

Provided for non-commercial research and education use.  
Not for reproduction, distribution or commercial use.



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>

## Hydrogen-Rich Saline Provides Protection Against Hyperoxic Lung Injury

Qiang Sun, Ph.D.,\* Jianmei Cai, Ph.D.,† Shulin Liu, Ph.D.,\* Yun Liu, M.D.,\* Weigang Xu, M.D.,\* Hengyi Tao, M.D.,\*<sup>1</sup> and Xuejun Sun, Ph.D., M.D.\*<sup>1</sup>

\*Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai, PR China; and †Department of Neurology, Changhai Hospital, 174 Changhai Road, Shanghai, PR China

Submitted for publication August 21, 2010

**Background.** Hydrogen has been proven to be a novel antioxidant through its selectively reducing of the hydroxyl radical. In this study, we investigated the effects of hydrogen-rich saline on the prevention of acute lung injury induced by hyperoxia (HALI) in rats.

**Materials and Methods.** Physiologic saline, hydrogen-rich saline, or nitrogen-rich saline was administered through intraperitoneal (i.p.) injection during exposure to hyperoxia (10 mL/Kg), respectively.

**Results.** Severity of HALI was assessed by the volume of pleural effusion, wet-to-dry weight ratio (W/D), and histologic analysis. Apoptosis in lung cells was determined with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive staining. The content of pro-inflammatory cytokine interleukin IL-1b and TNF-a in the lung tissues were detected by enzyme-linked immunosorbent assay (ELISA). Hydrogen-rich saline treatment provides protection against HALI by inhibiting lipid, DNA oxidation, and tissue edema. Moreover, hydrogen-rich saline treatment could inhibit apoptosis and inflammation while no significant reduction was observed in nitrogen-rich saline treated animals.

**Conclusion.** The results of this study demonstrate that hydrogen-rich saline ameliorated hyperoxia-induced acute lung injury by reducing oxidative stress and inflammatory cascades in lung tissue. © 2011 Elsevier Inc. All rights reserved.

**Key Words:** hydrogen; acute lung injury; oxidative stress; inflammation; apoptosis.

### INTRODUCTION

Hyperoxic acute lung injury (HALI), caused by prolonged supplement of very high concentrations of oxygen (fractional concentrations of oxygen > 50%), is a clinical syndrome characterized by endothelial and epithelial injury and enhanced alveolar capillary protein leak [1–6]. It is generally accepted that increased generation of reactive oxygen species (ROS) plays an important role in lung injury during exposure to hyperoxia [7–9]. To evaluate antioxidant defenses, a principal focus of prior studies has been on antioxidant enzymes such as superoxide dismutase (SOD) [10,11], GSH peroxidase (GPx) [12, 13], and peroxiredoxin 6 [14, 15] utilizing both overexpression and suppression of activity. In addition, subsequent reports indicated that IL-1, tumor necrosis factor (TNF)-a and IL-6 induce tolerance [1, 16]. As yet, there are no specific treatments for HALI available and new effective treatment are needed for clinical settings.

Hydrogen is a gaseous molecule without known toxicity, which could react with hydroxyl radical, has been considered as a novel antioxidant [17]. Both *in vivo* and *in vitro* studies support the protective effect of hydrogen on ischemia-reperfusion injuries caused by oxidative stress in brain [18], liver [19], heart [20–22], and intestine [23], as well as anti-inflammatory effect on acute pancreatitis [24], colon inflammation [25], liver inflammation [26].

Our previous study has demonstrated that hydrogen-rich saline could reduce lung injury induced by intestinal ischemia/reperfusion in rats [27]. This raises the possibility that the hydrogen-rich saline might lead to protection against HALI. Therefore, the present study investigated the possible therapeutic effects of hydrogen-rich saline on lung injury induced by hyperoxia in rats.

<sup>1</sup> To whom correspondence and reprint requests should be addressed at Department of Diving Medicine, Faculty of Naval Medicine, Second Military Medical University, Shanghai 200433, PR China. E-mail: taohengyi@hotmail.com and sunxjk@hotmail.com.

## MATERIALS AND METHODS

### Animals

Adult male Sprague-Dawley rats weighing 220–250 g were used in all experiments. The animals were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle and free access to food and distilled water. 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).

