

## Original Article

# Hydrogen saline is protective for acute lung ischaemia/reperfusion injuries in rats

Jianxin Shi<sup>1</sup>, Feng Yao<sup>1</sup>, Chenxi Zhong, Xufeng Pan, Yu Yang and Qiang Lin\*

Department of Thoracic Surgery, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai 200030, People's Republic of China

**Background:** Protective effects of saturated hydrogen (H<sub>2</sub>) saline on cardiac ischaemia–reperfusion (I/R) injury have been demonstrated previously. This study was designed to show that hydrogen-rich saline is protective in preventing lung I/R injury in rats.

**Methods:** Adult male Sprague-Dawley rats underwent 45 min occlusion of the right lung roots and 120 min reperfusion. Rats were divided randomly into three groups: sham-operated control group, I/R plus saline treatment, and I/R plus hydrogen-rich saline treatment (0.6 mmol/L, 0.5 ml/kg/d). Three days of intraperitoneal injection of hydrogen-rich saline before the reperfusion combined with immediate administration of hydrogen-rich saline after the reperfusion were performed. Following reperfusion, the lung tissue and the pulmonary artery was immediately obtained and the W/D ratio, pulmonary artery contraction and relaxation ability, H-E staining, TUNEL staining, caspase-3, MDA, 8-OHdG content and measurement of such biomarkers as WBC, CRP were measured or carried out.

**Results:** Hydrogen saline significantly protected vasoactivity of the pulmonary artery, reduced pulmonary oedema, decreased lung malondialdehyde (MDA), 8-OHdG concentration, alleviated lung epithelial cell apoptosis and lowered the level of such biomarkers as WBC, CRP, ALT and TBiL.

**Conclusions:** It is concluded that hydrogen-rich saline is a novel, simple, safe and effective method to attenuate pulmonary I/R injury.

(Heart, Lung and Circulation 2012;21:556–563)

© 2012 Published by Elsevier Inc on behalf of Australian and New Zealand Society of Cardiac and Thoracic Surgeons (ANZSCTS) and the Cardiac Society of Australia and New Zealand (CSANZ).

**Keywords.** Ischaemia/reperfusion; Oxidative stress; Hydrogen-rich saline; Antioxidant

## Introduction

In recent years, with the development of complicated lung transplantation, major cardiac surgeries, lung ischaemia/reperfusion (I/R) injury has become an important factor affecting the outcomes [1]. Due to the dual blood supply system and the availability of oxygen from alveolar ventilation, the mechanisms of ischaemia–reperfusion injury in the lungs are more complicated than in other organs. The lung is especially susceptible to I/R injury and effective preventive measures prior to the surgery could significantly improve the outcomes. One of the most important mechanisms of I/R injury is oxidative stress caused by excess accumulation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, and hydroxyl radical, which will severely damage vital macromolecules like DNA and proteins, leading to cell apoptosis, necrosis and severe organ dysfunction [2].

Among all the ROS, •OH and ONOO<sup>−</sup>, in particular are highly unstable and react with the first structure they encounter, usually the lipid component of the cell, causing cell dysfunction or death.

A recent article demonstrated that molecular hydrogen (H<sub>2</sub>) is a novel antioxidant agent which selectively neutralises •OH and ONOO<sup>−</sup>, the most cytotoxic ROS, and alleviates I/R injury [3]. After the major breakthrough, a number of innovative researches were performed, demonstrating that hydrogen is highly effective in prevention of I/R injury [4–7], transplantation injury [8,9], atherosclerosis [10], hyperoxic lung injury [11] and other injuries related to oxidative stress.

In the current study, we studied whether hydrogen-rich saline, which is safe and economic, confers lung protection against I/R injury in rats.

## Materials and Methods

All the protocols were approved by the Second Military Medical University, China in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (Publication No. 96-01). Adult male Sprague-Dawley rats weighing 250–280 g were used in all experiments. The animals were housed in individual cages

Received 25 February 2012; received in revised form 20 April 2012; accepted 28 May 2012; available online 26 June 2012

\* Corresponding author at: Shanghai Chest Hospital, Shanghai Jiao Tong University, 241 West Huaihai Road, Shanghai 200030, People's Republic of China. Tel.: +86 13564661943.

E-mail address: sirchenxiao@126.com (Q. Lin).

<sup>1</sup> These authors contributed equally to this work.

© 2012 Published by Elsevier Inc on behalf of Australian and New Zealand Society of Cardiac and Thoracic Surgeons (ANZSCTS) and the Cardiac Society of Australia and New Zealand (CSANZ).

1443-9506/04/\$36.00  
http://dx.doi.org/10.1016/j.hlc.2012.05.782

in a temperature-controlled room with a 12 h light/dark cycle and free access to food and distilled water.

### *Hydrogen-rich Saline Production*

Hydrogen was dissolved in physiological saline for 6 h under high pressure (0.4 MPa) to a supersaturated level. The saturated hydrogen saline was stored under the atmospheric pressure at 4 °C in an aluminium bag with no dead volume. Hydrogen-rich saline was sterilised by gamma radiation and freshly prepared every week, which ensured that a concentration of 0.6 mmol/L was maintained. Gas chromatography was used to confirm the content of hydrogen in saline by the method described by Ohsawa et al. [3].

### *Experimental Protocol*

Animals were randomly assigned to three groups with 10 rats in each group: (1) Control group: the rats received peritoneal injection of normal saline and the surgery without right lung roots being occluded. (2) I/R group: lung injury group, the rats received the surgery. The right lung roots were occluded for 45 min and then were released for 120 min. In the end, the same amount of normal saline as the hydrogen-rich saline in the treatment group was injected into the rats intraperitoneally. (3) I/R + H<sub>2</sub> group: hydrogen administration group, the rats received intraperitoneal injection of hydrogen saline 0.5 ml/kg/d for 3 d before the surgery and after the reperfusion, hydrogen-rich saline 0.5 ml/kg was given immediately into the abdominal cavity.

