JOURNAL ARTICLE

Immersing lungs in hydrogen-rich saline attenuates lung ischaemia–reperfusion injury† FREE

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European Journal of Cardio-Thoracic Surgery, Volume 51, Issue 3, March 2017, Pages 442–448, https://doi.org/10.1093/ejcts/ezw342

Published: 24 October 2016 **Article history** ▾

Abstract

OBJECTIVES: Anti-oxidant effects of hydrogen have been reported in studies examining ischaemia–reperfusion injury (IRI). In this study, we evaluated the therapeutic efficacy of immersing lungs in hydrogen-rich saline on lung IRI.

METHODS: Lewis rats were divided into three groups: (i) sham, (ii) normal saline and (iii) hydrogen-rich saline. In the first experiment, the left thoracic cavity was filled with either normal saline or hydrogen-rich saline for 1 h. Then, we measured the hydrogen concentration in the left lung using a sensor gas chromatograph (N = 3 per group). In the second experiment, lung IRI was induced by occlusion of the left pulmonary hilum for 1 h, followed by reperfusion for 3 h. During the ischaemic period, the left thoracic cavity was filled with either normal saline or hydrogen-rich saline. After reperfusion, we assessed lung function, histological changes and cytokine production (N = 5–7 per group).

RESULTS: Immersing lungs in hydrogen-rich saline resulted in an elevated hydrogen concentration in the lung (6.9 ± 2.9 μmol/1

1β and interleukin-6) in the left lung were significantly lower in the hydrogen-rich saline group than in the normal saline group ($P < 0.05$).

CONCLUSIONS: Immersing lungs in hydrogen-rich saline delivered hydrogen into the lung and consequently attenuated lung IRI. Hydrogen-rich solution appears to be a promising approach to managing lung IRI.

Keywords: Lung ischaemia–reperfusion injury, Lung preservation, Hydrogen-rich solution

Topic: cytokine, ischemia, lung, reperfusion therapy, physiologic reperfusion, hydrogen, normal saline

Issue Section: EXPERIMENTAL

INTRODUCTION

Ischaemia–reperfusion injury (IRI) is one of the obstacles for successful lung transplantation. This problem persists even with the significant advancements that have been made in lung preservation and perioperative management [1]. In fact, up to 15–25% of lung transplant recipients experience graft complications associated with lung IRI [2, 3]. Among the wide variety of causes of lung IRI, reactive oxygen species (ROS), such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) play a crucial role. Released from macrophages, endothelial cells and other immune cells, ROS induce cytotoxic effects through lipid peroxidation, DNA oxidation and mitochondrial depolarization [4, 5].

In 2007, Ohsawa *et al.* [6] reported protective effects of hydrogen by selectively scavenging ROS levels. Hydrogen molecules can diffuse directly into tissues through cell membranes due to their small

supplementation has been reported in the brain, heart and intestine with favourable outcomes [7–9]. In lung transplantation studies, Kawamura and coworkers [10, 11] showed that treatment of the donor and recipient with inhaled hydrogen at a safe concentration (2%) improves lung functions by reducing oxidative stress.

Recently, multiple studies have evaluated the therapeutic efficacy of solubilized hydrogen as a simple and practical method to deliver hydrogen [12–15]. For example, bathing an isolated heart graft in a hydrogen-containing solution during a cold ischaemia period attenuates post-transplant injury of the heart [13]. These properties of solubilized hydrogen prompted us to attempt using the topical treatment of immersing lungs in a hydrogen-rich solution to reduce lung IRI.

In this study, we hypothesized that solubilized hydrogen could diffuse directly into the lung through the visceral pleura and attenuate lung IRI. Using a rat model, we evaluated the therapeutic efficacy of immersing lungs in hydrogen-rich saline.

