

Saturated hydrogen saline protects the lung against oxygen toxicity

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ABSTRACT

Exposure to high oxygen concentrations leads to acute lung injury, including lung tissue and alveolar edema formation, congestion, intra-alveolar hemorrhage, as well as endothelial and epithelial cell apoptosis or necrosis. Several studies have reported that molecular hydrogen is an efficient anti-oxidant by gaseous rapid diffusion into tissues and cells. Moreover, consumption of water with dissolved molecular hydrogen to a saturated level (hydrogen water) prevents stress-induced cognitive decline in mice and superoxide formation in mice. The purpose of the present study was to investigate the effect of saturated hydrogen saline on pulmonary injury-induced exposure to >98% oxygen at 2.5 ATA for five hours. Adult male Sprague-Dawley (SD) rats were randomly divided into three groups: control group, saline group and saturated hydrogen saline group. Hematoxylin and eosin (H&E) staining were used to examine histological changes. The lung **wet to dry (WD)** weight ratio was calculated. The concentration of protein and total cell counts in bronchoalveolar lavage fluid (BALF) were measured. Lactate dehydrogenase (LDH) in serum and BALF were measured by spectrophotometer. The light microscope findings showed that saturated hydrogen saline reduced the impairment when compared with the saline group: Saturated hydrogen saline decreased lung edema, reduced LDH activity in BALF and serum, and decreased total cells and protein concentration in BALF. These results demonstrated that saturated hydrogen saline alleviated hyperoxia-induced pulmonary injury, which was partly responsible for the inhibition of oxidative damage.

INTRODUCTION

Although essential for survival, oxygen may become toxic at an elevated partial pressure; this puts limitations on the application of oxygen. Hyperbaric oxygen therapy is administered for the treatment of tissue hypoxia, most commonly in an intensive care setting of respiratory-insufficient patients, though its potent toxicity is well-described (1). Moreover, the relationship between pulmonary oxygen toxicity and acute lung injury (ALI) and its most severe form, the acute respiratory distress syndrome (ARDS), has attracted medical research attention. Studies have

provided strong evidences for the similarities between pulmonary oxygen toxicity and ALI/ARDS. Understanding these similarities and the signaling pathways is important for the mechanisms of ALI/ARDS.

The pathological manifestations of oxygen-induced lung injury include interstitial and alveolar edema, congestion and intra-alveolar hemorrhage, hyaline membrane formation, lysis of alveolar type I and capillary endothelial cells at 2-4 ATA oxygen pressure. The pathophysiology of oxygen injury is characterized by lung inflammation, including activation and recruitment of neutrophils and

alveolar macrophages, tissue and alveolar edema, surfactant dysfunction, and excess production of free radicals and inflammatory cytokines (2-4). Although the exact mechanisms of oxygen-induced lung injury are still unknown, compelling evidence suggests that reactive oxygen species such as superoxide anions, hydroxyl radicals and hydrogen peroxide are important mediators of lung injury (5-7).

The currently accepted primary mechanism for oxygen toxicity is the “free-radical” theory, which ascribes tissue damage to the increased generation of oxygen-derived radicals during exposure to elevated oxygen tensions (8, 9). It has been postulated that the source of oxygen-derived radicals associated with oxygen toxicity may be the polymorphonuclear leukocyte (PMN) (10). Models of lung injury have demonstrated that inflammation of the lung can interact with hyperoxic injury to potentiate lung damage (11). In addition, toxic products from PMN or non-cellular systems (*e.g.*, xanthine plus xanthine oxidase) in high concentration can damage lung cells (12, 13). Endothelial and epithelial injury and cell death are major features of pulmonary oxygen toxicity. There are two classical types of cell death in pulmonary oxygen toxicity: necrosis or apoptosis (14). Apoptosis appears to be the major mode of cell death when cells experience lethal oxidative insult from exposure to oxidants, including H₂O₂ and superoxide (15, 16).

