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Dear Dr. W.J. Lennarz:

Enclosed is a manuscript entitled "Hydrogen-rich saline reduces lung injury induced by intestinal ischemia/reperfusion in rats" by Yan-Fei Mao, Xing-Feng Zheng, Jian-Mei Cai , Xin-Min You, Xiao-Ming Deng, John H Zhang, Lai Jiang and Xue-Jun Sun, which we are submitting for publication in Biochemical and Biophysical Research Communications. We found that hydrogen-rich saline attenuates lung injury induced by intestinal I/R and we believe that the paper may be of particular interest to the readers of your journal. Neither the entire paper nor any part of its content has been published or has been accepted elsewhere . It is not being submitted to any other journal .

I hope you will give favorable consideration to this manuscript and find the article acceptable for publication in your journal.

Sincerely,

Xuejun Sun, Ph. D.

**Hydrogen-rich saline reduces lung injury induced by intestinal ischemia/reperfusion in rats**

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## **Abstract**

**Objective:** Hydrogen has been reported to selectively reduce the hydroxyl radical, the most cytotoxic of reactive oxygen species. In this study we investigated the effects of hydrogen-rich saline on the prevention of lung injury induced by intestinal ischemia/reperfusion (I/R) in rats.

**Methods:** Male Sprague–Dawley rats (n=30, 200-220 g) were divided randomly into three experimental groups: sham operated, intestinal I/R plus saline treatment (5ml/kg, i.v.), and intestinal I/R plus hydrogen-rich saline treatment (5ml/kg, i.v.) groups. Intestinal I/R was produced by 90 min of intestinal ischemia followed by a 4 h of reperfusion. **Results:** Hydrogen-rich saline treatment decreased the neutrophil infiltration, the lipid membrane peroxidation, NF- $\kappa$ B activation and the pro-inflammatory cytokine interleukin IL-1 $\beta$  and TNF- $\alpha$  in the lung tissues compared with those in saline treated rat. **Conclusion:** Hydrogen-rich saline attenuates lung injury induced by intestinal I/R.

**Key words:** Intestinal ischemia/reperfusion; Oxidative stress; Lung injury; Antioxidant; Hydrogen-rich saline

## **1. Introduction**

Intestinal ischemia/reperfusion (I/R) injury can lead to indirect lung injury and even acute respiratory distress syndrome in patients <sup>[1]</sup>. Free radicals are considered to be involved in the reoxygenated tissues and responsible for the development of the acute respiratory distress syndrome in the pathological process of I/R injury, especially in the reperfusion phase <sup>[2-4]</sup>. The neutrophils in the lung activated by intestinal I/R can produce a large amount of reactive oxygen species and further cause direct oxidative damage to DNA, proteins, and lipids <sup>[5-7]</sup>.

Recently, it has been suggested that hydrogen (H<sub>2</sub>), a potent free radical scavenger, selectively reduced the hydroxyl radical, the most cytotoxic of reactive oxygen species, and effectively protected against tissue damage such as transient cerebral ischemia, neonatal cerebral hypoxia–ischemia, liver injury and myocardial injury induced by I/R <sup>[8-12]</sup>. Using intestinal injury rat models, Buchholz et al <sup>[13]</sup> have demonstrated that hydrogen inhalation could ameliorate the oxidative stress during transplantation-induced intestinal graft injury. Tsukamoto et al <sup>[14]</sup> confirmed that molecular hydrogen had a potential to reduce I/R injury. However, the potential effect of hydrogen on lung injury caused by intestinal I/R has not been examined. Therefore, the present study investigated the possible therapeutic effects of hydrogen-rich saline on lung injury induced by intestinal I/R in rats.

## **2. Materials and methods**

### **2.1. Animals**

Male Sprague–Dawley rats, weighing 200–220g, were provided by the Experimental Animal Center of Second Military Medical University. Rats were housed with free access to food and

water under a natural day/night cycle. Rats were acclimated for 7 days before any experimental procedures. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Second Military Medical University.