MATERIALS AND METHODS

Animals

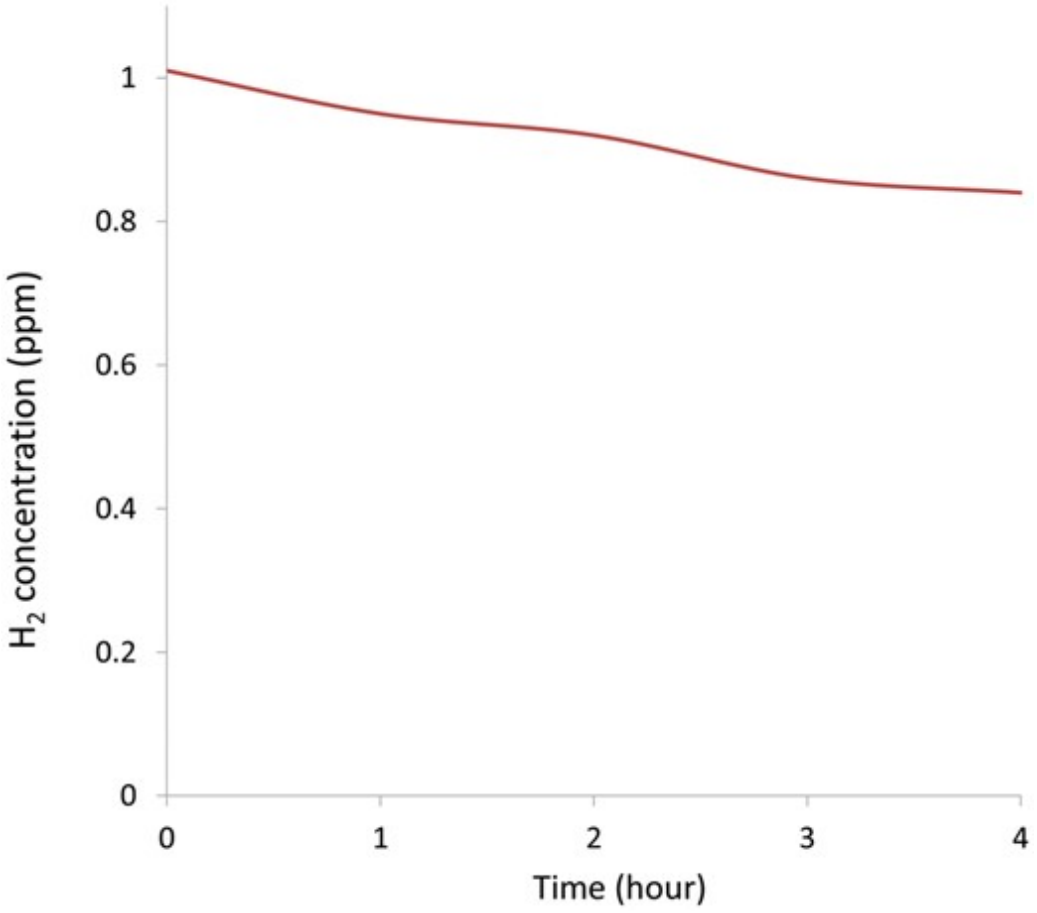
We used Lewis wild-type rats (Japan SLC, Hamamatsu, Japan), 12–13 weeks of age and weighting 290–320 g. All animal experiments were performed using animal protocols approved by the Kyoto University Institutional Animal Care and Use Committee.

Preparation of hydrogen-rich saline

Hydrogen-rich saline was prepared using a hydrogen-generating agent (MiZ Co., Ltd., Kanagawa, Japan) as previously described [14]. Briefly, the agent includes metal aluminium grains and calcium

at a room temperature (26 °C). The bottle was closed tightly to dissolve the generated hydrogen into the saline. Hydrogen-rich saline was made within 5 min and was stored in an aluminium bag with no dead volume to prevent spontaneous diffusion of solubilized hydrogen. The hydrogen concentration in the saline started at 1.0 ppm and maintained at the level for 4 h (Fig. 1). This level was similar to previous studies using solubilized hydrogen [12, 13]. To ensure that the hydrogen concentration was maintained, we prepared the hydrogen-rich saline freshly for every experiment. We also checked the hydrogen concentrations at hourly intervals. We measured the hydrogen concentration in the saline with a solubilized hydrogen analyser (ENH-1000, Sato Co., Kanagawa, Japan).

Figure 1:

