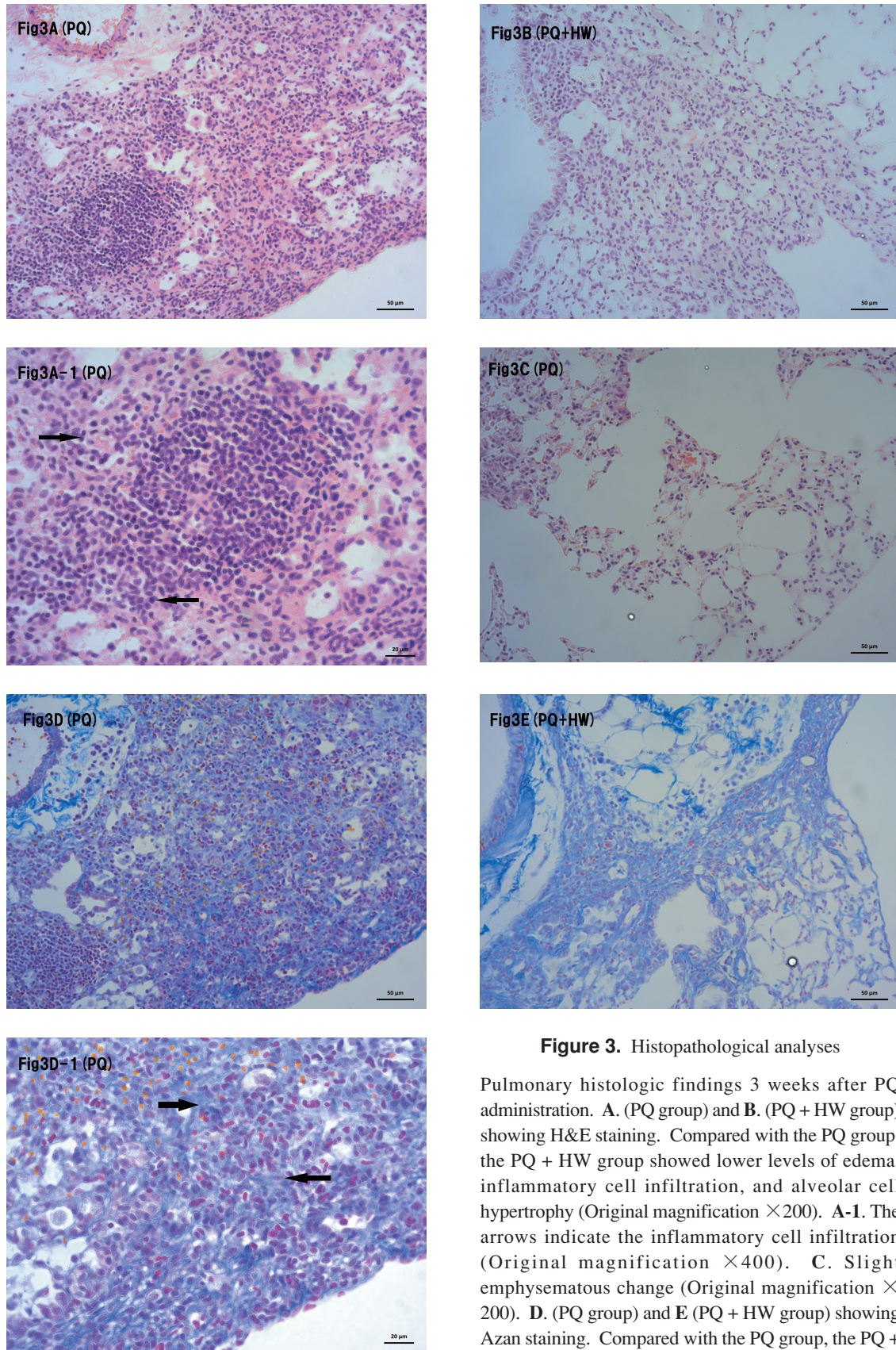
Lung function 3 weeks after the administration of PQ by Snap Shot showed: A. resistance (R), B. elastance (E), and C. compliance (C). \*P = 0.010



**Figure 2.** FOT

Lung function 3 weeks after the administration of PQ by FOT showed A. Newtonian resistance (Rn), B. tissue damping (G), C. tissue elastance (H), and D. hysteresivity (eta). \*P = 0.048





**Figure 3.** Histopathological analyses

Pulmonary histologic findings 3 weeks after PQ administration. **A.** (PQ group) and **B.** (PQ + HW group) showing H&E staining. Compared with the PQ group, the PQ + HW group showed lower levels of edema, inflammatory cell infiltration, and alveolar cell hypertrophy (Original magnification  $\times 200$ ). **A-1.** The arrows indicate the inflammatory cell infiltration (Original magnification  $\times 400$ ). **C.** Slight emphysematous change (Original magnification  $\times 200$ ). **D.** (PQ group) and **E.** (PQ + HW group) showing Azan staining. Compared with the PQ group, the PQ + HW group showed milder fibrosis (Original magnification  $\times 200$ ). **D-1.** Arrows indicate fibrosis (Original magnification  $\times 400$ ).