## **2.2. Drugs and materials**

The hydrogen-rich saline was prepared as described previously <sup>[12]</sup>. Briefly, hydrogen was dissolved in normal saline for two hours under high pressure (0.4 MPa) to the supersaturated level using a self-designed hydrogen-rich water-producing apparatus. The saturated hydrogen saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume and was sterilized by gamma radiation. Hydrogen-rich saline was freshly prepared every week to ensure a constant concentration. Malondialdehyde (MDA) and myeloperoxidase (MPO) assay reagents were obtained from Nanjing Jiangcheng Bioengineering Institute. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) Enzyme-Linked Immunosorbent Assay (ELISA) kits were purchased from R&D systems. NF- $\kappa$ B p65 Sandwich ELISA Kit was from Cell Signal Technology.

## **2.3. Experimental design**

Rats were fasted overnight and had free access to water for 16–18 h before the experiments. All animals spontaneously breathed room air throughout the experiment. The rats were anesthetized with 10% Chloralhydrate (0.4 ml/100 g body weight, i. p.) and placed in a supine position. Then the right carotid artery was cannulated to monitor arterial blood gas and to obtain blood samples. An incision was made on the midline of the abdomen under aseptic conditions and superior mesenteric artery was identified. Then the rats were randomly divided into three experimental groups ( $n=10$  in each group). The first group was the sham operation group, in which the rats

underwent surgical preparation including isolation of the superior mesenteric artery without occlusion. The second group was the intestinal I/R group plus saline treatment, in which intestinal ischemia was induced by isolation and occlusion of the superior mesenteric artery for 90 min followed by 4 h reperfusion. The superior mesenteric artery was occluded by atraumatic microvascular clamp and the ischemia was confirmed when the mesenteric pulsations ceased and the intestines became pale. Reperfusion was verified by the return of pulsation to the mesenteric vasculature after clamp removal. The third group was the intestinal I/R group plus hydrogen-rich saline treatment. Either 5ml/kg hydrogen-rich saline or the same volume of vehicle (saline for the second group) was injected into rats *via* the tail vein 10min before the start of reperfusion. Most rats required an additional bolus of 10% Chloralhydrate (0.2 ml/100 g body weight, i. p.) during the period of reperfusion to ensure stable anesthesia. All rats were sacrificed at the end of reperfusion. The specimens of the left lower lung tissues were fixed in 40 g/L paraformaldehyde for histological analysis. The left upper lung tissues were powdered using a mortar and pestle on dry ice and immediately stored at -70 °C for determining MDA and MPO. The specimens of the right lung tissues underwent an identical treatment as the left upper lung tissues in order to detect the protein level of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ) and NF- $\kappa$ B 65.

#### **2.4. Histopathologic observation**

For histopathological observation, the specimens of the left lower lung were harvested and flushed with normal saline, fixed with 10% formalin for 24 h, and embedded in paraffin; sections of 4  $\mu$ m were stained with hematoxylin and eosin (HE staining) for light microscope observation.

#### **2.5. Measurement of MDA and MPO in lung tissues**

Pulmonary MDA content and MPO activity were determined with chemical method described as the manufacturer's instructions (Nanjing Jiancheng Biochemistry Co., Nanjing, China). Lung tissue (100 mg, wet wt) was homogenized in 2 ml of 10 mM phosphate buffer (pH 7.4). After centrifugation at 12,000 g for 20 min, the MDA content and MPO activity in supernatant were measured using the corresponding kits.

## **2.6. Determination of TNF- $\alpha$ , IL-1 $\beta$ and NF- $\kappa$ B 65 levels in the lung tissues**

Lung tissues were collected and washed in normal saline , and then homogenized immediately on ice in 1 ml normal saline (4°C) . The homogenates were centrifuged at 3000 g at 4°C for 15 min . Levels of TNF- $\alpha$  and IL-1 $\beta$  were measured with a commercial ELISA kit following the instructions of the manufacturer. NF- $\kappa$ B 65 level was determined in the nuclear extracts of lung tissues by the Sandwich ELISA Kit. The absorbance was read on a microplate reader and the concentrations were calculated according to the standard curve. Protein content in the sample was determined by Commassie blue assay and the results were corrected per microgram of protein.