### *Establishment of Animal Models*

After intraperitoneal injection of ketamine 100 mg/kg to anaesthetise the rats, heparin 100 U/kg was injected through the tail vein. Then tracheotomy and endotracheal intubation were performed and mechanical ventilation for small animals (tidal volume 20 ml/kg, frequency of 60 times/min) was given. Thoracotomy was performed at the 4th intercostal space at the right edge of the sternum. Right lung roots (including the right main bronchus, right pulmonary artery and vein) were ligatured with a 2 mm-diameter thread. Ischaemia was sustained for 45 min. After 45 min, the lung roots were released so that the lung was refilled for 120 min reperfusion. Then the rats were sacrificed for further experiments.

### *Lung Tissue Wet Dry Ratio (W/D) Measurement*

After the rat was sacrificed, 1/3 of the left lung tissue was taken with a sharp blade. After surface liquid was absorbed with filter paper, wet weight was recorded with an analytical balance. And then the tissue was put into the oven (80 °C, 48 h) and dry weight was recorded. The ratio of W/D was calculated.

### *Haematoxylin–Eosin (HE) Staining*

After 120 min reperfusion, the lungs were harvested, sectioned, and immersion-fixed in 4% buffered paraformaldehyde. The paraffin was cut into 4 µm thick serial sections. The standard deparaffinisation

protocol was used. Then tissues were stained with haematoxylin–eosin. Slides were then assessed in a blinded fashion by a pathologist based on the following: neutrophilic infiltrate, haemorrhage, lymphohistiocytic infiltrate and apoptosis of pulmonary epithelial cells.

### *In Situ Apoptosis Assay*

TUNEL staining was performed on paraffin-embedded sections by using the *in situ* cell death detection kit (Roche). According to standard protocols, the sections were dewaxed and rehydrated at 60 °C. Then the sections were incubated in 20 µg/ml proteinase K working solution for 15 min at room temperature. The slides were rinsed three times with PBS and then were incubated in TUNEL reaction mixture for 1 h at 37 °C. Filter paper was used to dry the area around sample and converter-AP was added on samples for 1 h at 37 °C. After being rinsed with PBS (5 min, three times), sections were coloured in dark with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP). Four slide fields were randomly examined using a defined rectangular field area with magnification (20×). One hundred cells were counted in each field. The data were represented as the percentage of TUNEL-positive cells of total cell nuclei per field.

### *Pulmonary Artery Preparation and Examination*

After the rat was anaesthetised, the main pulmonary artery was taken and put into an oxygen saturated Krebs solution (pH 7.4) immediately. Then the vessel ring segments of a length of 3 mm were prepared and suspended in organ bath. Each ring was connected to a force-displacement transducer for isometric recording at a preload of 1 g. The rings were equilibrated for 1 h with three washouts in normal Krebs solution (pH 7.4, 37 °C, 95% O<sub>2</sub>, 5% CO<sub>2</sub>). After equilibration, norepinephrine (NE, 10<sup>−10</sup>, 10<sup>−9</sup>, 10<sup>−8</sup>, 10<sup>−7</sup>, 10<sup>−6</sup>, 10<sup>−5</sup> mol/L) was added to the bath. Once the stable contraction was reached, a concentration response curve to NE was constructed. Then the vessels were managed with 10<sup>−7</sup> mol/L NE and different concentrations of acetylcholine (10<sup>−9</sup>, 10<sup>−8</sup>, 10<sup>−7</sup>, 10<sup>−6</sup>, 10<sup>−5</sup>, 10<sup>−4</sup> mol/L) were added to the bath. Once the stable relaxation was reached, a concentration response curve to acetylcholine was constructed [12,13].

### *Caspase-3 Activity*

Caspase-3 activity was determined with a Fluorometric Assay Kit (BIOVISION Research Products 980 Linda Vista Avenue, Mountain View, CA 94043, USA), according to the manufacturer's instructions. In brief, 20–200 µg cell lysates of AAR were incubated in a 96-well plate with 2× Reaction Buffer (50 µl). The reaction was initiated by adding 1 mM DEVD-APC substrate (5 µl). After being incubated in the dark at 37 °C, the plate was read in a fluorometer equipped with a 400-nm excitation filter and a 505-nm emission filter. Fold-increase in caspase-3 activity was determined by comparing these results with the level of the control.

### Determination of Malondialdehyde (MDA) Concentration in the Lung

Lung tissue MDA concentration, a presumptive marker of oxidant-mediated lipid peroxidation, was quantified to estimate the extent of lipid peroxidation. Lung tissues were harvested 2 h after the reperfusion, snap frozen, and kept at  $-80^{\circ}\text{C}$  until analysis [9]. The tissue was homogenised, and tissue MDA concentration was determined using the manufacturer's kit direction (Jiancheng Company, Nanjing).