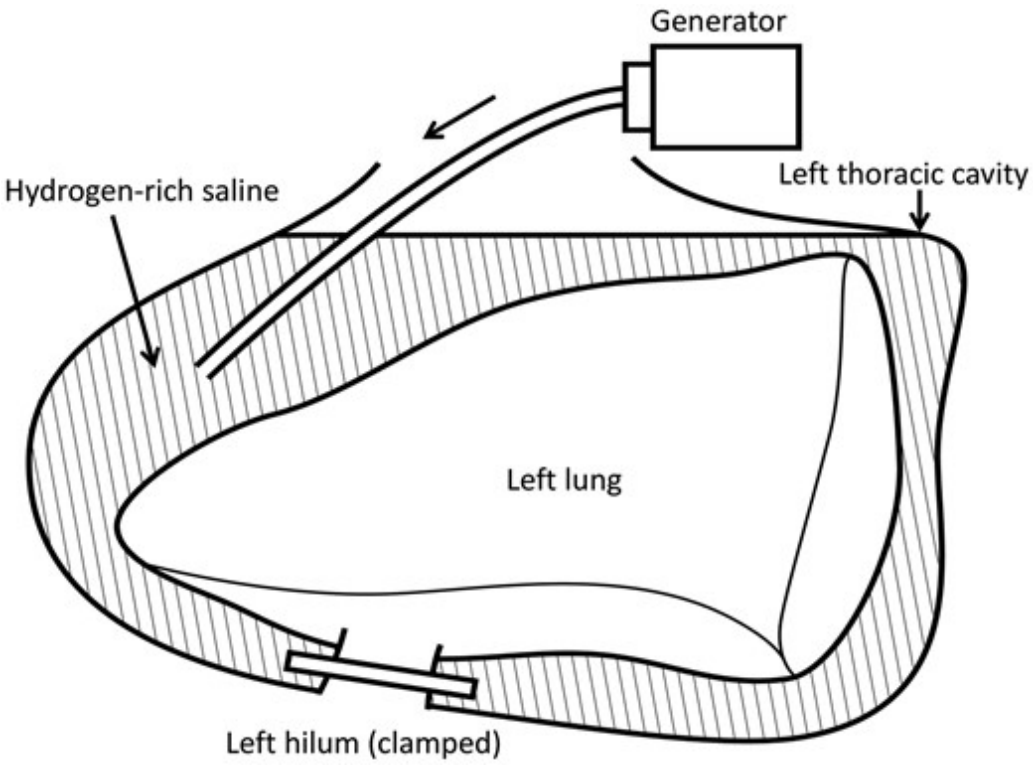


Time course of hydrogen concentrations in saline.

immersion in hydrogen-rich saline

Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (130 mg/kg) and placed on a heating pad to maintain body temperature during the experiment. Tracheotomy allowed mechanical ventilation with a tidal volume of 2.5 mL, respiratory rate of 60 breaths/min, positive end-expiratory pressure of 3 cmH_2O and a fraction of inspiratory oxygen (FiO_2) of 0.21. Through a left thoracotomy, we continuously filled the left thoracic cavity with normal saline or hydrogen-rich saline at a rate of 100 mL/h for 1 h (Fig. 2). Rats were randomly assigned to the following three groups (N= 3 per group): (i) Sham group (1 h left thoracotomy only), (ii) Normal saline group (1 h of left lung being immersed in normal saline), (iii) Hydrogen-rich saline group (1 h of left lung being immersed in hydrogen-rich saline). While immersing the left lung, we clamped the left hilum to prevent hydrogen delivery via both the left bronchi and left pulmonary vessels. After 1 h, the whole left lung (~0.4 g) was obtained and put into a small container (22 mL) for measurement of the hydrogen concentration. The hydrogen concentration was measured using a sensor gas chromatograph (Tracera, Shimadzu Co., Kyoto, Japan) according to instructions of Shimadzu Techno-Research Inc. (Kyoto, Japan). The hydrogen concentration of the tissue samples was measured after cleaning the tissue samples with normal saline before putting into the small container to avoid mixture of hydrogen-rich saline.

Figure 2:



Hydrogen supplementation using hydrogen-rich saline in the left thoracic cavity.

Experiment 2: Protective effects of immersing lungs in hydrogen-rich saline against lung ischaemia-reperfusion injury

Rats were randomly assigned to the following three groups ($N = 5–7$ per group): (i) Sham group (left thoracotomy + 3 h perfusion), (ii) Normal saline group (1 h ischaemia with normal saline + 3 h reperfusion) and (iii) Hydrogen-rich saline group (1 h ischaemia with hydrogen-rich saline + 3h reperfusion). The surgical procedures to induce lung IRI were performed as previously described, with several modifications [16]. Briefly, after the induction of sedation as described in Experiment 1, heparin (1000 unit/kg) was administered subcutaneously. Then, 5 min after heparin administration, to establish lung ischaemia, the left pulmonary hilum was occluded for 1 h using a microvascular clamp through a left thoracotomy. During

ventilation were 2.5 mL during the ischaemic period and 3.5 mL during the reperfusion period. The FiO_2 was 1.0 throughout Experiment 2. The respiratory rate and positive end-expiratory pressure were the same as in Experiment 1.

Pulmonary function

At the end of reperfusion, the right hilum was clamped. The left lung was ventilated with 100% oxygen for 3 min. Then, the left atrial blood was sampled to assess the pulmonary oxygenation of the left lung. We next measured left lung function (airway resistance and pulmonary compliance) with the right hilum clamped, using a flexiVent (SCIREQ, Montreal, Canada).