## **2.7. Statistical analysis**

Results were expressed as mean  $\pm$  SD. Statistical analysis was done using the SPSS11.0 software package. One way analysis of variance was used to establish whether the difference among the three groups was statistically significant. P value less than 0.05 was considered statistically significant.

## **3. Results**

### **3.1. Arterial blood gas analysis**

The results of arterial blood gas analysis of the three groups are shown in [Table 1](#). Compared with their baseline and ischemia stage, the saline-treated and hydrogen-rich saline-treated groups had significantly lower PaO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> values at the end of reperfusion ( $P<0.05$ ). It was also found that the two groups had significantly lower PaO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> values compared with the sham group at the end of reperfusion ( $P<0.05$ ). Furthermore, the PaO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> values in the Hydrogen-rich saline-treated group were significantly higher than those in the saline-treated group at the end of reperfusion ( $P<0.05$ ). The measurements indicated no significant differences in PaCO<sub>2</sub> and O<sub>2</sub>Sat among the three groups ( $P> 0.05$ ).

### **3.2. Histopathology of lung**

The effects of hydrogen-rich saline treatment on the histopathological changes of lungs in rats with intestinal I/R were shown in [Figure 1](#). Morphological study showed, after 90 min of intestinal ischemia and 4 h of reperfusion, the lung tissues of rats were severely damaged in the saline-treated group, with severe edema, severe alveolar hemorrhage and extensive inflammatory cell infiltration ([Figure 1B](#)). Moderate lung edema, hemorrhage and inflammatory cell infiltration were seen in hydrogen-rich saline-treated group ([Figure 1C](#)), suggesting that lung injury induced by intestinal I/R was reduced by hydrogen-rich saline treatment.

### **3.3. MPO and MDA in lung tissues**

The lung-tissue MPO and MDA assays revealed negligible lung injury in the sham group. However, compared to sham operation group, pulmonary MPO activity and MDA level increased in saline-treated group at the end of reperfusion ( $P<0.01$ ). It was noted that hydrogen-rich saline

treatment significantly decreased the MPO activity and MDA level compared to those of saline-treated rat lung tissues at the end of reperfusion (Figure 2, Figure 3).

#### **3.4. Effect of hydrogen-rich saline on TNF- $\alpha$ , IL-1 $\beta$ and NF- $\kappa$ B 65 levels**

ELISA detection showed that the levels of TNF- $\alpha$ , IL-1 $\beta$  and NF- $\kappa$ B 65 in lung tissue were markedly increased by 3.64-fold, 3.32-fold and 2.30-fold, respectively in the saline-treated group at 4 h after reperfusion when compared with sham-operated controls. Hydrogen-rich saline reduced the elevation of TNF- $\alpha$ , IL-1 $\beta$ , and NF- $\kappa$ B 65 in the lung tissues (Table 2).

#### **4. Discussion**

This study demonstrated that hydrogen-rich saline treatment reduced the severity of lung injury in a rat model of intestinal I/R. This observation is supported by the results from blood gases and histological findings. In addition, hydrogen-rich saline decreased MDA level and MPO activity in the lung tissues, accompanied by reduction of the cytokines (TNF- $\alpha$ , IL-1 $\beta$ ) and NF- $\kappa$ B 65.

Many studies have shown that intestinal I/R induce disruption of the intestinal mucosal barrier, allowing translocation of bacteria and endotoxin into the circulation, which may trigger a complex inflammatory response and frequently result in multiple organ dysfunction syndromes including acute lung injury [1]. Lung injury will usually lead to decreased oxygenation and further aggravate the systemic inflammatory response. The mechanism of lung injury induced by intestinal I/R is complicated. It has been suggested that pro-inflammatory molecules such as reactive oxygen species and cytokines released from post-ischemic intestine can increase permeability of the intestinal mucosa cells. Recent studies showed that the effect of oxidation and inflammatory

response (e.g., TNF- $\alpha$ , IL-1 $\beta$ ) is mediated by NF- $\kappa$ B activation [15].

