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## Hydrogen inhalation attenuates lung contusion after blunt chest trauma in mice



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### ABSTRACT

**Background:** Lung contusion caused by blunt chest trauma evokes a severe inflammatory reaction in the pulmonary parenchyma that may be associated with acute respiratory distress syndrome. Although hydrogen gas has antioxidant and anti-inflammatory effects and is protective against multiple types of lung injury at safe concentrations, the effects of inhaled hydrogen gas on blunt lung injury have not been previously investigated. Therefore, using a mouse model, we tested the hypothesis that hydrogen inhalation after chest trauma would reduce pulmonary inflammation and acute lung injury associated with lung contusion.

**Methods:** Inbred male C57BL/6 mice were randomly divided into 3 groups: sham with air inhalation, lung contusion with air inhalation, and lung contusion with 1.3% hydrogen inhalation. Experimental lung contusion was induced using a highly reproducible and standardized apparatus. Immediately after induction of lung contusion, mice were placed in a chamber exposed to 1.3% hydrogen gas in the air. Histopathological analysis and real-time polymerase chain reaction in lung tissue and blood gas analysis were performed 6 hours after contusion.

**Results:** Histopathological examination of the lung tissue after contusion revealed perivascular/intra-alveolar hemorrhage, perivascular/interstitial leukocyte infiltration, and interstitial/intra-alveolar edema. These histological changes and the extent of lung contusion, as determined by computed tomography, were significantly mitigated by hydrogen inhalation. Hydrogen inhalation also significantly reduced inflammatory cytokine and chemokine mRNA levels and improved oxygenation.

**Conclusion:** Hydrogen inhalation therapy significantly mitigated inflammatory responses associated with lung contusion in mice. Hydrogen inhalation therapy may be a supplemental therapeutic strategy for treating lung contusion.

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### Introduction

Lung contusion (LC) is a commonly diagnosed intrathoracic injury resulting from blunt chest trauma and is known as a

predominant or contributing factor in trauma-related deaths. Although the pathophysiology of LC is complex, the primary manifestation of LC is the destruction of the alveolar septum and pulmonary interstitium. Hemorrhage into the alveoli and

**Abbreviations:** CCL2, C-C motif chemokine 2; CT, Computed tomography; CXCL2, C-X-C motif chemokine 2; DAB, Diaminobenzidine; HE, Hematoxylin and eosin; IL-1 $\beta$ , Interleukin-1 $\beta$ ; IL-6, Interleukin-6; IL-10, Interleukin-10; LC, Lung contusion; LC Air, Lung contusion exposed to ambient air; LC H<sub>2</sub>, Lung contusion treated with 1.3% hydrogen inhalation for 6 hours; mRNA, Messenger RNA; NE, Neutrophil elastase; PaCO<sub>2</sub>, Arterial CO<sub>2</sub> pressure; PaO<sub>2</sub>, Arterial O<sub>2</sub> pressure; PBS, Phosphate buffered saline; PCR, Polymerase chain reaction; RPL4, Ribosomal protein L4; SEM, Standard error of the mean; TBS-T, Tris buffered saline with 0.1% Tween 20; TNF- $\alpha$ , Tumor necrosis factor- $\alpha$ .

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pulmonary interstitium is associated with the activation of proinflammatory cascades, resulting in increased permeability of the alveolar-capillary membranes and microvascular leakage that causes pulmonary edema, ventilation and/or perfusion mismatch, increased intrapulmonary shunting, airway pressure, and air velocity, and decreased functional residual air volume and lung compliance.<sup>1</sup> Thus, the clinical consequences of LC range from mild dyspnea to acute respiratory distress syndrome requiring prolonged mechanical ventilation and eventually leading to scarring and chronic respiratory failure.<sup>2</sup> In recent years, the prognosis of patients with LC has been improved due to the development of diagnostic tools and respiratory critical care.<sup>3,4</sup> However, game-changing therapeutic and pharmacologic approaches to treating LC have not been discovered.

Molecular hydrogen is a potent antioxidant and anti-inflammatory agent.<sup>5–7</sup> The efficacies of hydrogen inhalation for acute lung injury have been previously shown in various experimental models, including animal models of ventilator-induced lung injury,<sup>6</sup> hyperoxic lung injury,<sup>8</sup> hemorrhagic-shock-induced lung injury,<sup>9</sup> sepsis-induced lung injury,<sup>10</sup> ischemia-reperfusion injury,<sup>11</sup> radiation-induced lung injury,<sup>12</sup> and bronchial asthma.<sup>13</sup> The mechanisms underlying the anti-inflammatory effects of hydrogen, inhibition of inflammatory cytokines, and upstream signaling molecules have been suggested.