### Experimental Protocol

The animals were placed in cages in gastight 7-L Plexiglas chambers exposed continuously to hyperoxia (>98% O<sub>2</sub>) for 60 h. The animals were supplied food and water during the exposure. The gas in the chamber was continuously ventilated (with a flow rate of 1 L/min) to minimize PpCO<sub>2</sub> changes, and the temperature of the chamber was maintained at a range of 22–25°C. Once a day, a collection shelf beneath the cages was opened briefly to replace the bedding. O<sub>2</sub> concentrations in the chamber were determined with a gas spectrometer. Rats under normobaric room air served as sham. Animals were randomized into the following four groups and then they were allocated to the following protocols:

- (1) SHAM group: normobaric room air for 60 h ( $n = 12$ )
- (2) NORM group: normobaric room air for 60 h plus four intraperitoneal (i.p.) injection of hydrogen-rich saline (12, 24, 36, 48 h; (10 mL/Kg × 4);  $n = 12$ )
- (3) H<sub>2</sub> group: hyperoxia (>98% O<sub>2</sub>) for 60 h plus four i.p. injection of hydrogen-rich saline (12, 24, 36, 48 h; (10 mL/Kg × 4);  $n = 12$ )
- (4) CON group: hyperoxia (>98% O<sub>2</sub>) for 60 h plus four i.p. injection of physiologic saline ( $n = 12$ ).
- (5) N<sub>2</sub> group: hyperoxia (>98% O<sub>2</sub>) for 60 h plus four intraperitoneal injection of nitrogen-rich saline ( $n = 12$ )

### Hydrogen-Rich Saline Production

Hydrogen was dissolved in physiologic saline 6 h under high pressure (0.4 MPa) to a supersaturated level using hydrogen-rich saline-producing apparatus produced by our department. The saturated hydrogen saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. Hydrogen-rich saline was sterilized by gamma radiation. Hydrogen-rich saline was 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.* [17]. Nitrogen-rich saline was produced in the same manner as the hydrogen rich saline.

### Tissue Collection

After 60 h of exposure, the rats were anesthetized with pentobarbital sodium, the chest and peritoneal cavities were opened carefully, and the volume of pleural fluid was measured. The great vessels were transected in the abdomen, and the lungs were removed *en bloc* and drained of blood. The lungs were dissected from the hilar structures and blotted gently on moist gauze. One lung was cut in half, left lung weighed immediately (wet weight), and placed in a vacuum oven. The remaining right lung tissue was snap-frozen in liquid nitrogen and stored at –80°C for malondialdehyde (MDA) and superoxidase dismutase (SOD) concentration analysis, myeloperoxidase (MPO), TNF- $\alpha$  and IL-1 $\beta$  assays.

## Measurement of MDA and SOD in Lung Tissues

Pulmonary MDA and SOD content were determined with chemical method per the manufacturer's instructions (Nanjing Jiancheng Biochemistry Co., Nanjing, China). Lung tissue (100 mg, wet weight) was homogenized in 2 mL of 10 mM phosphate buffer (pH 7.4). After centrifugation at 12,000  $g$  for 20 min, the MDA and SOD content in the supernatant were measured using the corresponding kits. MDA content was measured with thiobarbituric acid (TBA) reaction. The method was used to obtain a spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 535 nm; estimated MDA level was expressed as nmol/mg-protein. SOD activity was measured using nitroblue tetrazolium (NBT) reduction assay following the reduction of nitrite by a xanthine-xanthine oxidase system, which is a superoxide anion generator. One unit of SOD is defined as the amount that shows 50% inhibition.

## Determination of TNF- $\alpha$ , IL-1 $\beta$ , MPO, and 8OHdG 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- $\alpha$ , IL-1 $\beta$ , MPO, and 8OHdG were measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit following the instructions of the manufacturer. 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 Coomassie blue assay and the results were corrected per microgram of protein.

## Hematoxylin and Eosin Staining

The specimens of the left 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.

## In Situ Apoptosis Assay

Lungs were perfused *via* transcardiac approach with PBS (50 mL) followed by 4% phosphate-buffered formalin. Perfusion-fixed lung tissues were further fixed overnight in the solution (4% paraformaldehyde in PBS), processed for embedding in paraffin, and cut into 4- $\mu$ m-thick serial sections. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on paraffin-embedded sections by using the *in situ* cell death detection kit (Roche, Indianapolis, IN, USA). According to standard protocols, the sections were dewaxed and rehydrated by heating the slides at 60°C. Then these sections were incubated in a 20  $\mu$ g/mL proteinase K working solution for 15 min at room temperature. The slides were rinsed three times with PBS before they were incubated in TUNEL reaction mixture for 1 h at 37°C. Dried area around sample by filter paper and added Converter-AP on samples for 1 h at 37°C. After rinsing with PBS (5 min, three times), sections were colored 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 ( $\times 200$ ). 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.