The signaling molecules TNF- $\alpha$  and IL-1 $\beta$ , discharged from activated macrophages and neutrophils, exert a considerable amplifying effect on the systemic inflammatory response. The severity of lung injury has been shown to correlate with TNF- $\alpha$  and IL-1 $\beta$  activity [16, 17]. In the present study, hydrogen-rich saline significantly decreased the TNF- $\alpha$  and IL-1 $\beta$  in lung tissues, which suggests that the preventive effect of hydrogen-rich saline on lung injury could be mediated by depression of TNF- $\alpha$  and IL-1 $\beta$ . In addition, we found that the NF- $\kappa$ B p65 expression was decreased by hydrogen rich-saline, which suggests that the NF- $\kappa$ B activation may be involved in the intestinal I/R and the subsequent pathological process of lung injury.

The abovementioned molecular events may be translated into oxidative stress and inflammation in lung tissues after intestinal I/R. Indeed, MPO accounts for 5% of dry weight of the neutrophils. Therefore, MPO activity could be used for estimating the number of neutrophils in the lung and is often used as a lung tissue injury indicator [18,19]. In this study, we found an enhanced MPO activity in lung tissues and administration of hydrogen-rich saline decreased MPO activity in rats undergoing intestinal I/R. A parallel event with inflammation, after intestinal I/R, is oxidative stress. It was previously reported that administration of edaravone could attenuate the lung injury induced by intestinal I/R [3], suggesting that reactive oxidant species participates in the pathogenesis of lung injury. In the pathological process, the rapid increase of oxygen free radicals, which damage the cell membrane, can cause peroxidation of unsaturated lipid. MDA is the ultimate product of unsaturated lipid peroxidation, whose content could reflect the content and extent of the radical lipid peroxidation. In this study, we found that MDA was increased in the lung tissues and

hydrogen rich-saline treatment significantly decreased the MDA content after intestinal I/R.

In conclusion, this study shows that hydrogen-rich saline attenuated the lung injury induced by intestinal I/R. Due to its efficacy, convenience and low costs, hydrogen-rich saline might be a potential therapy for multiple organ dysfunction syndromes including lung injury in the future.

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## Figure Legends

**Figure 1.** Photomicrographs of left lower lung sections from the sham group (A), saline-treated II/R group (B) and hydrogen-rich saline-treated II/R group (C) after 90 min of intestinal ischemia followed by 4 h of reperfusion. A: normal histopathology; B: the lung tissues revealed edema, alveolar hemorrhage and inflammatory cell infiltration; C: moderate edema, hemorrhage and inflammatory cell infiltration. Routine hematoxylin and eosin stained (200×).

**Figure 2.** Pulmonary myeloperoxidase activity. Either 5ml/kg hydrogen-rich saline or the same volume of vehicle (saline) was intravenously injected into rats via the tail vein 10 min before the start of reperfusion. Lung tissues were removed from rats 4 h after reperfusion. Data are expressed as mean  $\pm$  SD, n=10. \*\*P< 0.01.

**Figure 3.** Pulmonary malondialdehyde levels. Either 5ml/kg hydrogen-rich saline or the same volume of vehicle(saline) was intravenously injected into rats via the tail vein 10 min before the start of reperfusion. Lung tissues were removed from rats 4 h after reperfusion. Data are expressed as mean  $\pm$  SD, n=10. \*\*P< 0.01, \*P< 0.05.