Recently, it has been reported that molecular hydrogen is an efficient antioxidant by gaseous rapid diffusion into tissues and cells (17-21). Moreover, consumption of water with dissolved molecular hydrogen to a saturated level (hydrogen water) prevents stress-induced cognitive decline in mice and superoxide formation in mice (22). However, there is no direct evidence for the protective effect of hydrogen in pulmonary oxygen toxicity. Therefore, the purpose of the present study was to investigate the effect of saturated hydrogen saline on pulmonary oxygen toxicity.

MATERIALS AND METHODS

Animals

Thirty adult male Sprague-Dawley rats weighing 270±10 g were used for this study. All experimental procedures were conducted in accordance with the Guiding Principle in the Care and Use of Animals approved by the Institutional Animal Care and Use Committee of Secondary Military Medical University, ROC.

Experimental protocol

For the saturated hydrogen saline preparation, purified H₂ was dissolved into normal saline for two hours under 0.6 MPa. The saturated hydrogen saline was stored under atmospheric pressure at 4°C in an aluminum bag with no dead volume. Hydrogen-rich saline was freshly prepared every week, which maintained a continuous concentration of 0.6 mmol/L. All agents were intraperitoneally injected (6 ml/kg) 30 minutes before exposure. Rats were exposed to >98% O₂ at 2.5 ATA for five hours in a plexiglass chamber, described previously (23). During that time, the chamber was ventilated with pure oxygen, without interruption, to minimize CO₂ accumulation. O₂ levels were monitored hourly with an oxygen analyzer (Hangtianpengcheng, Beijing, China) to maintain the level of 98%. Controls were kept in room-air temperatures. Animals were killed by cervical dislocation, and lungs were fixed by perfusion of 10% buffered formalin at 20 cm H₂O pressure and embedded in paraffin, as described previously (23).

Rats were randomly divided into three groups as follows:

- control group, n=10 – exposed to room air;
- saline group, n=10 – received saline and exposed to 2.5ATA oxygen;
- saturated hydrogen saline group, n=10 – received saturated hydrogen saline and exposed to 2.5ATA oxygen.

Quantification of lung wet/dry weight ratio

Immediately following exposure, lungs were excised en bloc and dissected away from the heart and thymus. The middle lobe of right lung was immediately weighed and then placed in a drying oven at 60 °C for 96 hours to stabilize dry weight. The ratio of wet/dry weight was used to quantify lung water content.

Evaluation of lung injury

Protein concentration and LDH activity and total cells in bronchoalveolar lavage fluid (BALF) were measured for lung injury. After exposure, the low lobe of the right lung was inflated with paraformaldehyde for histological studies. Bronchoalveolar lavage (BAL) was performed on the left lung with 4 ml of a phosphate-balanced saline solution in 2.5-ml aliquots after cannulation of the left trachea. The collected BALF was centrifuged at 1000 g for 10 minutes, the supernatant was collected and stored at -20°C and -80°C for later protein assays and LDH activity, and the total cell count was determined on a fresh fluid specimen using a hemocytometer.

Total protein in BALF

To assess the permeability of the bronchoalveolar–capillary barrier and cellular infiltration in the alveolar space, **total protein content in BALF was measured by** the BCA protein assay reagents using BSA as a standard (Pierce, Rockford, Ill., USA).

Lactate dehydrogenase activity in BALF

The activity of LDH, a cytosolic enzyme used as an indicator for cellular oxidative damage, was measured at 490 nm using an LDH determination kit according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). LDH activity was expressed as U/L, using an LDH standard.

Lung histopathology

Immediately following exposure, animals were exsanguinated by abdominal aorta. The right lungs were removed and then transferred to 4% formaldehyde for 48 hours. The lungs were paraffin-

embedded, and butterfly-shaped sections of 5-mm thickness were cut and placed on glass microscope slides stained with hematoxylin and eosin (H&E). The H&E sections were imaged at microscopic levels.