## Statistical Analysis

Values were presented as mean  $\pm$  SD. Statistical analysis was done using the SPSS ver. 17.0 (SPSS Inc., Chicago, IL) by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* test.  $P$  value < 0.05 was considered statistically significant.

RESULTS

Pleural Fluid Volume and Lung Edema

To evaluate the extent of O<sub>2</sub>-induced lung damage, pleural fluid volume, and the wet-to-dry weight ratio were measured in SHAM, NORM, CON, H<sub>2</sub>, and N<sub>2</sub> groups right after 60 h of O<sub>2</sub> exposure. Pleural fluid volume and the wet-to-dry ratio increased significantly in rats exposed to O<sub>2</sub>. Measurements of pleural fluid volume and the wet-to-dry weight ratio in hyperoxia are summarized in Fig. 1. Compared with those of saline-treated rats, both pleural fluid volume and wet-to-dry weight ratio were significantly reduced by hydrogen-rich saline treatment (\**P* < 0.05). Nitrogen-rich saline showed no significant reduction of the pleural fluid volume and the wet-to-dry weight ratio (*P* = NS). Intraperitoneal injection of hydrogen-rich saline alone in NORM group did not cause any change of the pleural fluid volume and the wet-to-dry weight ratio compared to SHAM group (*P* = NS).

Histopathology of Lung

The effects of hydrogen-rich saline treatment on the histopathologic changes of lungs in rats are shown in

Fig. 1. Morphologic study showed, after 60 h of O<sub>2</sub> exposure, the lung tissues of rats were severely damaged in the CON group and N<sub>2</sub> group, with severe edema, severe alveolar hemorrhage, and extensive inflammatory cell infiltration. Moderate lung edema, hemorrhage, and inflammatory cell infiltration were seen in hydrogen-rich saline treated group, suggesting that hyperoxia lung injury was reduced by hydrogen-rich saline treatment. There was no significant histopathologic difference between SHAM and NORM groups.

SOD, MDA, and 8OHdG Measurements in Lung Tissues

As shown in Fig. 2, lung-tissue SOD, MDA, and 8OHdG assays revealed negligible lung oxidant stress in the SHAM group. However, compared with SHAM group, pulmonary SOD, MDA, and 8OHdG levels increased in saline-treated group in O<sub>2</sub> exposure groups (#*P* < 0.05 versus SHAM group). It was noted that hydrogen-rich saline treatment significantly decreased the SOD, MDA, and 8OHdG levels compared with those of saline-treated rats lung tissues after 60 h of O<sub>2</sub> exposure, thus, oxidative stress (\**P* < 0.05 versus CON group). Nitrogen-rich saline showed no significant