### Determination of 8-Hydroxy-desoxyguanosine (8-OHdG) Concentration in the Lung

After DNA was extracted from the cells with a DNA Extraction Kit (DNA Extractor Wb Kit, Wako Chemical; Osaka, Japan), it was digested by the method of Victoria et al. The samples were added to plate wells precoated with mouse monoclonal anti-8-OHdG antibody (Japan Institute for the Control of Aging, Fukuroi, Japan), then they were incubated for 45 min at  $37^{\circ}\text{C}$ . Similarly with the method described by Qian LR et al., after washed for three times, the wells were sequentially treated with Biotinylated rabbit-anti-mouse IgG for 30 min at  $37^{\circ}\text{C}$  and Streptavidin-Horseradish Peroxidase (HRP) for 30 min at  $37^{\circ}\text{C}$ . A substrate containing 3,3',5,5'-tetramethylbenzidine (TMB) was added and the wells were incubated for 15 min at  $37^{\circ}\text{C}$ . Then reaction was terminated by the addition of a sulphuric acid. Finally the absorbance was read at a wavelength of 450 nm. Based on the previously established absorbance curve, the concentration of 8-OHdG was calculated.

### Determination of Biochemistry Markers in the Plasma

Blood samples were taken for biochemical analysis, including white blood cell count (WBC), C-reactive protein (CRP), prothrombin time (PT), serum amylase (Amy),

alanine aminotransferase (ALT), total bilirubin (TBiL), creatinine (Cr), blood urea nitrogen (BUN).

### Statistical Analysis

All results were expressed as means  $\pm$  SEM. For comparison of differences between groups, one-way ANOVA followed by Student-Newman-Keuls test was utilised. A value of  $P < 0.05$  was considered to denote statistical significance.

## Results

### Histopathological Examination by HE Staining

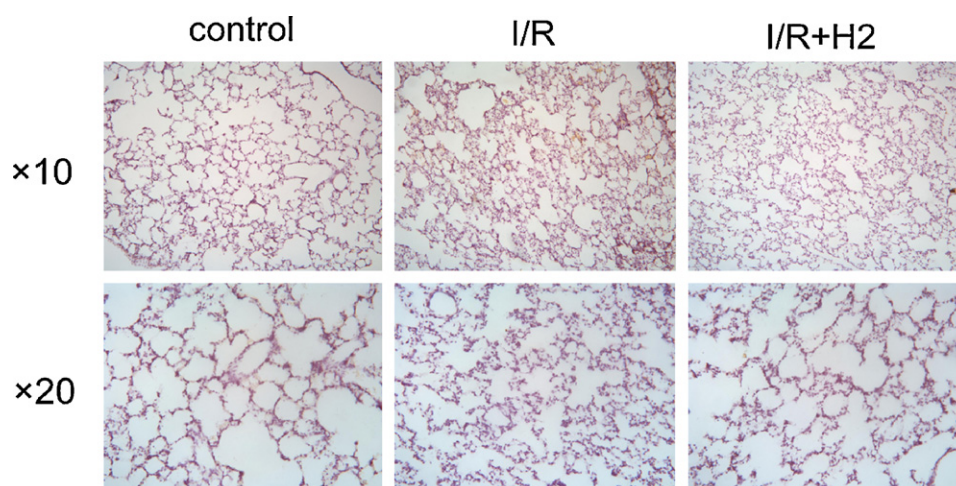
Pathological staining results showed that under light microscopy, lung tissues in the control group only showed a few inflammatory cells as the time went by, lung tissues in the I/R group manifested as alveolar wall destruction, capillary congestion, alveolar septal infiltration of inflammatory cells, a large number of inflammatory cells infiltration, alveolar exudation, haemorrhage and inflammatory exudate accumulation and lung tissues in the I/R + H<sub>2</sub> group showed mild alveolar septal infiltration of inflammatory cells, emergence of a few red blood cells and inflammatory cells in the alveolar cavity and no inflammatory exudate and bleeding (Fig. 1).

### Lung Tissue Wet Dry Ratio (W/D)

As shown in Fig. 2, for the control, I/R, and I/R + H<sub>2</sub> group, the ratios were  $4.18 \pm 0.40$ ,  $5.58 \pm 0.48$ ,  $4.75 \pm 0.32$  respectively. The ratio in I/R group was significantly higher than that in the control group ( $*P < 0.05$ ) and the I/R + H<sub>2</sub> group ( $\#P < 0.05$ ) (Fig. 2).

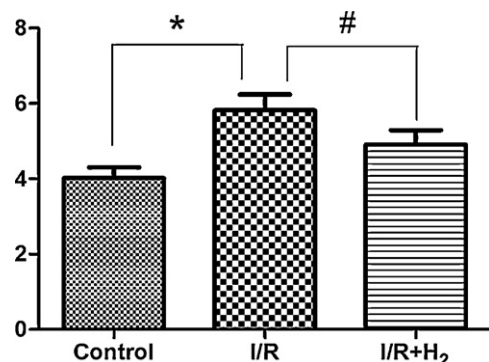
### Detection of Apoptotic Cells

As shown in Fig. 3, the number of TUNEL-positive cells were significantly increased in the I/R group. Hydrogen



**Figure 1.** HE staining of the lung tissues. Lung tissues in the control group showed a few inflammatory cells. Lung tissues in the I/R group manifested as alveolar wall destruction, capillary congestion, alveolar septal infiltration of inflammatory cells, a large number of inflammatory cells, alveolar exudation, haemorrhage and inflammatory exudate accumulation. Lung tissues in the I/R + H<sub>2</sub> group showed mild alveolar septal infiltration of inflammatory cells, emergence of a few red blood cells and inflammatory cells in the alveolar cavity and no inflammatory exudate and bleeding.





**Figure 2.** Lung tissue W/D ratio. Data are expressed as mean ± SEM. No statistical difference was found between the control group ( $4.18 \pm 0.40$ ) and the I/R + H<sub>2</sub> group ( $4.75 \pm 0.32$ ). \* $P < 0.05$  vs. control; # $P < 0.05$  vs. I/R + H<sub>2</sub> group.

saline comparably decreased the frequency of TUNEL-positive cells compared with that of the I/R group (\* $P < 0.05$  vs. control group; # $P < 0.05$  vs. I/R + H<sub>2</sub> group).