pulmonary injury. The Snap Shot results are shown in Figure 1. No significant difference in R was observed between the PQ + HW and PQ groups ( $0.507 \pm 0.03$  vs.  $0.577 \pm 0.054$ , respectively;  $P = 0.249$ ) (Figure 1A). E was significantly lower in the PQ + HW group than that in the PQ group ( $19.506 \pm 0.491$  vs.  $21.569 \pm 0.56$ , respectively;  $P = 0.010$ ) (Figure 1B). No significant difference was detected for C between the PQ + HW and PQ groups, although C was higher in the PQ + HW group compared with that of the PQ group ( $0.051 \pm 0.001$  vs.  $0.047 \pm 0.001$ , respectively;  $P = 0.0628$ ) (Figure 1C).

The results of FOT are shown in Figure 2. No significant difference in Rn was observed ( $0.297 \pm 0.025$  vs.  $0.307 \pm 0.033$ , respectively;  $P = 0.806$ ) (Figure 2A). No significant significance was observed for G and H (G,  $2.565 \pm 0.202$  vs.  $2.939 \pm 0.187$ , respectively,  $P = 0.204$ ; H,  $20.345 \pm 0.927$  vs.  $21.764 \pm 1.073$ , respectively,  $P = 0.326$ ) (Figures 2B and C), although eta was significantly lower in the PQ + HW group than that in the PQ group ( $0.120 \pm 0.009$  vs.  $0.146 \pm 0.008$ , respectively,  $P = 0.048$ ) (Figure 2D).

#### Histopathological analyses

Figure 3 shows the pulmonary histologic findings for the PQ + HW and PQ groups. In the PQ group, edema, inflammatory cell infiltration, and alveolar wall hypertrophy were observed (Figure 3A, A-1). In the PQ + HW group, some inflammatory cell infiltration was observed; however, compared with the PQ group, the infiltration was lower, the sites of origin were smaller (Figure 3B). In the PQ group, there are slight emphysematous changes (Figure 3C).

Partial fibrosis had begun developing in the PQ group (Figure 3D, D-1). Fibrosis was either not present or only mild in the PQ + HW group (Figure 3E).

## Discussion

Treatments such as gastrointestinal lavage, the administration of activated charcoal, hemodialysis, and direct hemoperfusion are often performed in cases of PQ poisoning. However, there is no evidence that these methods can ameliorate pulmonary injury.<sup>16</sup> Pulmonary injuries are substantially due to pulmonary fibrosis. Studies have pointed out that PQ-induced oxidative stress is involved in pulmonary fibrosis, therefore, a specific antidote to inhibit this mechanism is currently being investigated. Superoxide dismutase (SOD), liposomal encapsulated SOD, the anti-inflammatory actions of cyclophosphamide and glucocorticoids, the antioxidative

effects of vitamin C (ascorbic acid), vitamin E ( $\alpha$ -tocopherol), melatonin, glutathione (GSH), N-acetylcysteine, and iron chelators have all been studied. SOD, vitamin C, vitamin E, and GSH were unable to cross the cell membrane barrier, and their clearance appeared to be limited or not at all occurring.<sup>7</sup>

The effects of cyclophosphamide and glucocorticoids were negated in the largest randomized controlled study consisting of 298 patients.<sup>17</sup> Earlier *in vitro* and *in vivo* studies have shown that melatonin, N-acetylcysteine, and iron chelators prevent PQ poisoning; however, they have not yet been therapeutically used in humans.<sup>5,18-20</sup> Therefore, although an antidote for PQ has been shown to be effective in animal experiments and studies, no antidote for humans has been identified.

As PQ undergoes the redox cycle, it produces ROS, which has been previously shown to be correlated with ischemic reperfusion injury, Parkinson's disease, arteriosclerosis, metabolic syndrome, and aging. ROS possess hydroxyl radicals ( $\cdot\text{OH}$ ) and peroxynitrite ( $\text{ONOO}^-$ ), which have strong oxidative powers, as well as interact and damage DNA, proteins, and lipids. However, superoxides ( $\cdot\text{O}_2^-$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) have signal transduction functions at low concentrations,<sup>21-23</sup> making them extremely important. Antioxidants such as vitamin C, which has a strong reducing power, can eliminate all ROS, which disrupts the signal transduction pathways in the body. Furthermore, vitamin C produces substances with strong oxidative power after redox reactions, resulting in DNA damage.<sup>24,25</sup>

Because the reducing power of molecular  $\text{H}_2$  is weak, it only reduces ROS with a strong oxidizability such as hydroxyl radicals ( $\cdot\text{OH}$ ) and peroxynitrite ( $\text{ONOO}^-$ ).<sup>8</sup> Furthermore, because the amount of  $\text{H}_2$  is small, it can easily cross the cell membrane and reach the nucleus and mitochondria; thus, protecting the cell from oxidation.