Figure1  
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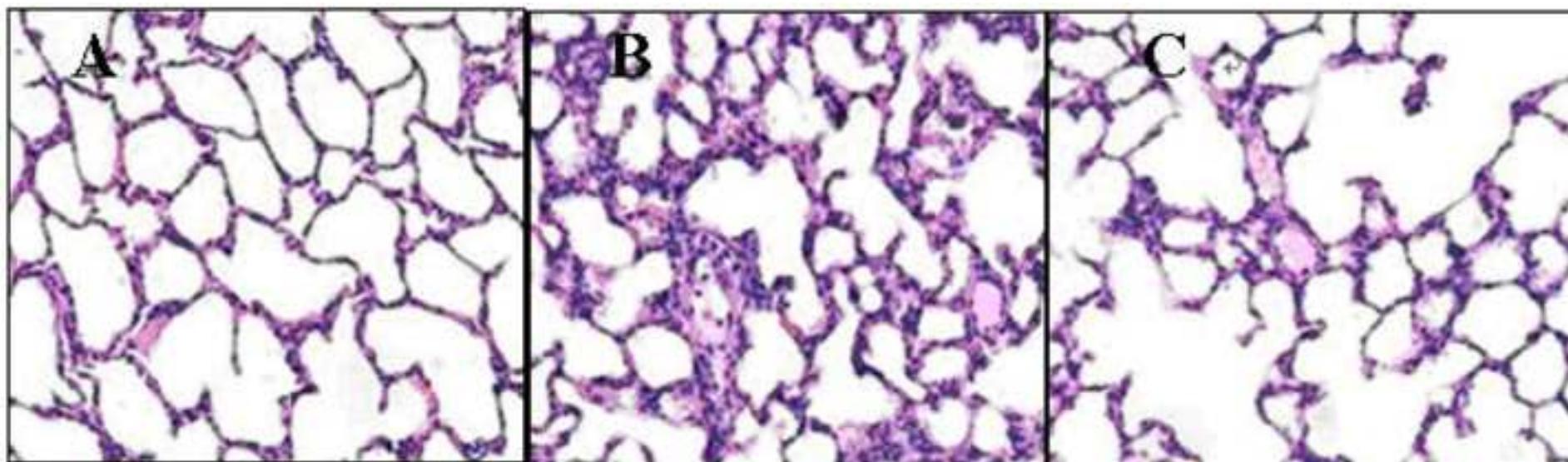