TUNEL staining

The In Situ Cell Death Detection Kit, AP (1168-4809910) (Roche) was employed to demonstrate cell death and DNA injury in lung tissue. The tissue sections were dewaxed and rehydrated according to standard protocols. Then, coronal sections were digested with proteinase K (20 mg/mL; Bio-Light) in 0.01 mol/L PBS at room temperature for 15 minutes. To interrupt digestion, the sections were dipped in 4% formaldehyde in 0.01 mol/L PBS for five minutes. After rinsing with PBS (five minutes, three times), the sections were immersed in TUNEL reaction mixture for one hour at 37 °C. In order to remove background staining caused by non-specific binding of antiluorescein-AP, the sections were treated with 1% BSA in PBS for 30 minutes at 37 °C. After rinsing with PBS (five minutes, three times), the sections were colorated in the dark with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Statistical analysis

All values are presented as mean ± SEM. Differences between groups were determined with one-way ANOVA followed by Student-Newman-Keuls test. A level of P<0.05 was considered statistically significant.

RESULTS

Morphology examination in lung

Lung slices from animals subjected to chronic oxygen toxicity with or without saturated hydrogen saline are shown in Figure 1 (*page 188*). The lung tissues in saline group were injured, with severe edema, alveolar wall thickening, alveolar hemorrhage, and infiltration of neutrophils into the lung interstitium and alveolar space. In the saturated hydrogen saline group, lung damage was alleviated, with results such as a reduction of infiltrated inflammatory cells and a marked improvement in lung architecture.

FIGURE 1

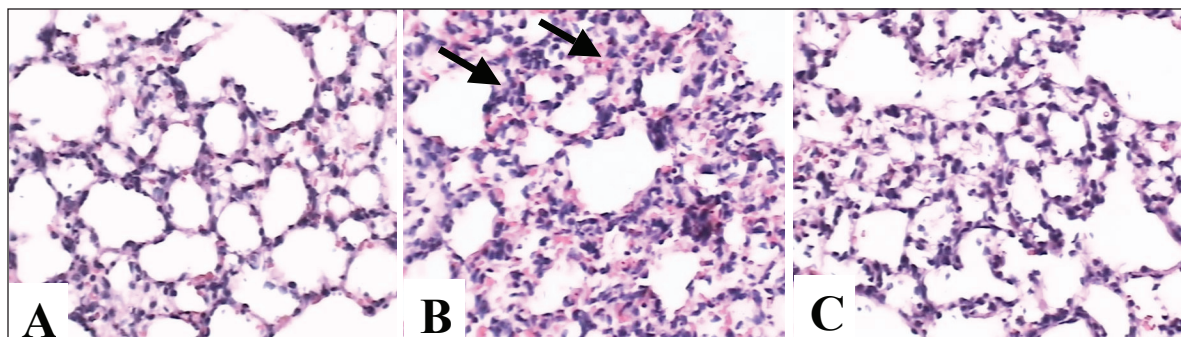


Figure 1 – Microphotographs of the lung tissue.

Lungs were obtained 24 hours after exposure; lung specimens were stained with hematoxylin and eosin. (A) control group; (B) saline group; (C) saturated hydrogen saline group. Original magnification: $\times 200$.

FIGURE 2

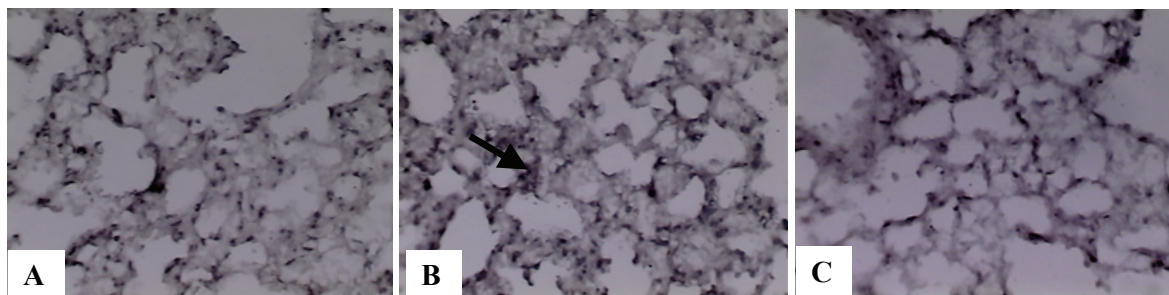
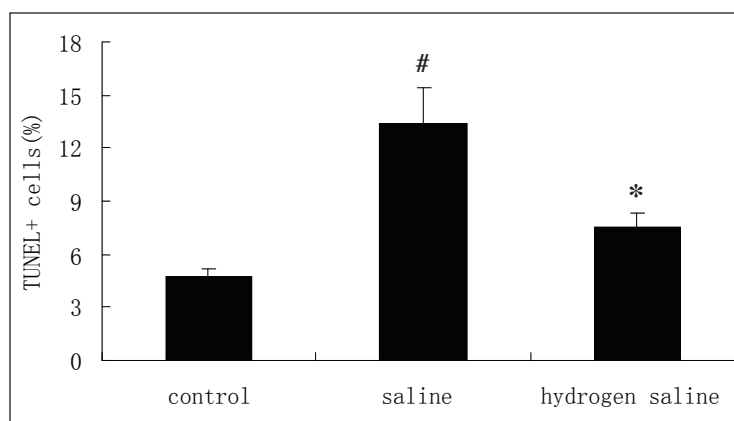