#### Pulmonary Artery Contraction and Relaxation Ability Test

Generally, pulmonary artery contractility in the control group was superior to that in the I/R group and I/R + H<sub>2</sub> group. Pulmonary artery contraction curve showed that under the NE concentration of  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$ , the vascular contraction ability in I/R + H<sub>2</sub> group was

significantly greater than that in the I/R group (\* $P < 0.05$ ) (Fig. 4A). Pulmonary artery relaxation curve showed that under the acetylcholine concentration of  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$ , the vascular relaxation ability in the I/R + H<sub>2</sub> group was significantly greater than that in the I/R group (\* $P < 0.05$ ) (Fig. 4B).

#### Determination of 8-OHdG Concentration

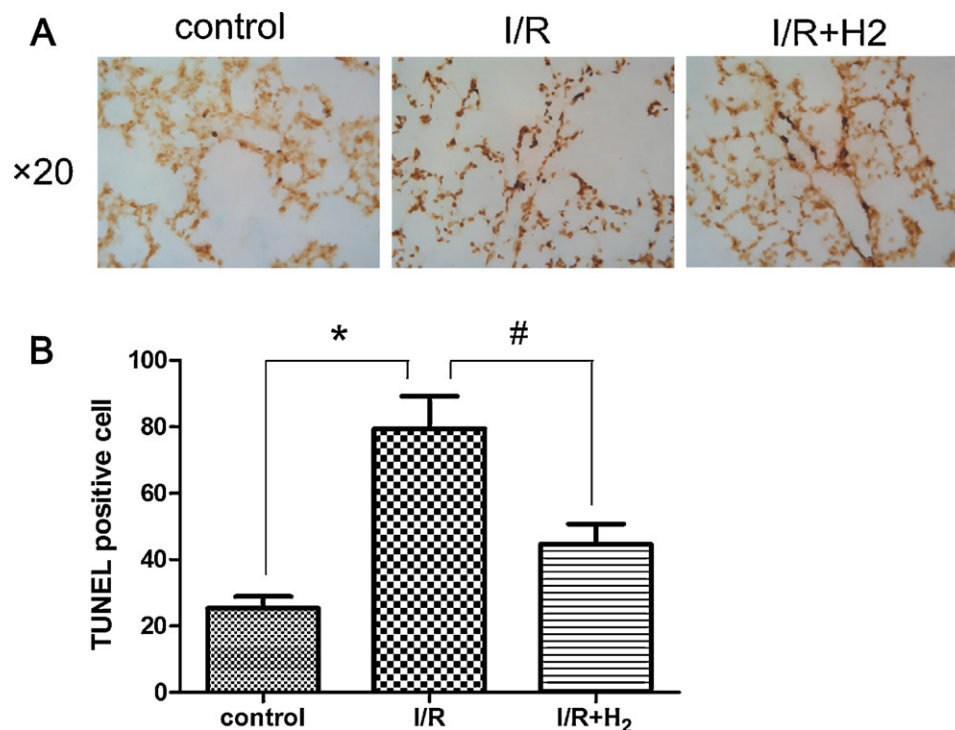
As shown in Fig. 5, the concentration of 8-OHdG was increased in the lung tissues in the I/R group. Hydrogen comparably decreased the 8-OHdG concentration relative to the control group ( $78 \pm 25$  vs.  $147 \pm 43$ , \* $P < 0.05$  vs. control group) (Fig. 5).

#### Lung Tissue MDA Concentration After Reperfusion

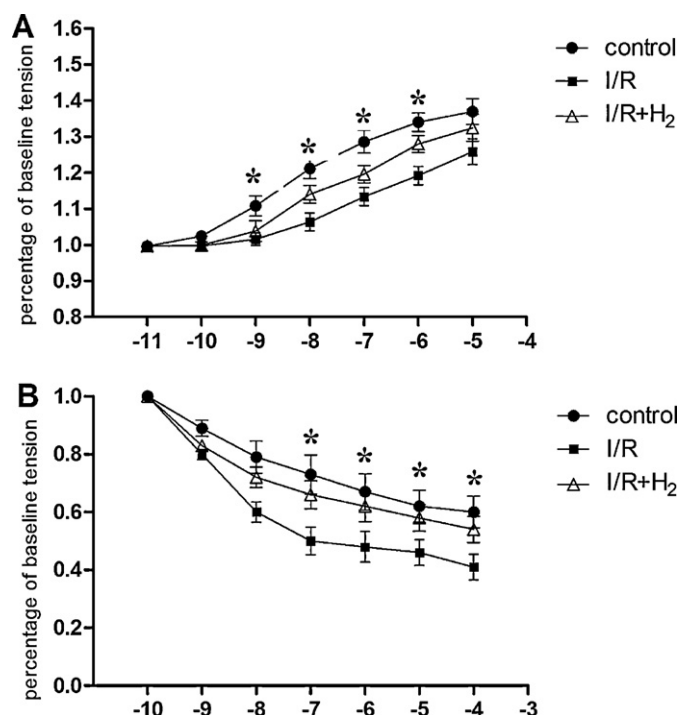
The lung MDA concentration was measured at 2 h of reperfusion (Fig. 6). Lung MDA concentration at 2 h of reperfusion in the I/R + H<sub>2</sub> group was significantly lower than that of the I/R group (\* $P < 0.05$ ). \* $P < 0.05$  vs. control group.

#### Caspase-3 Activity

The caspase-3 activity is shown in Fig. 7. I/R significantly increased caspase-3 activity compared to the non-ischaemic lung. Saturated hydrogen saline comparably inhibited caspase-3 activity compared to the I/R group (Fig. 7, # $P < 0.05$ ).