A rationale for the biomedical application of small gas molecules has emerged from promising preclinical data establishing the beneficial effects of hydrogen at low or near-physiological doses without toxicity in animal models of injury or disease. The lung and its supporting vasculature are the primary targets for inhalation therapies because inhaled gaseous molecules can reach the alveoli directly.<sup>14</sup> The proposed use of hydrogen gas as a molecular medicine or inhalation therapeutic for human disease has been found to be clinically feasible as long as the gas's flammability can be controlled.<sup>15,16</sup> Inhalation of hydrogen gas may be a straightforward and promising therapeutic option. Therefore, we tested the hypothesis that hydrogen inhalation after chest trauma would reduce pulmonary inflammation and acute lung injury associated with LC in a mouse model.

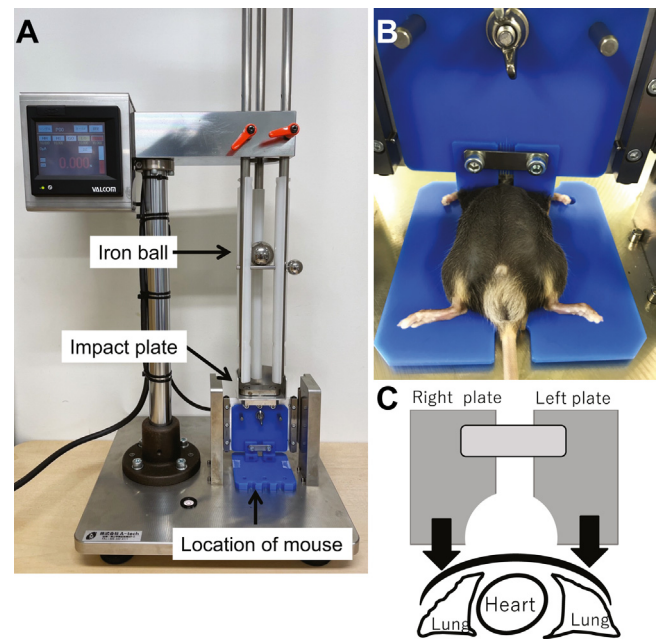
## Methods

### Animals

Six-week-old C57BL/6 male mice (weight  $23 \pm 1.4$  g, specific pathogen-free) were purchased from CLEA Japan Inc (Tokyo, Japan). Mice were maintained at 20° to 22°C with a 12-hour light/dark cycle and fed sterile food and water. All protocols followed Principles of Laboratory Animal Care (NIH Publication no. 86–23, revised 1985), and all research protocols were reviewed and approved by the Okayama University Animal Welfare Committee (OKU-2017294). This study was conducted in accordance with ARRIVE guidelines (<https://arriveguidelines.org/>).

### Lung injury model

Animals were anesthetized with 2.5% isoflurane, and LC was generated using an optimized energy shock to the chest wall with an experimental apparatus designed specifically for this purpose (Figure 1). In brief, mice were placed on a platform in the supine position, and all four legs were attached to the platform with adhesive tape. Two plates designed to avoid myocardial injury and apply energy to both lungs were placed in contact with the chest wall. A steel ball weighing 175 grams was dropped from a 15-cm height. The apparatus was designed to reduce variation in the impact force by using a release mechanism with a stick that supports



**Figure 1.** Experimental apparatus for the mouse lung contusion model. The experimental apparatus used for the high weight-drop method is shown. (A) (A-tech Co., Ltd. Okayama, Japan). An iron ball was placed at a height of 15 cm. Variation in the impact force was reduced with a release mechanism requiring removal of a stick and by verifying that the force/energy applied was uniform (0.013–0.016N). (B) After anesthesia, the mice are laid on the apparatus, and a plate is placed on their chest. (C) A unique plate was created so that the impact was indirectly transmitted from the iron ball to the chest. To avoid shock to the heart, a space was maintained between the 2 shock plates, and the corners of the plates that would touch the chest wall were shaved.

the ball and must be pulled out to drop it, keeping the ball's speed and distance traveled while falling more uniform. The strength of the impact was measured with a sensor to verify uniform conditions. The potential energy can be calculated as  $\text{mass} \times \text{height} \times 0.98$ . Under these conditions, a reproducible lung contusion model was established with the application of 0.013N to 0.016N of force (Figure 1).

### Exposure to hydrogen

For hydrogen gas treatment, cylinders with air-based, high-pressure, premixed gases were purchased (Japan Fine Products, Kanagawa, Japan). The manufacturer confirmed the concentrations of hydrogen ( $\text{H}_2$ , 1.3%), oxygen ( $\text{O}_2$ , 21%), and nitrogen ( $\text{N}_2$ , 77.7%). In Japan, 1.3% is the highest concentration of hydrogen that can be mixed and bottled under high pressure with 21% oxygen for clinical use. Immediately after induction of lung contusion, 5 or fewer mice were placed in a gas-exposure chamber (a sealed acrylic box; L 40 cm  $\times$  W 20 cm  $\times$  H 20 cm) with either air or premixed 1.3% hydrogen in the air for 6 hours with free access to food and water and maintenance of temperature (acceptable range, 22°–24°C) and humidity (acceptable range, 40%–70%).<sup>7,17</sup>