Cytokine measurements in bronchoalveolar lavage and the lung

After measurement of pulmonary function, bronchoalveolar lavage (BAL) was performed with the right hilum clamped. We injected 1 mL of phosphate-buffered saline, pre-warmed to room temperature (26 °C) into the trachea. Returned fluid was centrifuged immediately (500 × *g*, 5 min, 4 °C) and the supernatant was collected for quantification of pro-inflammatory cytokines. In addition, 50 mg of the excised left lung was homogenized with 0.5 mL of phosphate-buffered saline. The homogenized samples were centrifuged (15 800 × *g*, 5 min, 4 °C) and collected for quantification of pro-inflammatory cytokines in the same manner as was done with the BAL samples. Interleukin (IL)-1β and IL-6 levels were measured in both the BAL fluid samples and homogenized lung samples using a commercially available enzyme-linked immunosorbent assay (ELISA) kit as specified by the manufacturer (R&D Systems, Inc., Minneapolis, USA).

Western blot analysis

lung was snap-frozen in liquid nitrogen, and stored at -80 °C until it was needed for analysis. Frozen lung tissue was suspended in ice-cold RIPA buffer (Nacalai tesque, Kyoto, Japan). Protein concentrations were determined using the Bradford method. Non-specific binding was blocked with 5% bovine serum albumin before overnight incubation with primary antibody for high mobility group box-1 (HMGB1, Sigma-Aldrich Japan, Tokyo, Japan). After incubation with a horseradish peroxidase-conjugated secondary antibody, blots were developed using the ECL system according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK) and visualized with a LAS 3000 (GE Healthcare Japan, Tokyo, Japan). Band densitometry analysis was performed using Image J1.47 analysis software (NIH, Bethesda, USA). β-actin expression was used as an internal loading control.

Pathological evaluation

The left lung blocks at the end of reperfusion were inflation-fixed through the trachea with 10% formalin for 24 h at room temperature (26 °C) and then embedded in paraffin.

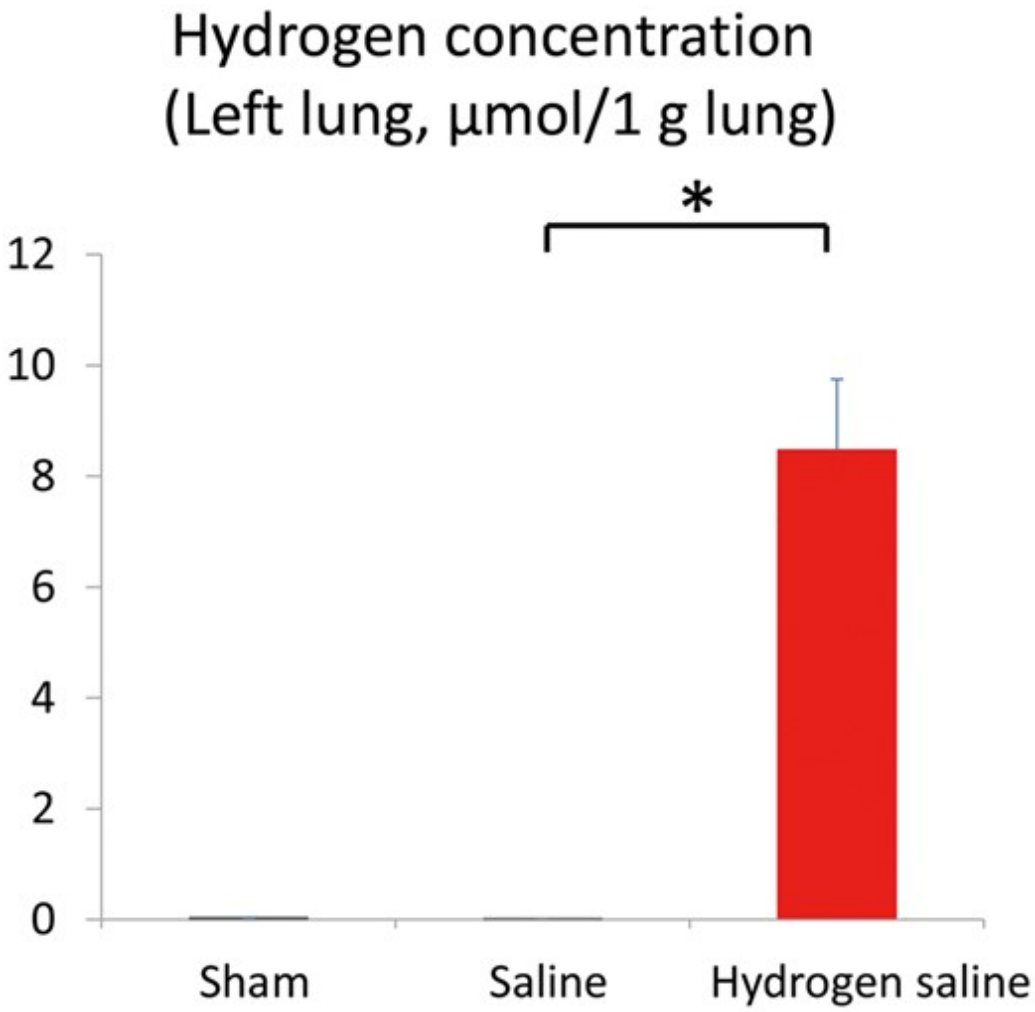
Statistical analysis

All values are presented as the mean ± the standard deviation. Data were evaluated using one-way analysis of variance to determine differences between the groups. A P-value < 0.05 was considered statistically significant.