Previous studies have shown that  $\text{H}_2$  is safe.  $\text{H}_2$  gas has been used for the prevention of decompression illness in divers.<sup>26-28</sup> Furthermore, there were various administration methods for  $\text{H}_2$  such as drinking HW water, the inhalation of  $\text{H}_2$  gas, and the intraperitoneal or intravenous administration of normal saline solution containing  $\text{H}_2$ , which have all been shown to have antioxidative efficacy.<sup>29</sup>

The administration of HW to animal models has been shown to be effective for the treatment of Parkinson's disease,<sup>30,31</sup> atherosclerosis,<sup>10</sup> metabolic syndrome,<sup>11</sup> and cisplatin induced nephrotoxicity.<sup>9,32</sup> The administration of hydrogen gas has been shown to improve prognoses for cerebral infarction,<sup>8</sup> myocardial infarction,<sup>33</sup> and



postcardiac arrest brain injury.<sup>34</sup> A randomized double-blind placebo-controlled trial of Parkinson's disease has resulted in amelioration, based on the Total Unified Parkinson's Disease Rating Scale.<sup>12</sup> Furthermore, clinical studies of ischemic-reperfusion injury in patients with acute myocardial infarction and patients with postresuscitation complications after cardiopulmonary resuscitation outside of the hospital are currently being conducted. The effects of HW on acute PQ poisoning have been shown in studies using rats.<sup>35</sup> In the study, both biochemical findings (amount of pleural effusion, number of cells, amount of protein and LDH in BALF [bronchoalveolar lavage fluid], and MDA levels in pulmonary tissue) and histologic findings indicate the effect 72 hours after PQ administration.

To study the effects of HW on mice, we used a flexiVent to examine the pulmonary functions and pulmonary histologic findings 3 weeks after the administration of PQ when pulmonary fibrosis occurs. Several studies have used flexiVent in assessing various models for pulmonary fibrosis. When pulmonary fibrosis is induced, a decrease in C and increases in R, E, Rn, G, H, and eta were observed.<sup>36-38</sup>

The present study used Snap Shot to show that E was significantly lower in the PQ + HW group compared with that in the PQ group. Although no statistically significant difference were observed, higher C values were observed in the PQ + HW group compared with those in the PQ group. This finding suggests that the lungs of the PQ + HW group had less resistance than those of the PQ group, thus facilitating in their inflation. However, this observation also reflects the condition of the respiratory system, including the lungs, trachea, and parenchymal organs; therefore, requires details that distinguish the central airway, peripheral tissue, and respiratory functions.

Rn is measured in the central airway by FOT. And H, G, and eta can be calculated through the peripheral airways. When fibrosis of the peripheral airways progresses, H, G, and eta increase. But there are many points that are not elucidated regarding eta. It is thought that eta is influenced by various pulmonary mechanical conditions because eta is the ratio G/H or the area in the pressure volume loop. Emphysematous changing or expansion of the peripheral airway with fibrosis has been observed in the chronic phase of PQ-induced lung injury. In this study, there were slight emphysematous changes in the histopathology in the PQ group (Figure 3C).<sup>39</sup> Because of this, it will, perhaps, be observed that G and

H do not change, but eta increased in FOT as a result of combined mechanical change, which is like combined pulmonary fibrosis and emphysema. Eta should not be evaluated alone, but the assessment of both G and H are more important.

In terms of the parameters that did not show a significant difference, we speculate that the low concentration of PQ was responsible for these findings. Approximately 0.5 mg/ml of PQ was administered; however, because 33% of the mice died within 5 days, a 0.25 mg/ml PQ concentration was utilized in the present study. This may have been the reason that the level of lung damage was relatively mild. The selection of amount and PQ administration method to induce pulmonary fibrosis was extremely difficult.

Histologic findings of PQ-induced pulmonary injury by H&E staining showed interstitial edema, pneumocyte hemorrhage, and interstitial inflammation in early stages and an increase in fibroblasts and hypertrophy of alveolar wall cells after 2 or 3 weeks. Furthermore, Azan staining revealed an accumulation of collagen, which is a characteristic of pulmonary fibrosis.<sup>40</sup>

In the present study, H&E and Azan staining revealed less inflammatory cell infiltration and fibrosis in the PQ + HW group compared with that in the PQ group. Because the histologic findings could not be quantified, the effects cannot be fully affirmed; however, the findings suggest that HW was effective for the treatment of PQ-induced pulmonary injury. These findings suggest that HW lessen PQ-induced pulmonary injury. Because HW is safe and imparts no side effects, its intake is easier than that of other medicines. Further studies are required to determine whether HW can be established as a treatment regimen for PQ poisoning in humans.

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