### Study protocol

Mice were randomly divided into 3 study groups of 8 mice each: (1) Sham group; animals received no insult or treatment; (2) LC group in the air: animals with LC were exposed to ambient air (control gas); (3) LC group in hydrogen: animals with LC were exposed to 1.3% hydrogen gas in the air. After the exposure to either air or 1.3% hydrogen in the gas-exposure chamber, the animals

were killed under general anesthesia 6 or 24 hours after blunt injury, followed by en bloc removal of all lobes of the right lung, which were snap frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . The left lung was collected for histopathological analysis. These time points were chosen based on preliminary experiments using sequential real-time polymerase chain reaction (PCR) analysis that showed that messenger RNA (mRNA) levels for proinflammatory cytokines, such as interleukin  $1\beta$  (IL- $1\beta$ ) and IL-6, peaked 6 hours after blunt lung trauma.

#### Computed tomography

As previously described, pulmonary computed tomography (CT) images were taken using the Latheta LCT200, a small animal CT system (Hitachi, Ltd. Tokyo, Japan).<sup>18,19</sup> The mouse was sedated and imaged with the following settings: imaging state, lungs; pixel size,  $24\ \mu\text{m}$ ; slice thickness,  $96\ \mu\text{m}$ ; slice spacing,  $96\ \mu\text{m}$ ; x-ray voltage, low; tomographic image scale,  $-640$  to  $-60$ . Three mice from each group were analyzed.

#### Arterial blood gas analysis

Arterial blood was collected from the abdominal aorta after anesthetizing the mouse with 2.5% isoflurane by inhalation in room air as previously described ( $N = 8$  for each treatment group).<sup>20</sup> Blood gas sampling was performed with the ABL90 FLEX (Radiometer, Tokyo, Japan).

#### Hematoxylin and eosin staining

The left lung was fixed with 4% paraformaldehyde dissolved in phosphate-buffered saline for 2 days, embedded in paraffin, then sliced into  $5\text{-}\mu\text{m}$  sections. Hematoxylin and eosin staining was performed using standardized protocols in the Central Research Laboratory at Okayama University.

Twenty high-magnification images (total magnification  $400\times$ ) of hematoxylin and eosin-stained tissue were captured randomly from each tissue section and blindly reviewed by one of the authors (N.T.) without knowledge of the experimental group. An acute lung injury score, which quantitated the extent of histologic lung injury, was determined based on alveolar congestion, hemorrhage, infiltration or aggregation of neutrophils in the airspace or the vessel walls, and thickness of the alveolar wall/hyaline membrane formation.<sup>21</sup> Each of these 4 items were scored (0–4) as follows: 0, minimal (little) damage; 1, mild damage; 2, moderate damage; 3, severe damage; and 4, maximal damage. Eight mice were analyzed for each treatment group.

#### Immunohistochemistry for neutrophils and cleaved caspase-3

Paraffin-embedded lung tissue sections ( $5\ \mu\text{m}$ ) were immunostained with anti-neutrophil elastase antibodies and for cleaved caspase-3 (Abcam, Tokyo, Japan) using an ABC Kit (Vector Laboratories, Inc, Burlingame, CA). Sections were deparaffinized, rehydrated, and treated for antigen retrieval with  $10\ \text{mmol/L}$  citric acid pH 6.0 at  $120^{\circ}\text{C}$  for 10 minutes in a pressure cooker. Endogenous peroxidase inhibition was performed with 0.3% hydrogen peroxide in phosphate-buffered saline for 20 minutes at room temperature. Blocking treatment was performed with 10% goat serum in tris buffered saline with 0.1% Tween 20 to prevent nonspecific binding of antibodies. The primary antibodies were diluted by Can Get Signal immunostaining Solution A (Toyobo, Osaka, Japan), applied to the sections, incubated overnight at  $4^{\circ}\text{C}$ , and then washed with 0.1%

Tween 20. Biotin-conjugated secondary antibodies were diluted in Can Get Signal immunostaining Solution A, applied to the sections, and incubated for 2 hours at room temperature. After washing, ABC reagent was applied to the sections and then incubated for 30 minutes at room temperature per the manufacturer's instructions. For 3,3'-diaminobenzidine (DAB) staining, one DAB tablet (10 mg per tablet, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) was dissolved in 50 mL of 0.05 mol/L Tris-HCl buffer pH 7.6 with  $10\ \mu\text{L}$  of 30% hydrogen peroxide as per the manufacturer's instructions. Sections were incubated in DAB solution for 10 minutes at room temperature, washed under running water, counterstained with hematoxylin, dehydrated, cleared, and coverslipped. Images were taken with the Mantra Quantitative Pathology Imaging System (PerkinElmer, Inc, Waltham, MA), and cells were counted in the alveoli and interstitium automatically using the InForm 2.4.10 software (Akoya Biosciences, Inc, Menlo Park, CA). Three images immunostained with anti-neutrophil elastase antibodies were taken randomly from each section at  $200\times$  magnification. In the InForm software, a computer learning system was used to learn the characteristics of the alveolar epithelium and the alveolar interstitial tissues and exclude tracheal epithelial cells. The cells were identified with hematoxylin staining, and the immunostaining was visualized with DAB stain. Immunostaining for cleaved caspase-3 captured at  $400\times$  magnification with 5 images from each section. Apoptotic cells, defined as cleaved caspase-3 positive cells with characteristic nuclear disruption, were counted with the sample identity masked. Eight mice were analyzed for each treatment group.