**Figure 3.** TUNEL staining of the lung tissues. (A) The number of TUNEL-positive cells (brown) in H<sub>2</sub> group were significantly greater in the I/R group. (B) Apoptotic cell counting showed that hydrogen saline significantly decreased the percentage of TUNEL-positive cells compared with the I/R group (\* $P < 0.05$  vs. control group; # $P < 0.05$  vs. I/R + H<sub>2</sub> group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



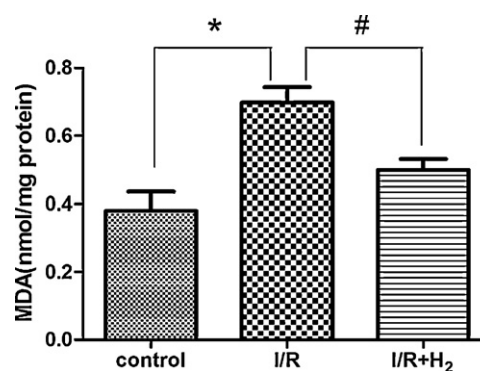
**Figure 4.** Pulmonary artery contraction and relaxation test. (A) Pulmonary artery contraction curve showed that under the NE concentration of  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$ , the vascular contraction ability in the I/R + H<sub>2</sub> group was significantly greater than that in the I/R group (\* $P < 0.05$ ). (B) Pulmonary artery relaxation curve showed that under the acetylcholine concentration of  $10^{-7}$ ,  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$ , the vascular relaxation ability in the I/R + H<sub>2</sub> group was significantly greater than that in the I/R group (\* $P < 0.05$ ).

#### Changes of Biochemistry Markers in the Plasma

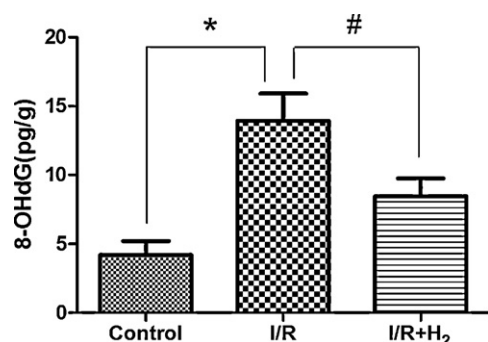
In this study, we evaluated WBC, CRP, Amy, ALT, TBiL and BUN in the plasma after the reperfusion as it is shown in Table 1 and Fig. 8. We found that hydrogen saline could significantly lower the level of WBC, CRP, ALT and TBiL. The differences were statistically significant ( $^{\#}P < 0.05$  vs. I/R + H<sub>2</sub> group).

#### Discussion

In this study, we successfully established the animal model of lung ischaemia/reperfusion injury, and demonstrated



**Figure 6.** MDA concentration in the lung tissues. Lung MDA concentration at 2 h of reperfusion in the I/R group was significantly higher than that of the I/R + H<sub>2</sub> group ( $^{\#}P < 0.05$ ). \* $P < 0.05$  vs. control group.



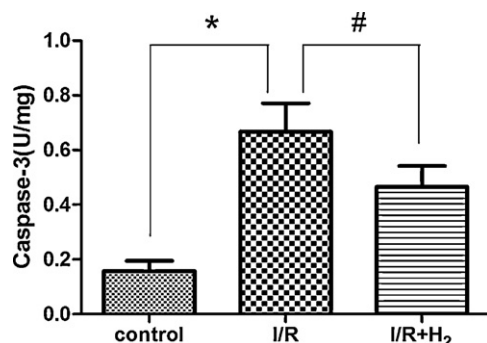
**Figure 5.** 8-OHdG concentration in the lung tissue. Lung 8-OHdG concentration at 2 h of reperfusion in the I/R + H<sub>2</sub> group was significantly lower than that of the I/R group ( $^{\#}P < 0.05$ ). Lung 8-OHdG concentration at 2 h of reperfusion in the I/R group was significantly higher than that of the control group (\* $P < 0.05$ ).

that saturated hydrogen saline was highly effective to protect the lung from I/R injury and improve the general conditions by alleviating oxidative stress state and

**Table 1.** Biochemistry Markers Changes.

Index	Control	I/R	I/R + H <sub>2</sub>
WBC ( $10^9/L$ )	$12.0 \pm 4.6$	$22.2 \pm 8.4^*$	$16.4 \pm 9.7$
CRP (mg/L)	$3.6 \pm 3.4$	$12.0 \pm 9.8^*$	$5.0 \pm 2.8$
ALT (U/L)	$26.4 \pm 5.5$	$53.6 \pm 10.6^*$	$38.8 \pm 9.7$
TBil ( $\mu\text{mol/L}$ )	$18.1 \pm 8.3$	$55.7 \pm 9.4^*$	$21.7 \pm 11.6$

\*  $P < 0.05$  vs. I/R + H<sub>2</sub> group.