Figure 2 – TUNEL staining.

(A) control group; (B) saline group; (C) saturated hydrogen saline group. Original magnification: $\times 200$.



Effects of saturated hydrogen saline on cell apoptosis

The TUNEL demonstrated apoptotic cell death in saline treatment animals, and saturated hydrogen saline reduced the number of apoptotic cells (*Figure 2*, this page).

Effect of saturated hydrogen saline on lung edema

The wet/dry ratio of the saline group is significantly higher than that of the control group ($p < 0.05$). Compared to the saline group, saturated hydrogen saline significantly decreased the wet/dry ratios (*Figure 3*, facing page) ($p < 0.05$).

FIGURE 3

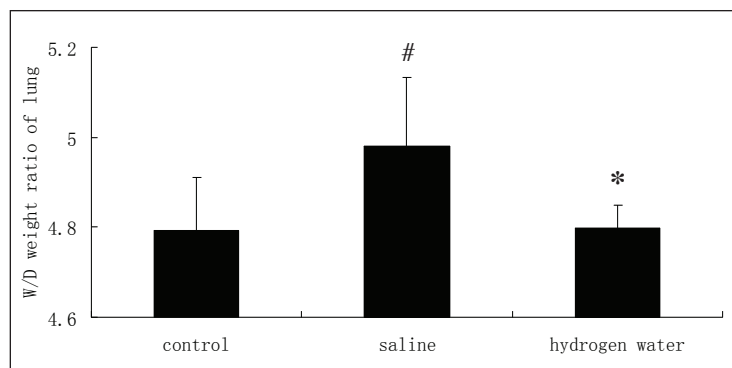
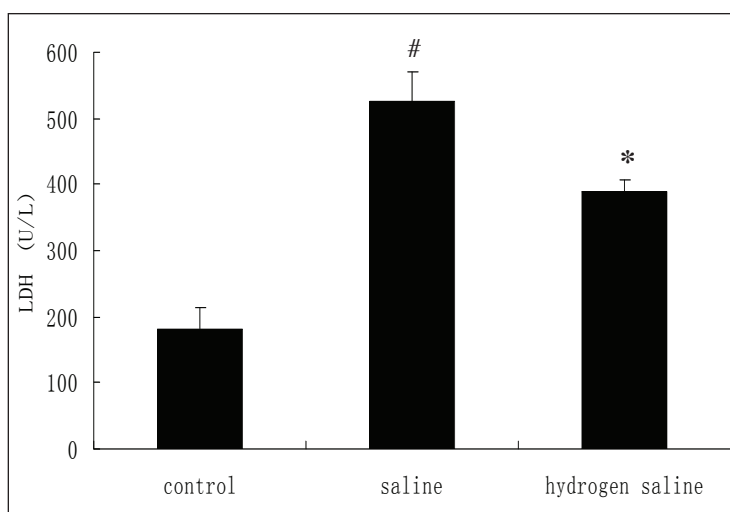


Figure 3 – The wet/dry ratio of lung weight (W/D) determined after exposure. Saturated hydrogen saline attenuated the increase in W/D ratio in saline group. Numbers in the bars indicate the group size.

* $p < 0.05$ compared with the saline group; # $p < 0.05$ compared with the control group.