#### SYBR Green 2-step real-time reverse transcriptase polymerase chain reaction

To assess mRNA levels in the tissue, portions of the right lung were placed in liquid nitrogen and ground to powder. The RNA extraction was performed with the Nucleospin RNA kit (Takara Bio, Inc, Kusatsu, Japan) using powdered lung tissue (30 mg) according to the manufacturer's instructions. Total RNA ( $1\ \mu\text{g}$ ) was reverse transcribed with ReverTraAce qPCR RT Master Mix (TOYOBO Inc., Osaka, Japan). Messenger RNA levels for IL-6, IL- $1\beta$ , IL-10, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), C-C motif chemokine 2 (CCL2), chemokine (C-X-C motif) ligand-2 (CXCL2) and ribosomal protein L4 were assessed using SYBR Green, 2-step, real-time, reverse-transcription PCR as previously described.<sup>22,23</sup> The mixture for SYBR Green PCR was prepared using THUNDERBIRD SYBR qPCR MIX (TOYOBO, Inc, Osaka, Japan) and primers (Supplementary Table). The thermal cycling protocol activated the polymerase for 10 minutes at  $95^{\circ}\text{C}$ , followed by 40 cycles of  $95^{\circ}\text{C}$  for 15 seconds and  $60^{\circ}\text{C}$  for 1 minute in a StepOnePlus Realtime PCR machine (Thermo Fisher Scientific, Waltham, MA). Eight mice were analyzed for each treatment group.

#### Statistical analysis

All data are presented as mean  $\pm$  SE. Statistical analysis was performed with a one-way analysis of variance followed by Bonferroni correction for multiple comparisons. Student's  $t$  test was used for 2-group comparisons. All analyses were performed using STATA/SE software version 17.0 (StataCorp, LLC, College Station, TX). All tests presented were 2-sided.  $P$  values of pooled analysis were used instead of rejecting the null hypothesis that 2 variances are equal when  $P \geq .05$ . However, when  $P < .05$ , we rejected the null hypothesis that the 2 variances are equal.