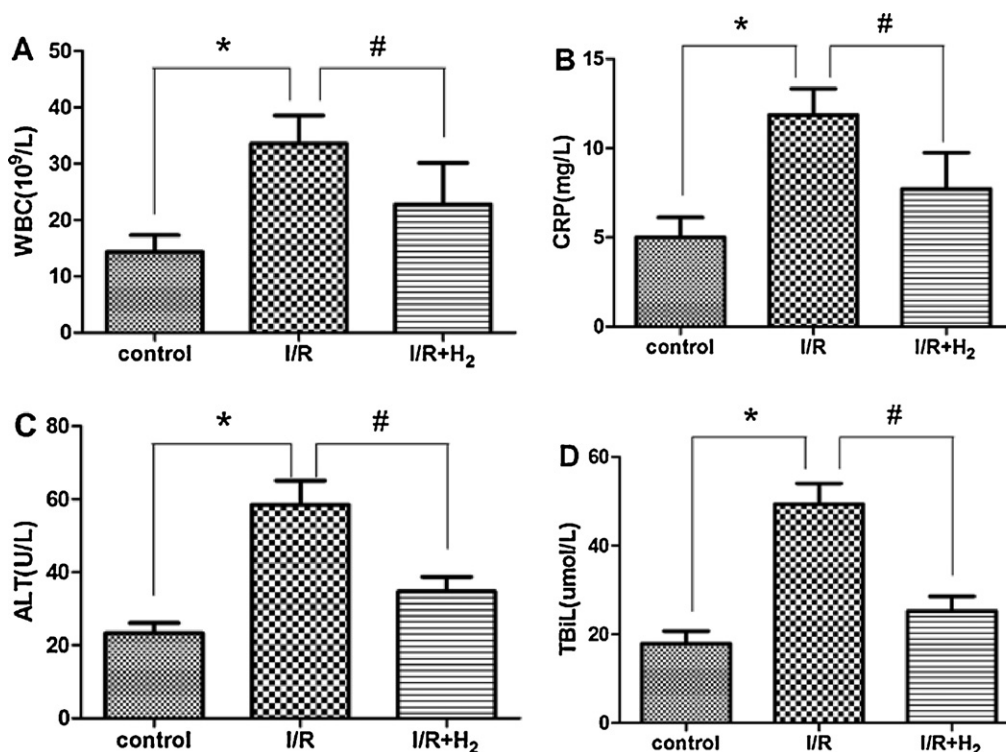
**Figure 7.** Caspase-3 activity of the lung tissues. Caspase-3 activity significantly increased 24 h after the I/R injury. Hydrogen-rich saline significantly lowered the caspase-3 activity in the lung (#*P* < 0.05). \**P* < 0.05 vs. control group.

ameliorating damages caused by excessive ROS. Hydrogen saline significantly reduced pulmonary oedema demonstrated by lung tissue W/D ratio, protected pulmonary artery vasoactivity showed by improved artery contraction and relaxation abilities, reduced pulmonary epithelial cell apoptosis displayed by HE and TUNEL staining and caspase-3 inactivity, improved oxidative stress showed by lowering MDA and 8-OHdG and improved general conditions, alleviated the injury to other remote organs best demonstrated by such biochemistry biomarkers as WBC, CRP, ALT and TBiL.

Oxidative stress is characterised by the formation of reactive oxygen species such as superoxide anion, hydrogen peroxide and hydroxyl radical [2]. During ischaemia/reperfusion process, a series of changes bring about excessive ROS. Among all the ROS,  $\cdot\text{OH}$  and  $\text{ONOO}^-$ , regarded as the most toxic to the cell, can cause enormous damages to the cell, leading to the final organ dysfunction or even failure.

In clinical practice, lung transplantation and routine open heart surgery with cardiopulmonary bypass will cause pulmonary ischaemia and reperfusion, which can lead to pulmonary dysfunction, resulting in a high morbidity and mortality [1,14]. Ischaemia–reperfusion-induced lung injury is characterised by vessel endothelial cell damage, increased vessel permeability and regulating dysfunction, alveolar damage, lung oedema, and hypoxaemia occurring within 72 h after lung transplantation [1]. Besides, local ischaemia reperfusion can induce remote organ injuries, thus worsening the general conditions [15]. Over the past decade, a better understanding of the mechanisms underlying ischaemia–reperfusion injury and the development of new agents specifically for the lung protection against I/R injury have attracted great attention worldwide. Several strategies have also been introduced into clinical practice for the prevention and treatment of ischaemia–reperfusion-induced lung injury with various degrees of success.

Among all the already discovered mechanisms such as oxidative stress, sodium pump inactivation, intracellular calcium overload, iron release, oxidative stress caused by



**Figure 8.** Biochemical markers. Hydrogen saline significantly lowered the level of WBC, CRP, ALT and TBiL. \**P* < 0.05 vs. control group; #*P* < 0.05 vs. I/R + H<sub>2</sub> group.

excessive ROS produced during ischaemia/reperfusion was considered as one of the most important mechanisms in I/R lung injury. When ROS produced exceed the cell antioxidant abilities, they may lead to mitochondrial membrane damage and release of cytochrome *c* which will initiate caspase-mediated programmed cell apoptosis [16]. During the early reperfusion, increased ROS up-regulate inflammatory mediators expression such as cytokines and adhesion molecules, which may cause polymorphonuclear neutrophils infiltration, leading to inflammation, thrombosis, complement activation, and acute tissue damages [17,18].

In recent years, sufficient experimental evidences have documented that without influencing other less potent ROS, important in intracellular signalling, molecular hydrogen possesses the ability to selectively neutralise ONOO<sup>−</sup> and •OH, the most cytotoxic ROS, which can damage cellular macromolecules aggressively and indiscriminately. Thus, H<sub>2</sub> can protect cells from oxidative stress injuries. The therapeutic effects of hydrogen have been shown in various types of oxidative stress related injuries such as ischaemia/reperfusion injury [3], type 2 diabetes [19], neurodegeneration [20], atherosclerosis [10], Parkinson's disease [21], and so on. In particular, hydrogen has ideal protective effects against ischaemia/reperfusion injury in various tissues and organs, such as the heart [4,7,22], liver [18,23], intestine [8,24,25], kidney [6,26], lung [9], brain [27,28] and spinal cord [5,29].