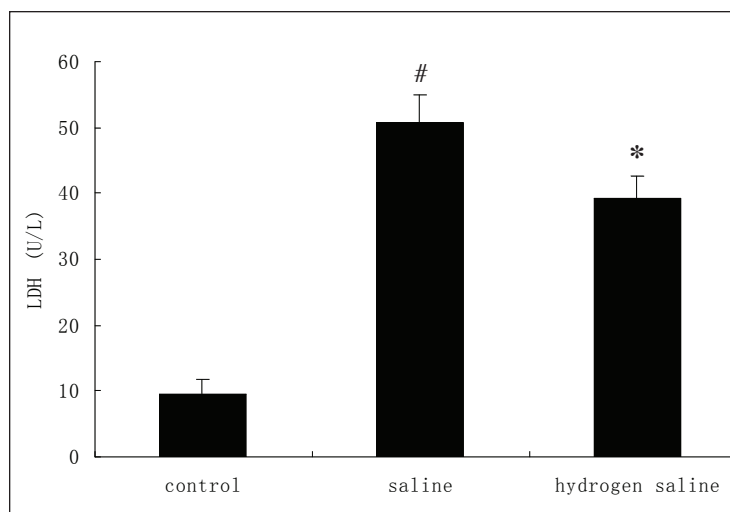
FIGURE 4a



Effects of saturated hydrogen saline on LDH in BALF and serum

The concentration of LDH in plasma was significantly higher in the saline group than that in the control group (*Figure 4a*, left). The saturated hydrogen saline group had significantly decreased LDH level in plasma compared to the saline group ($p < 0.05$). However, compared to the control group, the LDH level in the BAL fluid and plasma in the saturated hydrogen saline group were not significantly changed ($p > 0.05$). The LDH level in the BAL fluid was significantly higher in the saline group than that in the control group ($p < 0.05$). In contrast, a significant decrease of LDH in the BAL fluid was found in the saturated hydrogen saline group (*Figure 4b*, left) compared to that in the saline group.

FIGURE 4b



Effects of saturated hydrogen saline on total cells and protein concentration in BALF

Total cells and protein concentration in the BAL fluid were determined as an indicator of lung hyperpermeability induced by hyperoxic exposure. Results demonstrate that total cells and protein in the BAL fluid in the saline group were significantly increased, compared to the control group ($p < 0.05$) (*Figure 5* and *Figure 6*, page 190). Conversely, in the saturated hydrogen saline group, total cells and protein were much

Figure 4 – Lactate dehydrogenase activity in serum (a) and BALF (b) from each group of rats after five hours' exposure to $>98\%$ O₂ at 2.5 ATA. Data are mean \pm SEM. # $p < 0.05$ compared with the control group; * $p < 0.05$ compared with the saline group.

FIGURE 5

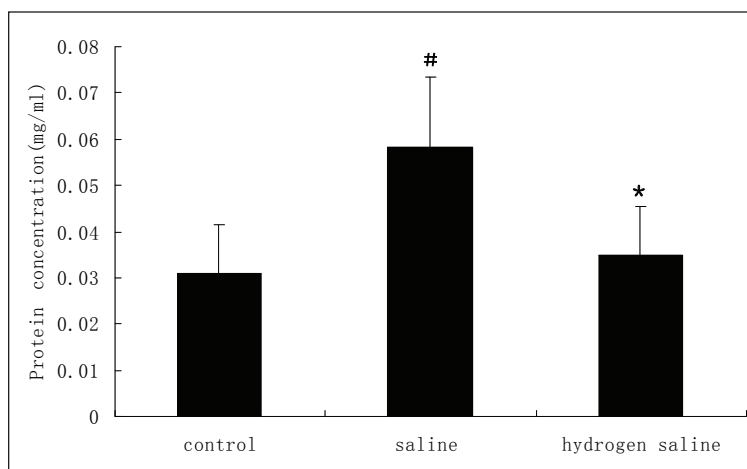


Figure 5 – Protein concentration in BAL from each group. Data are mean \pm SEM.

[#] $p < 0.05$ compared with the control group;
^{*} $p < 0.05$ compared with the saturated hydrogen saline group.