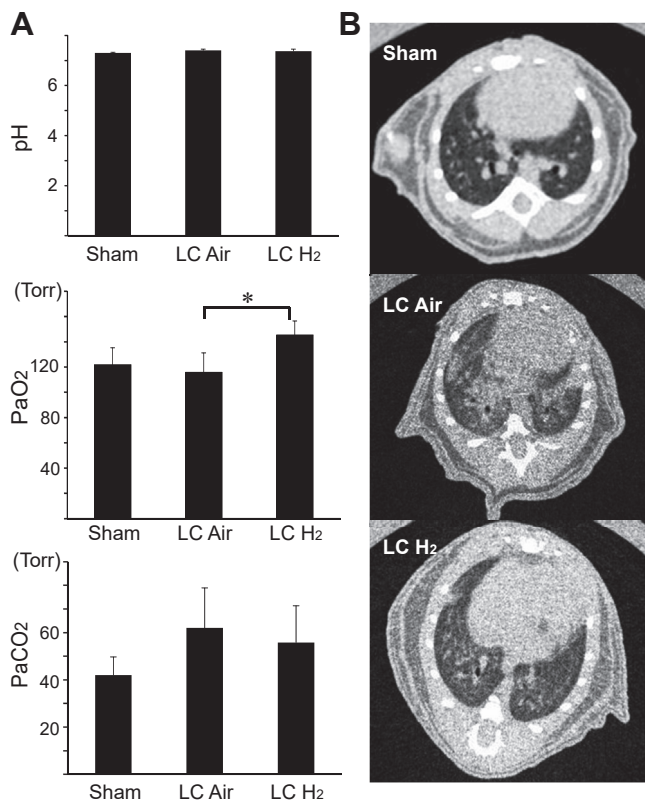
## Results

### Hydrogen inhalation mitigated deterioration of lung function associated with LC

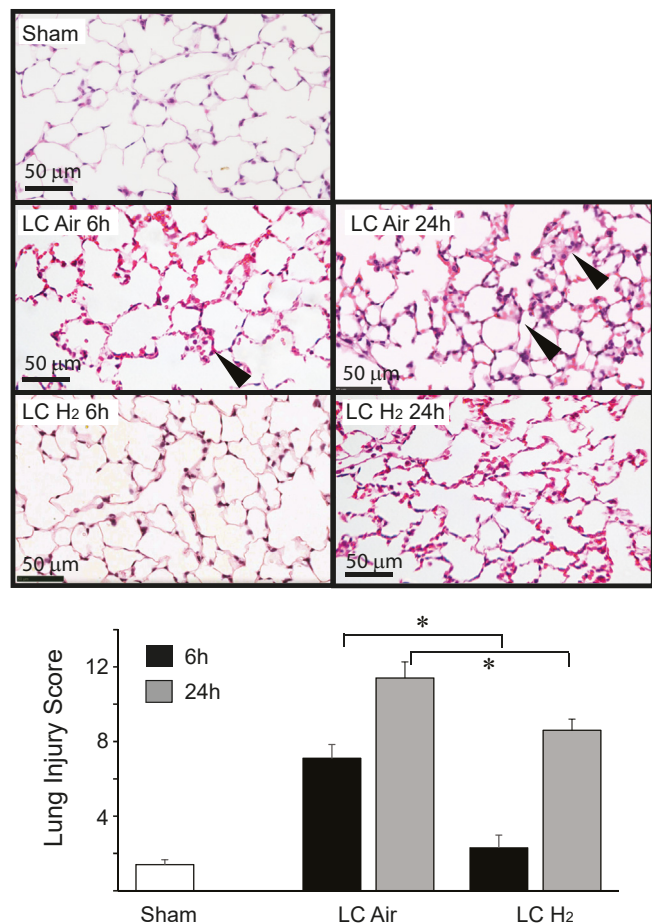
The effects of hydrogen inhalation on lung function, as indicated by blood gas analysis, after LC was assessed. However, there were no differences among experimental groups in blood pH or PaCO<sub>2</sub>. The PaO<sub>2</sub> was 115.7 Torr 6 hours after blunt lung trauma and air exposure. Oxygenation was significantly better when the mice were exposed to 1.3% hydrogen after trauma, and PaO<sub>2</sub> was 145.3 Torr (Figure 2, A). Radiological analysis using chest CT at 6 hours demonstrated infiltration shadows, pleural effusions, and atelectasis in the lung fields after blunt trauma indicative of LC. Hydrogen inhalation mitigated the appearance of these features in the lung fields on CT images compared to the air group (Figure 2, B).

### Hydrogen ameliorated histopathological changes in the lung after blunt chest trauma

The experimentally administered blunt chest trauma resulted in the thickening of the alveolar septal wall and infiltration of inflammatory cells within 6 hours after the trauma. Hydrogen administration significantly suppressed these histopathological



**Figure 2.** Blood gas analysis and chest imaging. (A) Arterial blood gas analysis. There were no differences in pH or PaCO<sub>2</sub> among the groups. Blood oxygenation determined by PaO<sub>2</sub> was higher after lung contusion (LC) in the hydrogen group than in the air group.  $N = 8$  for each group. (B) Radiological analysis using chest computed tomography at 6 hours after LC demonstrating infiltration shadows, pleural effusions, and atelectasis in the lung fields. Hydrogen inhalation (LC treated with 1.3% hydrogen inhalation for 6 hours) resulted in an improvement of the lung fields on computed tomography images as compared with exposure to ambient air (LC exposed to ambient air) after LC. Images are representative of at least 3 independent experiments. LC Air, lung contusion exposed to ambient air; LC H<sub>2</sub>, lung contusion treated with 1.3% hydrogen inhalation for 6 hours. \* $P < .05$  between lung contusion exposed to ambient air and lung contusion treated with 1.3% hydrogen inhalation for 6 hours.