In 2010, a study showed that inhaled hydrogen gas could prevent lung transplantation induced I/R injury and significantly improve the function of lung grafts [9]. In this study, orthotopic left LTxs were performed in syngenic Lewis rats. Grafts were perfused with and stored in low potassium dextran solution at 4 °C for 6 h. The recipients received 100% O<sub>2</sub> or 98% O<sub>2</sub> with 2% N<sub>2</sub>, 2% He, or 2% H<sub>2</sub> during surgery and 1 h after reperfusion. Commonly, ischaemia/reperfusion corresponds to anoxia-reoxygenation in organ transplantation. However, the lung is different because it contains oxygen in the alveoli during ischaemic preservation. In the lung, the oxidative stress resulting from ischaemia should be distinguished from the oxidative stress resulting from hypoxia [1]. Therefore, we considered that different inhaled gas would have some unexpected effects in the experiment, which was apparently neglected. Therefore, in order to exclude the gas influence in the process of ischaemia and reperfusion, saturated hydrogen saline becomes an ideal option. Besides, saturated hydrogen saline is more economic and safe compared with the gas.

Up to now, most of the studies which demonstrated the antioxidant effects of hydrogen have attributed to its ability of scavenging highly toxic ROS as was evidenced in the study of 2007 [3]. However, the exact underlying mechanisms are still not very clear. Several pathways have been introduced. Several studies have demonstrated that hydrogen could inhibit NF- $\kappa$ B activation, thus ameliorating inflammatory responses and protecting the cells [30–32]. Another study showed that molecular hydrogen suppressed Fc epsilon RI-mediated signal transduction and prevented degranulation of mast cells [33]. In our

study, we found that hydrogen inhibited cell apoptosis and incapacitated the most important effect enzyme in the process of apoptosis, caspase-3 [34]. Therefore, it is highly reasonable to speculate that hydrogen may directly or indirectly inhibit caspase-3 activity to achieve apoptosis inhibition effects. However, based on the already involved pathways, hydrogen seems to exert its protective effects nonspecifically and to have little impact on the normal physiological process. We suppose that hydrogen may have a significant influence on the key link in the whole process from receiving the injury to the final cell death. Caspase-3 is undoubtedly an important element which needs further and deeper study in the future.

As a novel antioxidant, hydrogen possesses a number of advantages. (1) Hydrogen can easily penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus, where it exerts its antioxidant effects. (2) It is nontoxic to the organisms, which has been proven by hyperbaric diving for decades. (3) Due to its selectivity, it has less impact on other less active but very important ROS within the cells. However, hydrogen gas is highly flammable, which may easily cause explosion. Therefore, it is not ideally safe and convenient to preserve and use in clinical practice. We dissolve hydrogen gas into normal saline to produce saturated hydrogen saline, which has the similar protective effects of hydrogen gas, but is safer and more convenient in practice, thus being highly promising in the future.

In conclusion, saturated hydrogen saline is highly effective in preventing the lung from ischaemia/reperfusion injury possibly by inhibiting caspase-3 activity, and is a very promising therapy in clinical practice in the future.

## References

- [1] de Perrot M, Liu M, Waddell TK, Keshavjee S. Ischemia-reperfusion-induced lung injury. *Am J Respir Crit Care Med* 2003;167:490–511.
- [2] McCord JM. Oxygen-derived free radicals in postischemic tissue injury. *N Engl J Med* 1985;312:159–63.
- [3] Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007;13:688–94.
- [4] Zhang YF, Sun QA, He B, Xiao JA, Wang ZN, Sun XJ. Anti-inflammatory effect of hydrogen-rich saline in a rat model of regional myocardial ischemia and reperfusion. *Int J Cardiol* 2010;148:91–5.
- [5] Huang Y, Xie KL, Li JP, Xu N, Gong G, Wang G, et al. Beneficial effects of hydrogen gas against spinal cord ischemia-reperfusion injury in rabbits. *Brain Res* 2011;1378:125–36.
- [6] Wang F, Yu GA, Liu SY, Li JB, Wang JF, Bo LL, et al. Hydrogen-rich saline protects against renal ischemia/reperfusion injury in rats. *J Surg Res* 2010;167:E339–44.
- [7] Sun Q, Kang ZM, Cai JM, Liu W, Liu Y, Zhang JH, et al. Hydrogen-rich saline protects myocardium against ischemia/reperfusion injury in rats. *Exp Biol Med* 2009;234:1212–9.
- [8] Buchholz BM, Kaczorowski DJ, Sugimoto R, Yang R, Wang Y, Billiar TR, et al. Hydrogen inhalation ameliorates oxidative