Figure2

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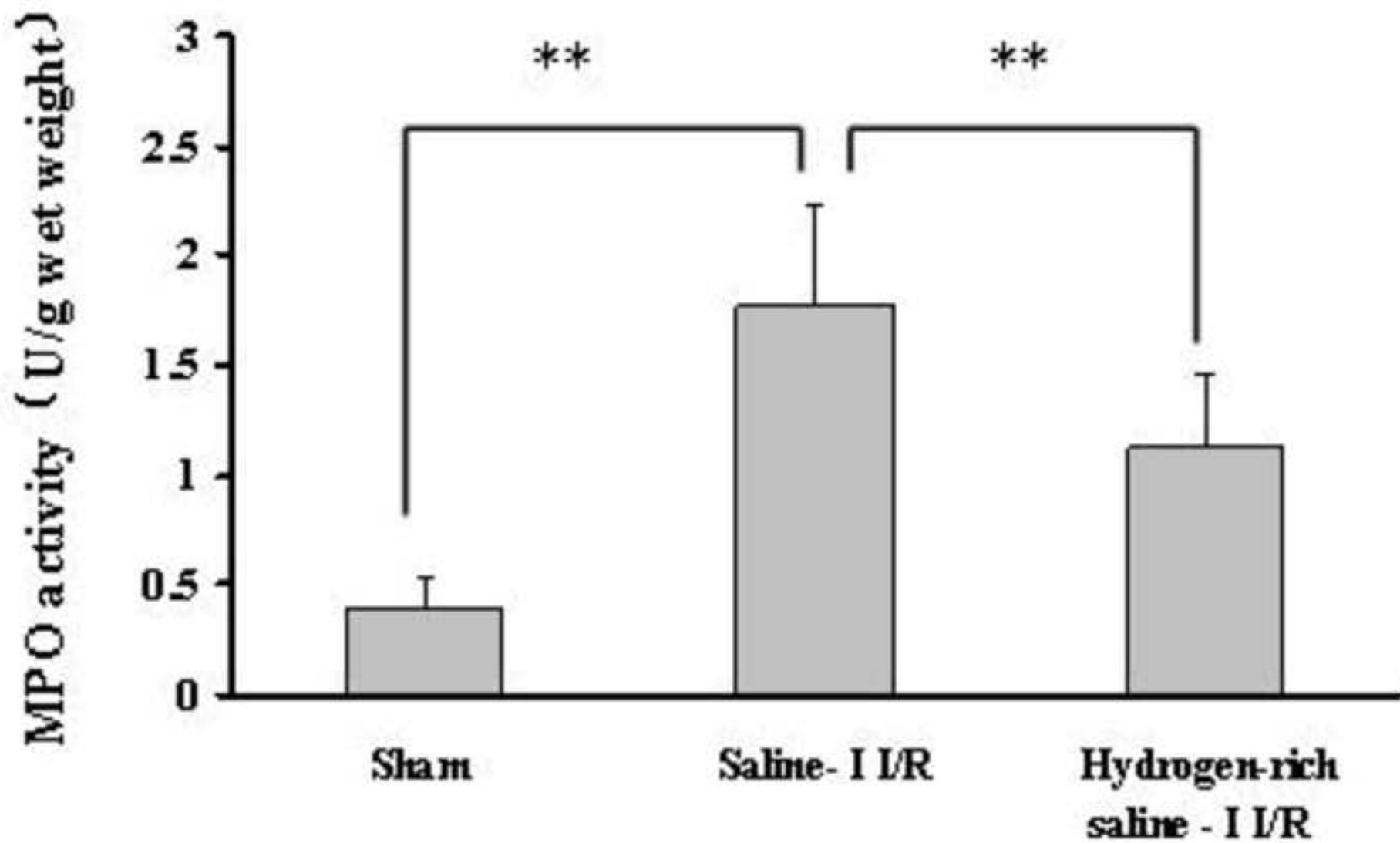


Figure3

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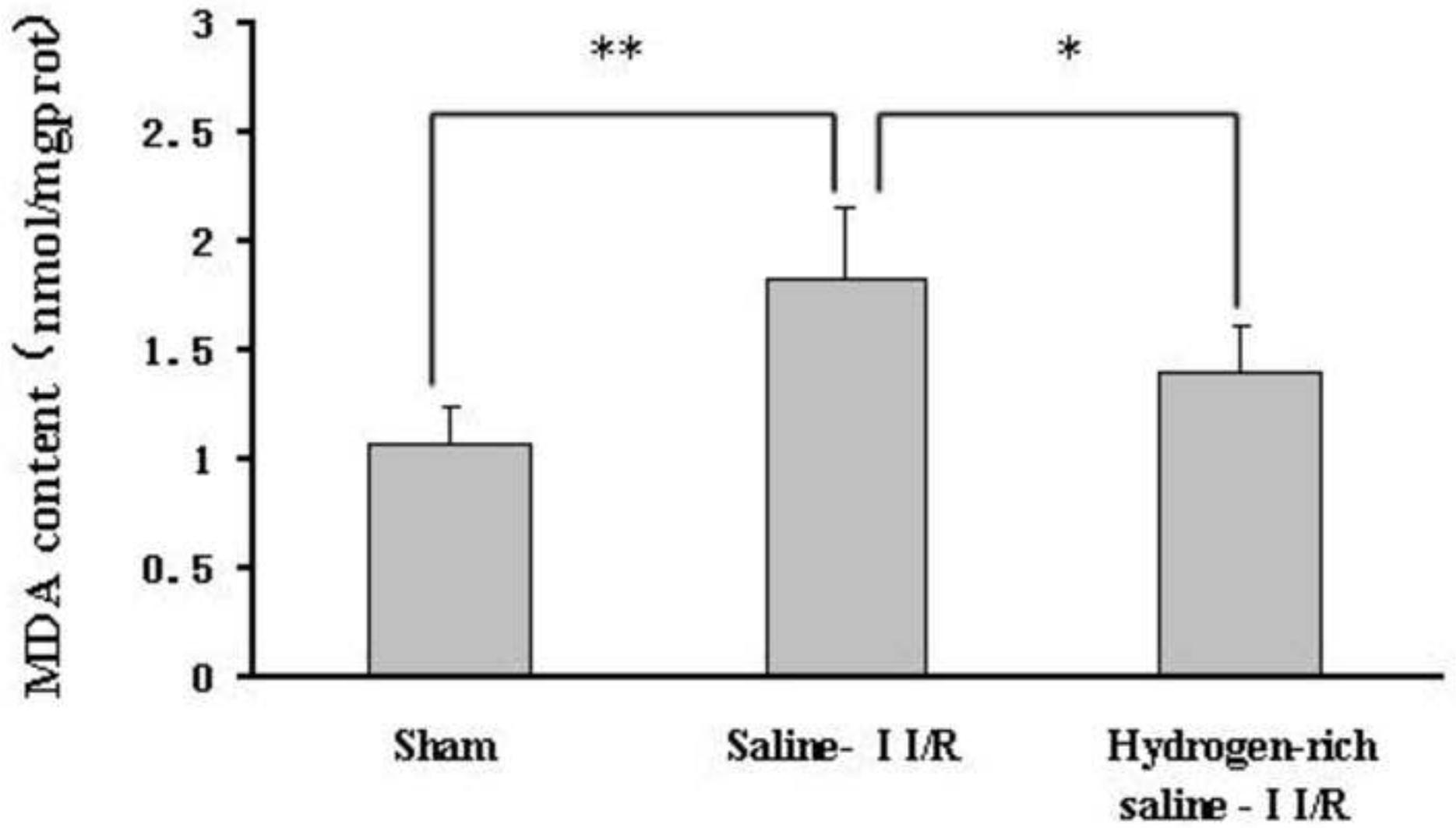