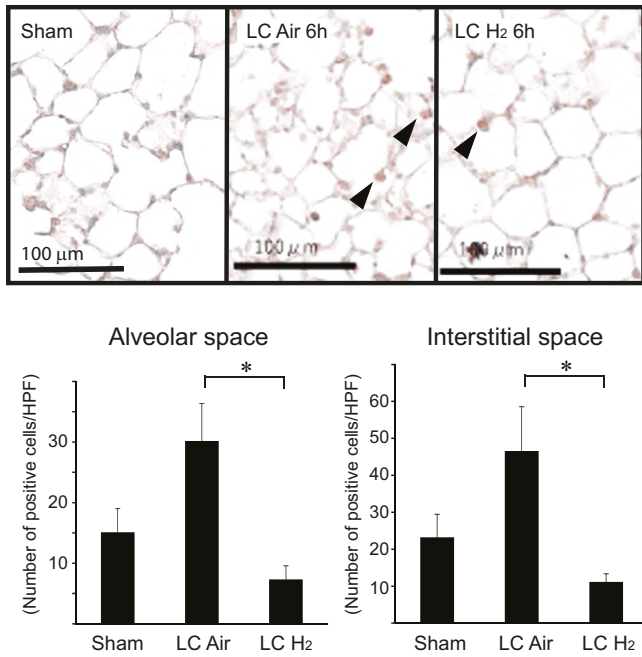
**Figure 3.** Histopathological analysis of the lung after blunt chest trauma. Lungs from sham mice and lungs collected 6 and 24 hours after blunt injury. (Top) Histopathological examination of the lung tissue with contusion revealed perivascular/intra-alveolar hemorrhage, perivascular/interstitial leukocyte infiltration, and interstitial/intra-alveolar edema. These changes were significantly ameliorated by hydrogen inhalation for 6 hours postinjury. Representative images from independent experiments are shown. (Bottom) Acute lung injury was scored in each sample (>20 images from  $n = 8$  for each group) lung injury score was significantly lowered by hydrogen inhalation both 6 and 24 hours after blunt trauma. Arrowhead showing hemorrhage/inflammatory cell infiltration. \*LC Air, lung contusion exposed to ambient air; LC H<sub>2</sub>, lung contusion treated with 1.3% hydrogen inhalation for 6 hours.  $P < .05$  between lung contusion exposed to ambient air and lung contusion treated with 1.3% hydrogen inhalation for 6 hours.

changes (Figure 3). Although the mean lung injury score of sham lungs was  $1.4 \pm 0.27$ , blunt trauma and LC resulted in an increase in the mean lung injury score to  $7.1 \pm 0.74$ . Hydrogen inhalation significantly lowered the lung injury score to  $2.3 \pm 0.68$  (Figure 3).

In addition, we evaluated lungs taken 24 hours after blunt injury and found that intra-alveolar and interstitial hemorrhage was more prominent in the lungs 24 hours after blunt injury compared to 6 hours after injury. The lung injury score in mice exposed to ambient air immediately after trauma was  $11.4 \pm 0.87$ . Hydrogen inhalation in the 6 hours after LC significantly reduced the lung injury score to  $8.6 \pm 0.6$  at 24 hours posttrauma (Figure 3).

### Hydrogen reduced neutrophil infiltration in the lung with blunt trauma

To further determine whether neutrophils were recruited from the circulation into the lungs in response to LC, neutrophil infiltration was investigated using anti-neutrophil elastase staining. In



**Figure 4.** Neutrophil staining. Staining with anti-neutrophil elastase antibodies to detect and quantitate neutrophil infiltration. The number of neutrophils was significantly elevated after pulmonary contusion. Hydrogen inhalation after injury resulted in significantly fewer neutrophils per high power field. Images are representative from independent animals. Arrowheads indicate stained neutrophils in the alveolar or interstitial space. *N* = 8 for each group. *LC Air*, lung contusion exposed to ambient air; *LC H<sub>2</sub>*, lung contusion treated with 1.3% hydrogen inhalation for 6 hours. \**P* < .05 between lung contusion exposed to ambient air and lung contusion treated with 1.3% hydrogen inhalation for 6 hours.

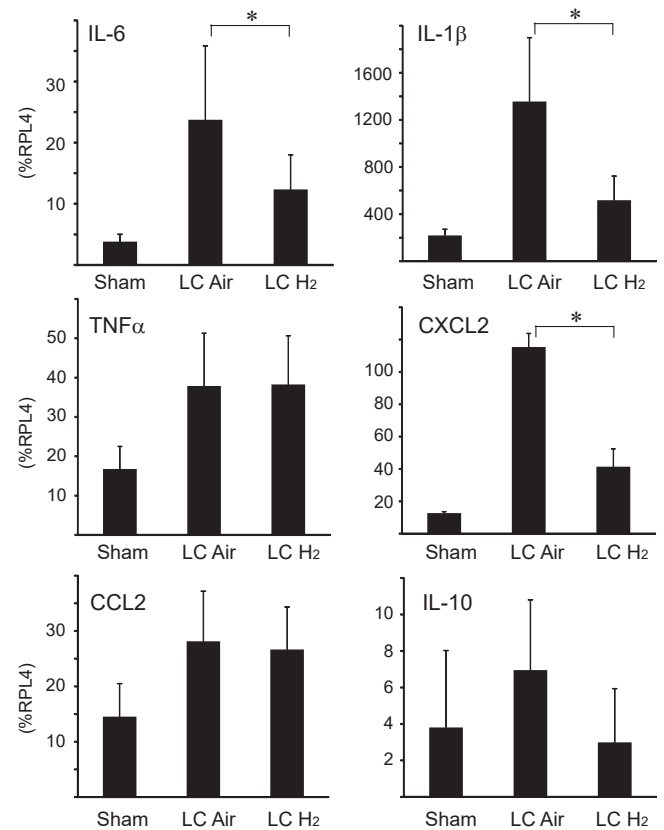
the lungs of mice with LC, there were 46.6 neutrophils in the alveolar space and 30.2 neutrophils in the interstitial space. Hydrogen inhalation after injury decreased the number of neutrophils to 11.1 cells in the alveolar space and 7.3 cells in the interstitial space (Figure 4).