RESULTS

Hydrogen concentration after immersion in hydrogen-rich saline

Figure 3:

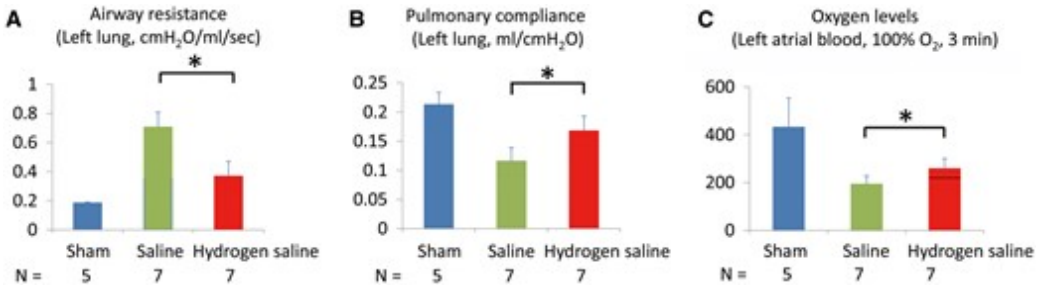


Hydrogen concentrations after immersing lungs in hydrogen-rich saline for 1 h (*N* = 3 per group). Sham: sham group; Saline: normal saline group; Hydrogen saline: hydrogen-rich saline group. *$P < 0.05$ in the saline group versus the hydrogen-rich saline group.

Effect of solubilized hydrogen on pulmonary functions after lung ischaemia-reperfusion injury

The results of the lung function analyses are shown in Fig. 4. Pulmonary compliance was significantly higher in the hydrogen-rich saline group than that in the normal saline group. Airway resistance was significantly lower in the hydrogen-rich saline group than that in the normal saline group. Additionally, oxygenation levels of the

Figure 4:

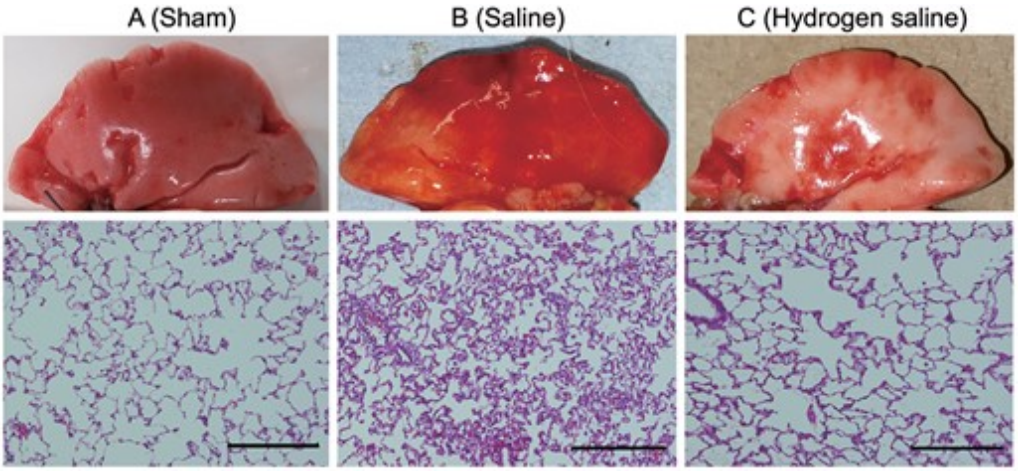


Left lung function after ischaemia-reperfusion injury. (**A**) Airway resistance. (**B**) Pulmonary compliance. (**C**) Oxygen levels in the left lung. Sham: sham group; Saline: normal saline group; Hydrogen saline: hydrogen-rich saline group; *N*: number of animal. **P*<0.05 in the saline group versus the hydrogen-rich saline group.

Histological findings after lung ischaemia-reperfusion injury

After reperfusion, the left lungs from the normal saline group showed alveolar wall swelling and accumulation of red blood cells. In contrast, lungs from the hydrogen-rich saline group showed less haemorrhagic changes compared with those in the normal saline group (Fig. 5A–C).