FIGURE 6

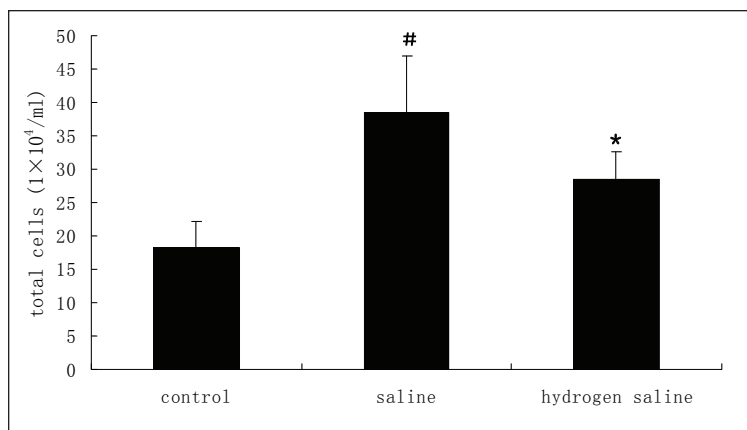


Figure 6 – Total cell counts in BAL from each group. Data are mean \pm SEM.

[#] $p < 0.05$ compared with the control group;
^{*} $p < 0.05$ compared with the saturated hydrogen saline group.

lower than those of the saline group ($p < 0.05$). In addition, there was not a significant difference between the control group and the saturated hydrogen saline group ($p > 0.05$).

DISCUSSION

In the present study, we have demonstrated for the first time that saturated hydrogen saline alleviated the lung injury induced by hyperbaric oxygen. Treatment with saturated hydrogen saline, a potent free-radical scavenger, decreased the wet/dry ratio of lung weight and protein concentration and LDH activity in the BAL fluid, as well as LDH in serum. In addition, saturated hydrogen saline decreased total cells in the BAL fluid and inhibited apoptosis.

The currently accepted primary mechanism for oxygen-induced cellular injury is enhanced oxygen-derived free radical generation, with subsequent oxidative attack upon basic cell constituents. Free radicals, such as superoxide and hydroxyl radicals, are important mediators of lung injury, whether they are produced inside lung parenchyma cells or by immigrant neutrophils (14). Ikuroh Ohsawa *et al.* have found that molecular hydrogen selectively reduces hydroxyl radicals (17). Hydrogen has already been used in diving for humans to help prevent decompression sickness in divers at the level of 2 MPa partial pressure of hydrogen, suggesting that 16 mM hydrogen in blood could be safe (25).

Hydrogen diffuses very rapidly into cells and tissues, and high efficacy is expected (26, 27). To date, hydrogen or hydrogen water has manifested substantial protection against ischemic hypoxia diseases including hepatic injury (24), nerve injury (28, 29), and intestinal graft injury (27). Hydrogen or hydrogen water is becoming more popular and important in many fields.

Consistent with the above-mentioned studies of hydrogen in ischemic and reperfusion injuries, this study demonstrated hydrogen saline reduced lung injury resulting from oxygen toxicity. Our results have showed that total BALF cell counts and proteins were significantly elevated in the saline group compared to the control group. This increase in the number of total cells and protein in BAL fluid may partially reflect the loss of integrity of the endothelial barrier. In addition, this oxidative damage is indicated by a significant elevation of LDH activity in the BALF and W/D ratio after exposure to oxygen. Saturated hydrogen saline markedly reduced total BALF cell counts and LDH activity in BALF and lung edema.

Under physiologic conditions, tissue homeostasis is controlled by the tight regulation of apoptosis and necrosis. Hyperoxia is lethal to cells and has been shown to cause the accumulation of apoptosis-inducing reactive oxygen intermediates, such as O⁻² and H₂O₂. In our study, to determine whether inhibition of apoptosis was one of the mechanisms of saturated hydrogen saline's protective effect, we examined lung cells apoptosis by TUNEL staining. Results showed that saturated hydrogen saline inhibited cell apoptosis, but the accurate mechanism of this protective effect needs further investigation.

In summary, we first determined that saturated hydrogen saline protects pulmonary against oxygen-induced injury. This shows the need for further studies in prevention and therapy for oxygen-induced injury. ■

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