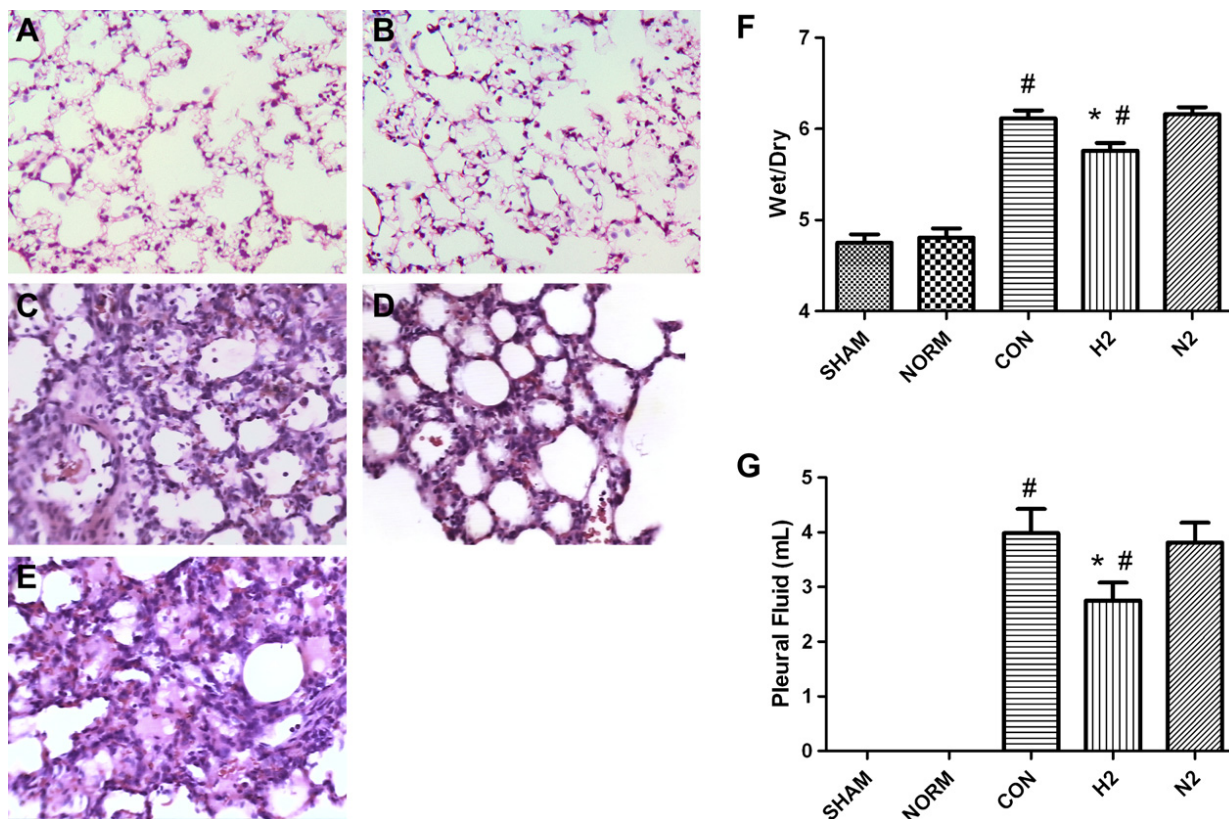
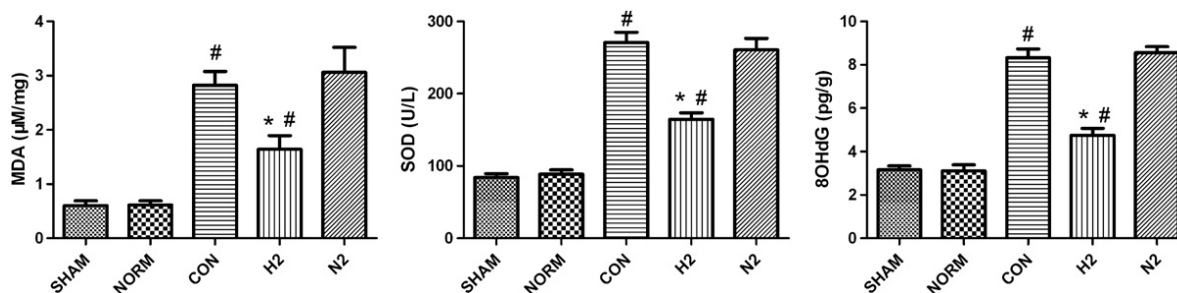


FIG. 1. Pleural fluid volume, wet-to-dry weight ratio, and histopathology analysis in SHAM, NORM, CON, H<sub>2</sub>, and N<sub>2</sub> groups. *F* = wet-to-dry weight ratio. *G* = Pleural fluid volume (*n* = 6, \**P* < 0.05 relative to CON group, #*P* < 0.05 relative to SHAM group). Data are expressed as ± SEM. (A)–(D) Photomicrographs of left lung sections. SHAM (A) and NORM (B): Normal histopathology; CON (C) and N<sub>2</sub> (E): the lung tissues revealed severe edema, alveolar hemorrhage, and inflammatory cell infiltration; H<sub>2</sub> (D): moderate edema, hemorrhage and inflammatory cell infiltration. Routine hematoxylin and eosin stained (×200).



**FIG. 2.** Results showing SOD, MDA, and 8OHdG levels in lungs of rats in SHAM, NORM, CON, H<sub>2</sub>, and N<sub>2</sub> groups. (A) Lung tissue SOD expression at the end of 60 h of O<sub>2</sub> exposure; (B) lung tissue MDA activity at the end of 60 h of O<sub>2</sub> exposure; (C) lung tissue 8OHdG level at the end of 60 h of O<sub>2</sub> exposure. Hydrogen-rich saline significantly reduced the elevation of SOD, MDA, and 8OHdG levels in the lung tissues; ( $n = 6$ ,  $*P < 0.05$  compared with CON group;  $\#P < 0.05$  relative to SHAM group).

reduction of SOD, MDA, and 8OHdG levels ( $P = NS$  versus CON group). There was no significant difference of SOD, MDA, and 8OHdG levels between SHAM group and NORM group ( $P = NS$ ).