Figure 5:

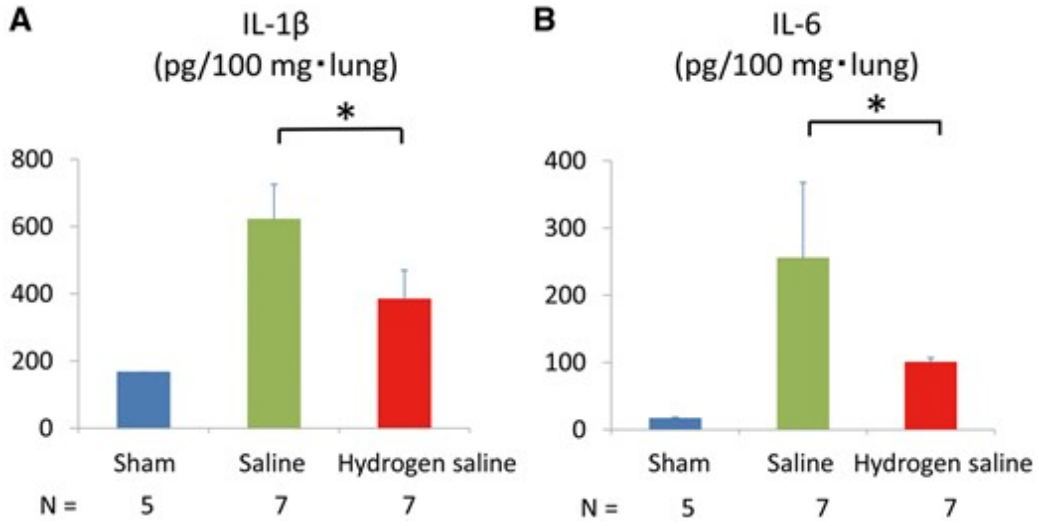


Representative sections of the injured lung (haematoxylin and eosin stain, scale bar=200 μm). (**A**) Sham: sham group. (**B**) Saline: normal saline group. (**C**) Hydrogen saline: hydrogen-rich saline group.

Effect of immersion in hydrogen-rich saline on inflammatory cytokine production

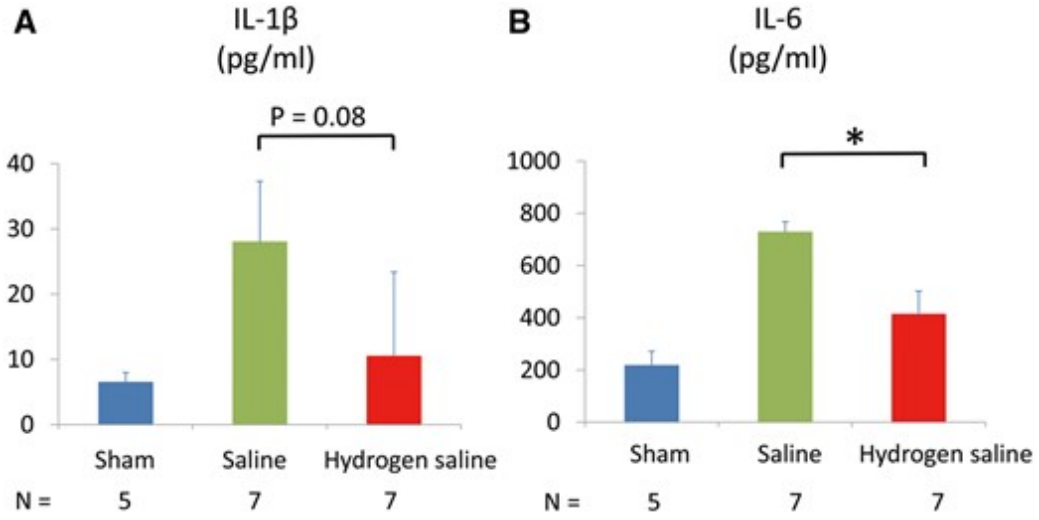
After reperfusion, the hydrogen-rich saline group showed a 2-fold decrease in IL-1β and a 3-fold decrease in IL-6 levels in the left lung, compared with those in the normal saline group (Fig. 6A and B). Similarly, the hydrogen-rich saline group showed a 3-fold decrease in IL-1β and a 2-fold decrease in IL-6 levels in the BAL fluids, compared with those in the normal saline group (Fig. 7A and B).

Figure 6:



Pro-inflammatory cytokine levels in the left lungs. (**A**) IL-1β. (**B**) IL-6. IL: Interleukin; Sham: sham group; Saline: normal saline group; Hydrogen saline: hydrogen-rich saline group; *N*: number of animal. **P*<0.05 in the saline group versus the hydrogen-rich saline group.