- stress in transplantation induced intestinal graft injury. *Am J Transplant* 2008;8:2015–24.
- [9] Kawamura T, Huang CS, Tochigi N, Lee S, Shigemura N, Billiar TR, et al. Inhaled hydrogen gas therapy for prevention of lung transplant-induced ischemia/reperfusion injury in rats. *Transplantation* 2010;90:1344–51.
  - [10] Ohsawa I, Nishimaki K, Yamagata K, Ishikawa M, Ohta S. Consumption of hydrogen water prevents atherosclerosis in apolipoprotein E knockout mice. *Biochem Biophys Res Commun* 2008;377:1195–8.
  - [11] Sun QA, Cai JM, Liu SL, Liu Y, Xu W, Tao H, et al. Hydrogen-rich saline provides protection against hyperoxic lung injury. *J Surg Res* 2010;165:E43–9.
  - [12] Pei JM, Chen M, Wang YM, Wen J, Zhu YL. Kappa-opioid receptor stimulation contributes to aortic artery dilation through activation of K(ATP) channel in the rats. *Sheng Li Xue Bao* 2003;55:91–5.
  - [13] Wang YX, Legzdins P, Poon JS, Pang CC. Vasodilator effects of organotransition-metal nitrosyl complexes, novel nitric oxide donors. *J Cardiovasc Pharmacol* 2000;35:73–7.
  - [14] Kagawa H, Morita K, Nagahori R, Shinohara G, Kinouchi K, Hashimoto K. Prevention of ischemia/reperfusion-induced pulmonary dysfunction after cardiopulmonary bypass with terminal leukocyte-depleted lung reperfusion. *J Thorac Cardiovasc Surg* 2010;139:174–80.
  - [15] Goksin I, Adali F, Enli Y, Akbulut M, Teke Z, Sackan G, et al. The effect of phlebotomy and mannitol on acute renal injury induced by ischemia/reperfusion of lower limbs in rats. *Ann Vasc Surg* 2011;25:1118–28.
  - [16] Kumar S, Harvey NL. Role of multiple cellular proteases in the execution of programmed cell death. *FEBS Lett* 1995;375:169–73.
  - [17] Entman ML, Michael L, Rossen RD, Dreyer WJ, Anderson DC, Taylor AA, et al. Inflammation in the course of early myocardial ischemia. *FASEB J* 1991;5:2529–37.
  - [18] Zhou W, McCollum MO, Levine BA, Olson MS. Inflammation and platelet-activating factor production during hepatic ischemia/reperfusion. *Hepatology* 1992;16:1236–40.
  - [19] Kajiyama S, Hasegawa G, Asano M, Hosoda H, Fukui M, Nakamura N, et al. Supplementation of hydrogen-rich water improves lipid and glucose metabolism in patients with type 2 diabetes or impaired glucose tolerance. *Nutr Res* 2008;28:137–43.
  - [20] Ohsawa I, Nishimaki K, Murakami Y, Suzuki Y, Ishikawa M, Ohta S. Age-dependent neurodegeneration accompanying memory loss in transgenic mice defective in mitochondrial aldehyde dehydrogenase 2 activity. *J Neurosci* 2008;28:6239–49.
  - [21] Fu Y, Ito M, Fujita Y, Ito M, Ichihara M, Masuda A, et al. Molecular hydrogen is protective against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease. *Neurosci Lett* 2009;453:81–5.
  - [22] Hayashida K, Sano M, Ohsawa I, Shinmura K, Tamaki K, Kimura K, et al. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia–reperfusion injury. *Biochem Biophys Res Commun* 2008;373:30–5.
  - [23] Fukuda KI, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. *Biochem Biophys Res Commun* 2007;361:670–4.
  - [24] Chen H, Sun YP, Hu PF, Liu WW, Xiang HG, Li Y, et al. The effects of hydrogen-rich saline on the contractile and structural changes of intestine induced by ischemia–reperfusion in rats. *J Surg Res* 2011;167:316–22.
  - [25] Zheng XF, Mao YF, Cai JM, Li YH, Liu WW, Sun P, et al. Hydrogen-rich saline protects against intestinal ischemia/reperfusion injury in rats. *Free Radic Res* 2009;43:478–84.
  - [26] Shingu C, Koga H, Hagiwara S, Matsumoto S, Goto K, Yokoi I, et al. Hydrogen-rich saline solution attenuates renal ischemia–reperfusion injury. *J Anesth* 2010;24:569–74.
  - [27] Xiang J, Tang YP, Wu P, Gao JP, Cai DF. Chinese medicine Nao-Shuan-Tong attenuates cerebral ischemic injury by inhibiting apoptosis in a rat model of stroke. *J Ethnopharmacol* 2010;131:174–81.
  - [28] Cai JM, Kang ZM, Liu K, Liu WW, Li RP, Zhang JH, et al. Neuroprotective effects of hydrogen saline in neonatal hypoxia–ischemia rat model. *Brain Res* 2009;1256:129–37.
  - [29] Huang Y, Xie KL, Li JP, Xu N, Gong G, Wang GL, et al. Beneficial effects of hydrogen gas against spinal cord ischemia–reperfusion injury in rabbits. *Brain Res* 2010;1378:125–36.
  - [30] Song GH, Tian H, Liu J, Zhang HL, Sun XJ, Qin SC. H(2) inhibits TNF-alpha-induced lectin-like oxidized LDL receptor-1 expression by inhibiting nuclear factor kappa B activation in endothelial cells. *Biotechnol Lett* 2011;33:1715–22.
  - [31] Wang C, Li JA, Liu QA, Yang R, Zhang JH, Cao YP, et al. Hydrogen-rich saline reduces oxidative stress and inflammation by inhibit of JNK and NF-kappa B activation in a rat model of amyloid-beta-induced Alzheimer's disease. *Neurosci Lett* 2011;491:127–32.
  - [32] Huang CS, Kawamura T, Peng XM, Tochigi N, Shigemura N, Billiar TR, et al. Hydrogen inhalation reduced epithelial apoptosis in ventilator-induced lung injury via a mechanism involving nuclear factor-kappa B activation. *Biochem Biophys Res Commun* 2011;408:253–8.
  - [33] Itoh T, Fujita Y, Ito M, Masuda A, Ohno K, Ichihara M, et al. Molecular hydrogen suppresses Fc epsilon RI-mediated signal transduction and prevents degranulation of mast cells. *Biochem Biophys Res Commun* 2009;389:651–6.
  - [34] Chang HY, Yang X. Proteases for cell suicide: functions and regulation of caspases. *Microbiol Mol Biol Rev* 2000;64:821–46.