#### Effect of Hydrogen-Rich Saline on TNF- $\alpha$ , IL-1 $\beta$ , and MPO Levels

ELISA detection showed that the levels of TNF- $\alpha$ , IL-1 $\beta$ , and MPO in lung tissue were markedly increased by 2.9-fold, 6.4-fold, and 1.4-fold, respectively, in the CON group compared with SHAM group. Hydrogen-rich saline significantly reduced the elevation of TNF- $\alpha$ , IL-1 $\beta$ , and MPO in the lung tissues (Fig. 3). Nitrogen-rich saline showed no significant reduction of TNF- $\alpha$ , IL-1 $\beta$ , and MPO levels ( $P = NS$  versus CON). Intraperitoneal injection of hydrogen-rich saline alone in NORM group showed no significant changing of TNF- $\alpha$ , IL-1 $\beta$ , and MPO levels compared with SHAM group ( $P = NS$ ).

#### Detection of Apoptotic Cell Death

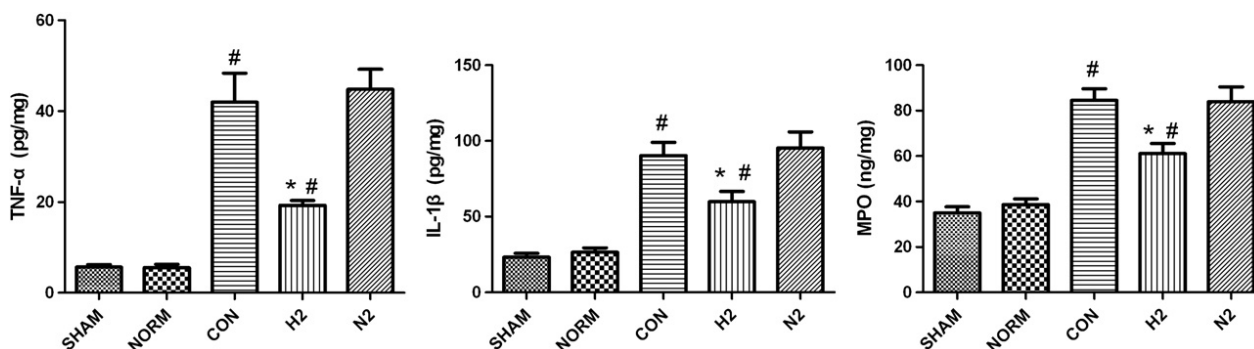
As shown in Fig. 4, the number of TUNEL-positive cells was increased in the lung tissues after 60 h of O<sub>2</sub> exposure. Hydrogen-rich saline treatment comparably decreased the percent of TUNEL-positive cells relative

to the CON group ( $*P < 0.05$ ). Nitrogen-rich saline showed no significant reduction of TUNEL-positive cells ( $P = NS$  versus CON group). NORM group showed no significant increasing number of TUNEL-positive cells compared with SHAM group ( $P = NS$ ).