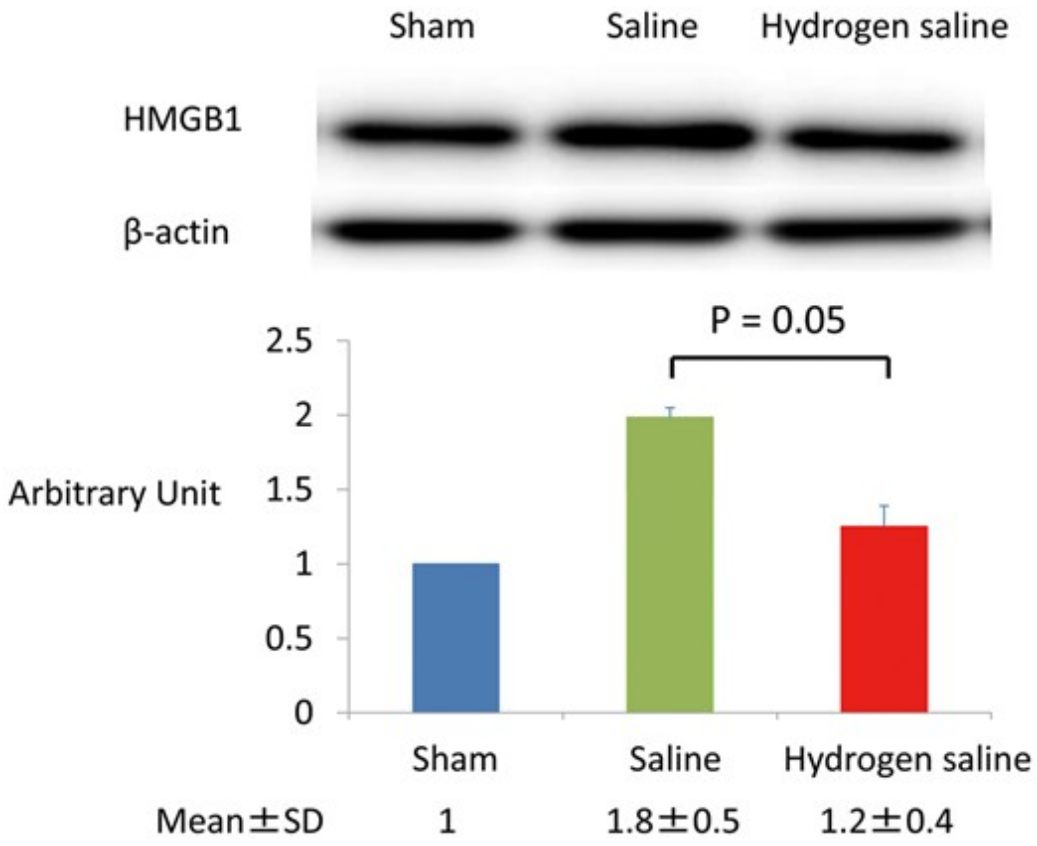
Figure 7:



Pro-inflammatory cytokine levels in the bronchoalveolar lavage fluid. (**A**) IL-1β. (**B**) IL-6. IL: interleukin; Sham: sham group; Saline: normal saline group; Hydrogen saline: hydrogen-rich saline group; *N*: number of animal. **P*<0.05 in the saline group versus the hydrogen-rich saline group.

expression after lung IRI. HMGB1 is a non-histone DNA binding protein normally present in the nucleus of cells. Ali *et al.* [18] reported that HMGB1 expression in the injured lung was positively correlated with the severity of lung IRI. The HMGB1 band in the hydrogen-rich saline group was less pronounced compared with the normal saline group.

Figure 8:



Western blotting analysis of HMGB1 after lung ischaemia-reperfusion injury. (**A**) Representative images for HMGB1 in the left lung. (**B**) Quantification of the expression of HMGB1 (*N* = 4 per group). Sham: sham group; Saline: normal saline group; Hydrogen saline: hydrogen-rich saline group; HMGB1: high mobility group box-1; SD: standard deviation; *N*: number of animal.

DISCUSSION

effectively to the lung, passing through the visceral pleura. Second, immersing the lungs in hydrogen-rich saline during ischaemia inhibited the levels of pro-inflammatory cytokines and expression of HMGB1. These finding suggested that immersing lungs in hydrogen-rich saline attenuated lung IRI.