*Hydrogen inhalation reduced mRNA levels for Inflammatory cytokines*

The mRNAs for pro-inflammatory cytokines, IL-6 and IL-1β, increased 6.24- fold and 3.25-fold, respectively, after LC, compared with sham lungs. Hydrogen inhalation significantly reduced this upregulation to 3.25- and 2.37-fold, respectively. The CXCL2 mRNA levels were upregulated (9.05-fold) after LC compared to sham lungs. Hydrogen inhalation significantly inhibited this expression change and limited the increase to 3.25-fold. The mRNAs for TNF-α, CCL2, and IL-10 were markedly elevated in lungs with contusion 6 hours after blunt trauma, but hydrogen did not significantly alter mRNA expression for these molecules (Figure 5).

*Hydrogen inhalation reduced cleaved caspase-3 expression*

Apoptosis in the lungs after LC was examined by staining for cleaved caspase-3. Lung contusion resulted in a marked increase in cleaved-caspase-3-positive alveolar cells 6 hours after blunt injury, indicating increased apoptosis. Apoptosis was significantly attenuated by hydrogen inhalation (Figure 6).

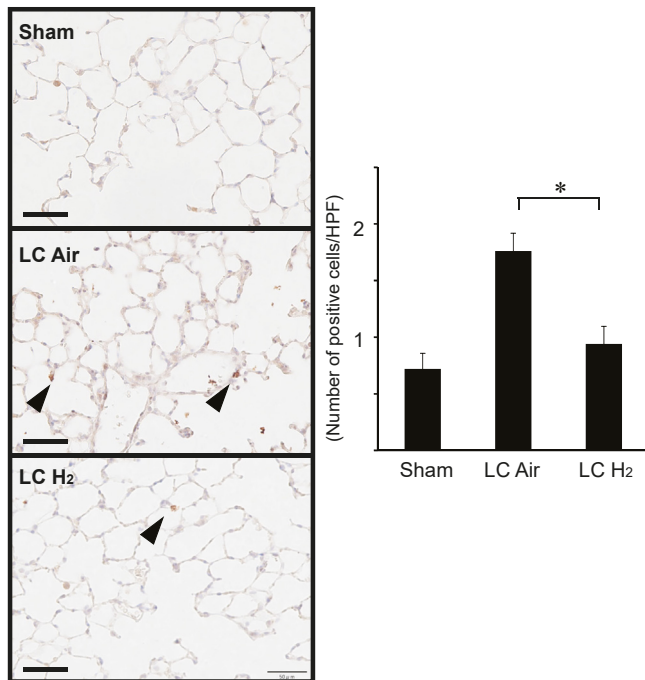


**Figure 5.** Quantitative reverse transcription-polymerase chain reaction for inflammatory mediators and transcripts in lung tissues with blunt chest trauma. Hydrogen inhalation significantly dampened increases in expression of messenger RNAs for inflammatory mediators interleukin 6 (IL-6), IL-1β, and chemokine (C-X-C motif) ligand-2. There were no differences in TNF-α, C-C motif chemokine 2, IL-10 and between the lung contusion (LC) exposed to ambient air and LC treated with 1.3% hydrogen inhalation for 6 hours groups (*n* = 8 for each group). *CCL2*, C-C motif chemokine 2; *CXCL2*, chemokine (C-X-C motif) ligand-2; *IL-10*, interleukin 10; *LC Air*, lung contusion exposed to ambient air; *LC H<sub>2</sub>*, lung contusion treated with 1.3% hydrogen inhalation for 6 hours; *TNF-α*, tumor necrosis factor-α. \**P* < .05 between LC exposed to ambient air and LC treated with 1.3% hydrogen inhalation for 6 hours.

**Discussion**

This study is novel because it is the first to demonstrate that hydrogen inhalation reduces LC after traumatic injury. The mouse model we developed adequately reproduced lung tissue injury due to LC, as evidenced by leukocyte recruitment and alveolar cell apoptosis, followed by the subsequent upregulation of inflammatory mediators. As seen in previous studies, hydrogen inhalation suppressed neutrophil migration and attenuated the expression of inflammatory cytokines.<sup>24</sup> Our laboratory also previously showed that hydrogen inhalation inhibits tissue macrophage activation in the lung.<sup>7</sup> We believe this study is an important addition to exploring the clinical applications of hydrogen gas as it expands the target pathologies for hydrogen therapy to include blunt lung injury.