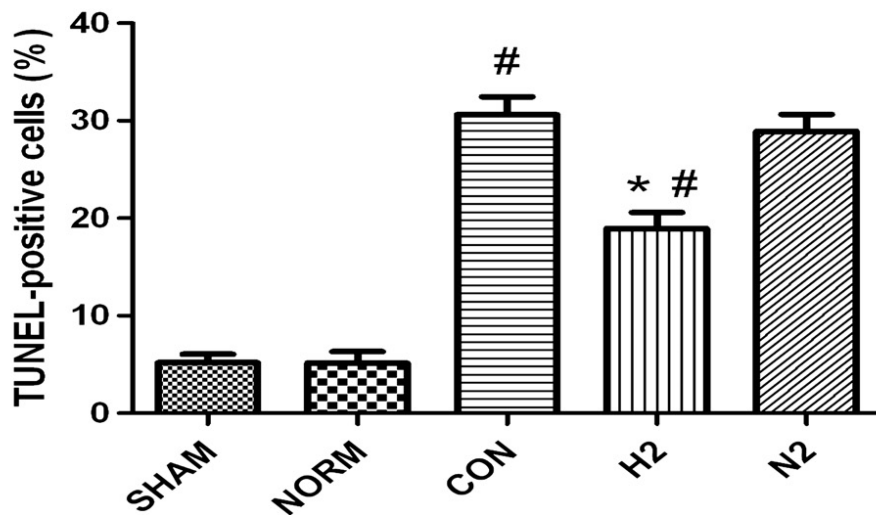
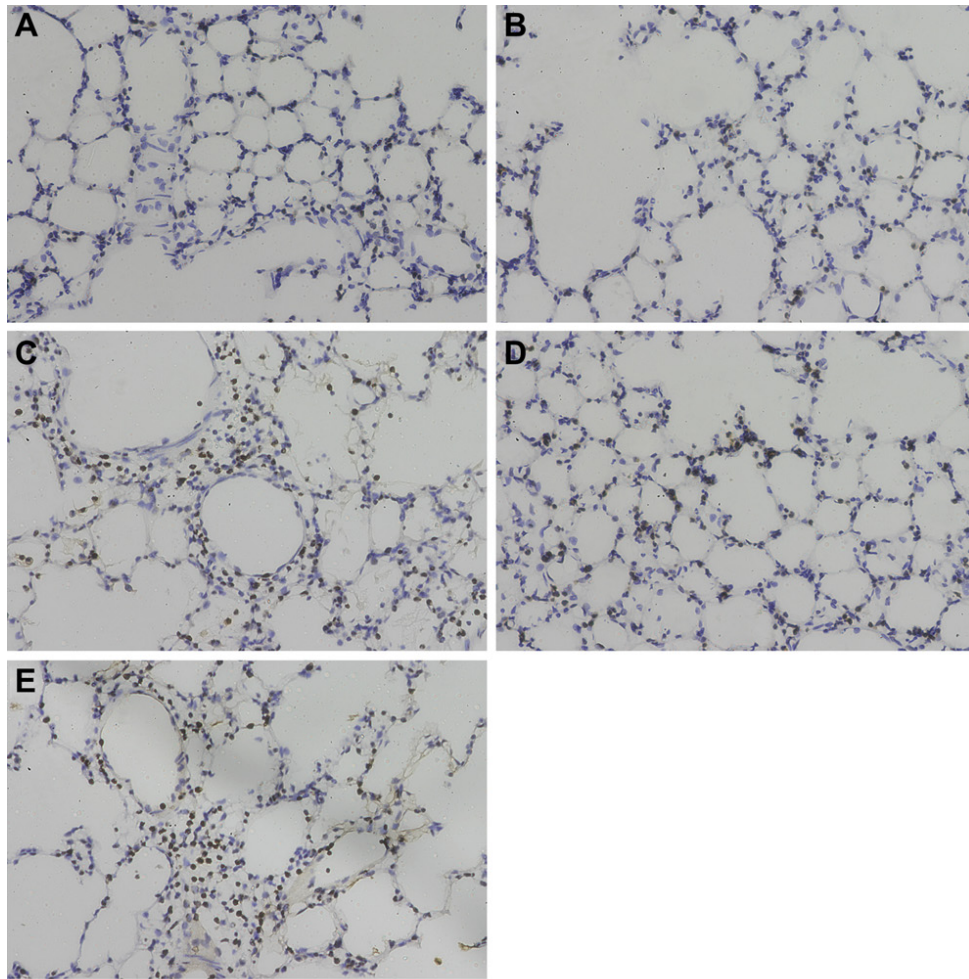
#### DISCUSSION

As we aware that this is the first study that demonstrated hydrogen-rich saline significantly prevent HALI. The protective effect is supported by reduced lung injury as measured by diminished volume of pleural effusion, lower level of W/D, lung cell apoptosis, and marked preservation of lung tissue structure macroscopically and microscopically. In addition, hydrogen-rich saline has been shown to significantly ameliorate the increased MDA and 8OHdG levels and MPO activity in the lung tissues, accompanied by reduced content of pro-inflammatory cytokine IL-1 $\beta$  and TNF- $\alpha$ .

Hyperoxia-induced lung injury can be considered as a bimodal process resulting (1) from direct oxygen toxicity and (2) from the accumulation of inflammatory mediators within the lungs [28]. First, oxygen toxicity is believed to be mediated by the production and accumulation of excessive ROS, at levels exceeding



**FIG. 3.** TNF- $\alpha$ , IL-1 $\beta$ , and MPO levels in the SHAM, NORM, CON, H<sub>2</sub>, and N<sub>2</sub> groups 60 h after O<sub>2</sub> exposure. Hydrogen-rich saline significantly reduced the elevation of TNF- $\alpha$ , IL-1 $\beta$ , and MPO in the lung tissues; ( $n = 6$ ,  $*P < 0.05$  compared with CON group;  $\#P < 0.05$  relative to SHAM group).