Therapeutic effects of hydrogen are principally mediated by direct scavenging of free radicals and inducing anti-oxidative enzymes [11–13]. Due to its small molecular weight, hydrogen has a large diffusion capacity. This is a favourable characteristic of hydrogen molecules because they can diffuse directly through tissues, unlike other antioxidant agents. Hydrogen gas inhalation as a clinical application may require careful management because of its potential flammability, especially when oxygen supplementation is simultaneously required. On the other hand, solubilized hydrogen is non-flammable and easier to administer. For example, Noda *et al.* [13] reported that a hydrogen-rich water bath during cold preservation significantly attenuated myocardial injury and inflammatory events due to cold ischaemia and reperfusion. In addition, Shigeta *et al.* [14] have revealed that luminal injection of hydrogen-rich solution could reduce oxidative stress and consequently ameliorate intestinal IRI using a rat model. Moreover, hydrogen dissolved in eye drops can pass through the cornea and attenuate ocular IRI when the hydrogen containing eye drops were continuously administered [15]. However, no previous study has evaluated therapeutic efficacy of immersing lungs in hydrogen-containing solutions. Our study is the first to suggest that solubilized hydrogen can pass through the visceral pleura and diffuse into the lung because the bronchi and the vessels of the left lung were blocked during the lung immersion.

In the second experiment, we evaluated the therapeutic efficacy of a hydrogen-containing solution in attenuating lung IRI. A previous study showed that lung IRI is accompanied by activation of alveolar macrophages, which stimulate pro-inflammatory cytokine release

our study demonstrated that lung IRI resulted in impaired lung functions and elevated levels of IL-1β and IL-6. On the other hand, immersing lungs in hydrogen-rich saline resulted in decreased pro-inflammatory cytokine levels. Previous reports showed that treatment with hydrogen-rich solution significantly suppressed pro-inflammatory cytokine mRNA levels, such as IL-1β, IL-6 or TNF-α, in cardiac and intestinal grafts [13, 14]. In addition, Li *et al.* [21] revealed that intraperitoneal administration of hydrogen-rich saline significantly reduced IL-6 and TNF-α expression in rat kidneys. Our results are consistent with these previous studies and suggest that storing lungs in hydrogen-rich saline has an anti-inflammatory property.

In addition, HMGB1 expression after lung IRI was decreased in the hydrogen-rich saline group. In the current study, we confirmed that the HMGB1 expression in the saline group was higher than that in the sham group. Moreover, HMGB1 expression in lungs from the hydrogen-rich saline group was decreased compared with the normal saline group. Although we did not investigate the precise mechanisms between hydrogen and HMGB1, a previous study showed that hydrogen can down-regulate HMGB1 expression in septic mice [22]. Thus, hydrogen may up-regulate expression of anti-inflammatory proteins, which in turn, down-regulates HMGB1 and attenuates lung IRI. As HMGB1 is released from damaged cells with tissue injury, the HMGB1 levels in BAL fluid or in the blood may be positively correlated with the severity of lung IRI as well. Therefore, we are planning to evaluate these levels in our next study.

In the present study, we applied solubilized hydrogen during an ischaemic period and we confirmed that lung IRI was attenuated. Previous studies have demonstrated that the main portion of mitochondrial damage occurs during ischaemia [19, 23]. Studies of lung transplantation have demonstrated that application of hydrogen gas before or during ischaemia effectively attenuates lung IRI [11, 24]. Therefore, we speculated that application of hydrogen-rich solution

However, the precise cellular sites or timing of ROS production are not fully understood [5]. Thus, optimizing the timing of hydrogen delivery will be an important focus in the future.

There were several limitations of this study worth mentioning. First, we did not use a transplant model in this study. Lung transplantation obligates cold preservation and warm reperfusion of donated lungs. Temperature can affect not only the dissolved hydrogen concentration, but also the rate of protein turnover [26]. In addition, inhaled hydrogen would be more rationale given the nature of lung physiology and anatomy. As a next step, we are planning on using an orthotopic transplantation model to evaluate the efficacy of the combination of inhaled hydrogen and storage in hydrogen-rich organ preservation solution. That research may reveal if hydrogen works in a dose-dependent manner as well. Second, we did not investigate anti-oxidative properties of solubilized hydrogen. Previous studies evaluate oxidative damage by measuring the levels of malondialdehyde and 8-hydroxydeoxyguanosine [12, 14]. We are planning to evaluate these anti-oxidative markers after immersing lungs in hydrogen-rich solutions in our next study.

In conclusion, solubilized hydrogen can directly diffuse into the lungs through the visceral pleura. In addition, immersing lungs in hydrogen-rich saline during an ischemic period attenuated lung IRI. Immersing lungs in hydrogen-rich saline could be an easy and effective method to deliver hydrogen into lung allografts.

Conflict of interest: none declared.

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† A portion of this study was presented at the 36th Annual Meeting of the International Society for Heart and Lung Transplantation, Washington, DC, USA, 27–30 April 2016.