Several animal models have been developed to study the pathophysiology of LC, particularly in large animals such as canines, swine, simians, dogs, pigs, and monkeys.<sup>25-32</sup> Although these models have important applications, they are hindered by technical difficulties with frequent mortality, high experimental cost, the requirement for a large-scale experimental setting, and the presence of penetrating trauma with concomitant blunt trauma. Another disadvantage of large animal models of LC for mechanistic



**Figure 6.** Caspase-3 staining. There was an increase of caspase-3 positive alveolar cells 6 hours after lung contusion. Images are representative of experiments in 8 different animals. ( $N = 8$  for each group; images were taken at  $400\times$  magnification). Arrowheads indicate apoptotic cells. *HPF*, high power field; *LC Air*, lung contusion exposed to ambient air; *LC H<sub>2</sub>*, lung contusion treated with 1.3% hydrogen inhalation for 6 hours.  $*P < .05$  between lung contusion exposed to ambient air and lung contusion treated with 1.3% hydrogen inhalation for 6 hours.

investigations is the lack of molecular probes and other cell- and mediator-specific reagents, which are much more widely available for small animals such as mice and rats. Additional advantages of small animal models include ease of maintenance and manipulation, a shorter life cycle, lower cost, and abundant genetic resources.

There are currently few reliable and reproducible mouse models for isolated bilateral lung contusion.<sup>33–35</sup> Previously, blunt chest trauma was induced in mice with a single blast wave centered on the thorax,<sup>36–38</sup> direct impact of the lungs,<sup>39</sup> or by shooting the side of the chest with a cortical contusion impactor.<sup>34</sup> The reproducible mouse model established here exhibited LC's major hallmarks, including histological alterations, inflammation, apoptosis, neutrophil activation and immigration into the lungs, and impaired gas exchange. Balls of differing weights might be used to produce different degrees of pulmonary damage to study diagnostic and histological correlates of LC. This model will also allow us to examine recovery from lung contusion during a longer period, as evidenced by analysis of mice 24 hours post-trauma, and will facilitate future research.

The accumulation of neutrophils in the lung is a key event in the initial development of acute lung injury, and extravascular leakage of activated neutrophils from the circulation to the site of injury requires activation of innate immunity and cytokine expression. In this mouse model, hydrogen reduced lung neutrophil infiltration after LC. These effects of hydrogen may be partially due to the inhibition of overexpression of CXCL2 and inflammatory cytokines such as IL-6 and IL-1 $\beta$  that are involved in early neutrophil mobilization.<sup>40,41</sup> Although previous studies showed that hydrogen increases the expression of IL-10, a potent, inducible transcription

factor with antioxidant, anti-inflammatory, and antiapoptotic properties,<sup>42–44</sup> the expression of these protective genes was not enhanced by hydrogen in this study. We do not have a clear explanation for why hydrogen did not mitigate the overexpression of TNF $\alpha$  and CCL2 in this model, whereas previous studies demonstrate that hydrogen effectively controls these inflammatory mediators.<sup>6</sup>

In the clinical setting, hydrogen can be administered at a safe density through a ventilation circuit. Several clinical studies have demonstrated that hydrogen inhalation therapy is applicable for humans and beneficial in treating acute cerebral infarction,<sup>45</sup> post-cardiac arrest syndrome.<sup>46</sup> Thus, hydrogen as a therapeutic strategy is gaining ever-growing attention, with several manufacturers producing hydrogen-generating machines for biomedical applications.

#### Study limitations

This study has several limitations. First, we evaluated the effects of hydrogen only at limited time points during the acute phase after injury. The effects of hydrogen are diverse and include inhibition of fibrosis and apoptosis. Longer-term observation is needed to further examine the potential therapeutic effects of hydrogen in treating LC. Additionally, we did not perform a power calculation to estimate sample size when planning the experiments; therefore, some observations may be underpowered. Third, this exploratory study did not examine molecular mechanisms of hydrogen's protective effects thoroughly. Because the pathophysiology of LC contains multiple and complex processes, this LC model may not be suitable for studying the mechanisms underlying all the potential pharmacological efficacies of hydrogen. Nonetheless, we believe that this study is an addition to the inhaled therapeutics field, opening a window into novel therapies for LC in the clinical setting.

In conclusion, we have established a novel and highly reproducible mouse model of LC due to blunt chest trauma by documenting clinically relevant histopathological changes, inflammation, and loss of lung function after a standardized injury. Hydrogen inhalation significantly mitigated LC-induced lung injuries in this mouse model. Hydrogen therapy reduced overexpression of IL-6, IL-1 $\beta$ , and CXCL2, neutrophil infiltration, and alveolar cell apoptosis in the lungs, consequently preventing hypoxemia. Hydrogen inhalation after blunt chest trauma may be effective in treating LC. This study provides a basic rationale for the clinical application of hydrogen during trauma care.

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#### Conflict of interest/Disclosure

The authors have no conflicts of interests or disclosures to report.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.surg.2023.04.029>.

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