**FIG. 4.** Detection of apoptotic cell death by TUNEL staining in the SHAM, NORM, CON, H<sub>2</sub>, and N<sub>2</sub> groups 60 h after O<sub>2</sub> exposure. (A) TUNEL staining of lung sections in SHAM group. (B) TUNEL staining of lung sections in NORM group. (C) TUNEL staining of lung sections in CON group. (D) TUNEL staining of lung sections in H<sub>2</sub> group. (E) TUNEL staining of lung sections in N<sub>2</sub> group. Relative to the CON, hydrogen-rich saline significantly reduced the number of TUNEL-positive cells (brown staining). Values are mean  $\pm$  SEM; \* $P$  < 0.05 compared with CON group. # $P$  < 0.05 relative to SHAM group;  $n$  = 6 for each group.

the capacity of the lung antioxidant defense mechanisms [7]. Hydrogen could selectively react with exclusively detrimental ROS, such as hydroxyl radical and peroxynitrite, exerting protective effects, while not interaction with other physiological ROS, such as superoxide anion and H<sub>2</sub>O<sub>2</sub>, which possess physiologic roles [17]. In our present study, along with the reducing of end-product of lipid oxidation (MDA) and DNA oxidation (8OHdG), the SOD activity was also comparably low in the hydrogen treatment group, which suggests the degree of oxidative stress was down-regulated by hydrogen. In addition, exposure to hyperoxia triggers an inflammatory response, which exacerbates oxidative toxicity [29]. Upon exposure to hyperoxia, ROS evoke pulmonary cells to increase the secretion of chemoattractants and other proinflammatory cytokines that lead to leukocyte recruitment to the lung. Recruited leukocytes are significant sources of additional ROS, and the interactions between ROS and leukocytes establish a vicious cycle that initiates and/or exacerbates lung injury. According to our findings, hydrogen-rich saline decreased MPO levels, a marker of neutrophil recruitment compared with animals treated with saline, suggesting that hydrogen reduces neutrophil recruitment to the lung, protecting against lung injury. Elevation of proinflammatory cytokine IL-1b and TNF- $\alpha$  represents one of the first pulmonary inflammatory responses to hyperoxia, which leads to pulmonary tissue damage [30]. Hydrogen significantly reduced these proinflammatory cytokines in lung tissues, suggesting that the preventive effect of hydrogen on lung injury may be mediated by suppression of the excessive inflammatory response and its downstream cascade. Moreover, no significant lung injury was found in the NORM group, which indicates that applying the four-dose hydrogen-rich saline will be safe.

Furthermore, we tested the nitrogen-rich saline in HALI model as negative control. Nitrogen-rich saline was also a de-oxygenated solution in the same manner as that we prepared the hydrogen-rich saline. According to our findings, neither oxidative stress nor inflammatory response was reduced by nitrogen-rich saline treatment, which helps us to conclude that the observed protection by hydrogen-rich saline is being mediated *via* a hydrogen-dependent manner.

In conclusion, the results of this study demonstrate that hydrogen-rich saline ameliorated hyperoxia-induced acute lung injury by reducing oxidative stress and inflammatory cascades in lung tissue. Although the intensive mechanism involved in the protective role of hydrogen remains to be determined because of its safety, efficacy, and convenience, peritoneal injection of hydrogen offers a simple, easy to use, safe, and economic novel approach for future HALI protection.

## ACKNOWLEDGMENTS

This study was supported by Ph.D. InnoFund of Second Military Medical University (2009).