Table 1. Results of arterial blood gas analysis

Parameters	Sham		Saline- I/R		Hydrogen-rich saline - I/R	
	Baseline	I/R	Baseline	I/R	Baseline	I/R
PaO <sub>2</sub> (mmHg)	96.7±4.3	96.2 ±5.9	97.3 ± 6.4	83.5±11.9 **	97.2±10.1	89.8±13.1*†
O <sub>2</sub> Sat (%)	95.4±2.7	94.9±2.5	97.0±2.0	95.7±3.4	94.5±2.9	96.2±2.5
PaCO <sub>2</sub> (mmHg)	41.3 ±5.2	43.2±5.7	41.2±3.5	37.6±4.3	39.3±5.0	38.3±7.1
HCO <sub>3</sub> <sup>-</sup> (mmol/L)	24.5 ±4.7	21.4±5.3	24.1±4.9	14.3 ±4.2 **	24.7 ±4.4	17.8 ±3.6*†

Data are expressed as mean ± SD, n=10. \*P<0.05, \*\*P<0.01 compared with the corresponding baseline; †P<0.05 compared with saline-I/R group.

**Table2.** Levels of TNF- $\alpha$ , IL-1 $\beta$  and NF- $\kappa$ B 65 in rat lung tissues

Parameters	Sham	Saline- II/R	Hydrogen - rich saline - II/R
TNF- $\alpha$ (pg/mg protein)	6.01 $\pm$ 2.57	21.9 $\pm$ 5.74**	15.34 $\pm$ 4.81**††
IL-1 $\beta$ (pg/mg protein)	3.55 $\pm$ 1.10	11.82 $\pm$ 3.75**	8.08 $\pm$ 3.34**†
NF- $\kappa$ B 65 (pg/mg protein)	41.22 $\pm$ 10.31	94.65 $\pm$ 21.35**	69.12 $\pm$ 15.49**††

Data are expressed as mean  $\pm$  SD, n=10. \*P<0.05, \*\*P<0.01 compared with sham group; †

P<0.05, †† P<0.01 compared with saline-treated II/R group.