## REFERENCES

1. Kolliputi N, Waxman AB. IL-6 cytoprotection in hyperoxic acute lung injury occurs *via* suppressor of cytokine signaling-1-induced apoptosis signal-regulating kinase-1 degradation. *Am J Respir Cell Mol Biol* 2009;40:314.
2. Barazzone C, Horowitz S, Donati YR, et al. Oxygen toxicity in mouse lung: Pathways to cell death. *Am J Respir Cell Mol Biol* 1998;19:573.
3. O'Reilly MA, Staversky RJ, Huyck HL, et al. Bcl-2 family gene expression during severe hyperoxia-induced lung injury. *Lab Invest* 2000;80:1845.
4. Saikumar P, Dong Z, Weinberg JM, et al. Mechanisms of cell death in hypoxia/reoxygenation injury. *Oncogene* 1998;17:3341.
5. Waxman AB, Einarsson O, Seres T, et al. Targeted lung expression of interleukin-11 enhances murine tolerance of 100% oxygen and diminishes hyperoxia-induced DNA fragmentation. *J Clin Invest* 1998;101:1970.
6. De Paepe ME, Mao Q, Chao Y, et al. Hyperoxia-induced apoptosis and Fas/FasL expression in lung epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L647.
7. Pace PW, Yao LJ, Wilson JX, et al. The effects of hyperoxia exposure on lung function and pulmonary surfactant in a rat model of acute lung injury. *Exp Lung Res* 2009;35:380.
8. Suttorp N, Simon LM. Lung cell oxidant injury. Enhancement of polymorphonuclear leukocyte-mediated cytotoxicity in lung cells exposed to sustained *in vitro* hyperoxia. *J Clin Invest* 1982;70:342.
9. Reddy NM, Kleeberger SR, Kensler TW, et al. Disruption of Nrf2 impairs the resolution of hyperoxia-induced acute lung injury and inflammation in mice. *J Immunol* 2009;182:7264.
10. Robbins CG, Horowitz S, Merritt TA, et al. Recombinant human superoxide dismutase reduces lung injury caused by inhaled nitric oxide and hyperoxia. *Am J Physiol* 1997;272:L903.
11. Asikainen TM, Huang TT, Taskinen E, et al. Increased sensitivity of homozygous Sod2 mutant mice to oxygen toxicity. *Free Radic Biol Med* 2002;32:175.
12. Cheng WH, Ho YS, Ross DA, et al. Overexpression of cellular glutathione peroxidase does not affect expression of plasma glutathione peroxidase or phospholipid hydroperoxide glutathione peroxidase in mice offered diets adequate or deficient in selenium. *J Nutr* 1997;127:675.
13. Klivenyi P, Andreassen OA, Ferrante RJ, et al. Mice deficient in cellular glutathione peroxidase show increased vulnerability to malonate, 3-nitropropionic acid, and 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. *J Neurosci* 2000;20:1.
14. Wang Y, Phelan SA, Manevich Y, et al. Transgenic mice overexpressing peroxiredoxin 6 show increased resistance to lung injury in hyperoxia. *Am J Respir Cell Mol Biol* 2006;34:481.
15. Wang Y, Feinstein SI, Manevich Y, et al. Lung injury and mortality with hyperoxia are increased in peroxiredoxin 6 gene-targeted mice. *Free Radic Biol Med* 2004;37:1736.
16. White CW, Ghezzi P, Dinarello CA, et al. Recombinant tumor necrosis factor/cachectin and interleukin 1 pretreatment decreases lung oxidized glutathione accumulation, lung injury, and mortality in rats exposed to hyperoxia. *J Clin Invest* 1987;79:1868.
17. Ohsawa I, Ishikawa M, Takahashi K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. *Nat Med* 2007;13:688.
18. Cai J, Kang Z, Liu K, et al. Neuroprotective effects of hydrogen saline in neonatal hypoxia-ischemia rat model. *Brain Res* 2009;1256:129.

19. Fukuda K, Asoh S, Ishikawa M, et al. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. *Biochem Biophys Res Commun* 2007;361:670.
20. Buchholz BM, Kaczorowski DJ, Sugimoto R, et al. Hydrogen inhalation ameliorates oxidative stress in transplantation-induced intestinal graft injury. *Am J Transplant* 2008;8:2015.
21. Hayashida K, Sano M, Ohsawa I, 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.
22. Sun Q, Kang Z, Cai J, et al. Hydrogen-rich saline protects myocardium against ischemia/reperfusion injury in rats. *Exp Biol Med (Maywood)* 2009;234:1212.
23. Zheng X, Mao Y, Cai J, et al. Hydrogen-rich saline protects against intestinal ischemia/reperfusion injury in rats. *Free Radic Res* 2009;43:478.
24. Chen H, Sun YP, Li Y, et al. Hydrogen-rich saline ameliorates the severity of l-arginine-induced acute pancreatitis in rats. *Biochem Biophys Res Commun* 2010;393:308.
25. Kajiya M, Silva MJ, Sato K, et al. Hydrogen mediates suppression of colon inflammation induced by dextran sodium sulfate. *Biochem Biophys Res Commun* 2009;386:11.
26. Gharib B, Hanna S, Abdallahi OM, et al. Anti-inflammatory properties of molecular hydrogen: investigation on parasite-induced liver inflammation. *C R Acad Sci III* 2001;324:719.
27. Mao YF, Zheng XF, Cai JM, et al. Hydrogen-rich saline reduces lung injury induced by intestinal ischemia/reperfusion in rats. *Biochem Biophys Res Commun* 2009;381:602.
28. Zaher TE, Miller EJ, Morrow DM, et al. Hyperoxia-induced signal transduction pathways in pulmonary epithelial cells. *Free Radic Biol Med* 2007;42:897.
29. Barazzone C, White CW. Mechanisms of cell injury and death in hyperoxia: Role of cytokines and Bcl-2 family proteins. *Am J Respir Cell Mol Biol* 2000;22:517.
30. Guthmann F, Wissel H, Schachtrup C, et al. Inhibition of TNF $\alpha$  *in vivo* prevents hyperoxia-mediated activation of caspase 3 in type II cells. *Respir Res* 2005;6:10.