Hydrogen-Rich Saline Provides Protection Against Hyperoxic Lung Injury

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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)-α 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.

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-1β and TNF-α 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.

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

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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 gas-tight 7-L Plexiglas chambers exposed continuously to hyperoxia (>98% O2) 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 P(CO2) 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. O2 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) H2 group: hyperoxia (>98% O2) 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% O2) for 60 h plus four i.p. injection of physiologic saline (n = 12).
(5) N2 group: hyperoxia (>98% O2) 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 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 Ohswa 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 and the volume of pleural fluid was measured. The great vessels were dissected from the hilum 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 superoxide dismutase (SOD) concentration analysis, myeloperoxidase (MPO), TNF-α and IL-1β 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-α, IL-1β, 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-α, IL-1β, 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 mL were stained with hematoxylin and eosin Q4 (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-μm-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). According to standard protocols, the sections were dehydrated and rehydrated by heating the slides at 60°C. Then these sections were incubated in a 20 μg/mL protease 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-indolyl phosphate (BCIP). Four slide fields were randomly examined using a defined rectangular field area with magnification (×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 ± 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 O2-induced lung damage, pleural fluid volume, and the wet-to-dry weight ratio were measured in SHAM, NORM, CON, H2, and N2 groups right after 60 h of O2 exposure. Pleural fluid volume and the wet-to-dry ratio increased significantly in rats exposed to O2. 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 O2 exposure, the lung tissues of rats were severely damaged in the CON group and N2 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 O2 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 O2 exposure, thus, oxidative stress (*P < 0.05 versus CON group). Nitrogen-rich saline showed no significant

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**FIG. 1.** Pleural fluid volume, wet-to-dry weight ratio, and histopathology analysis in SHAM, NORM, CON, H2, and N2 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 N2 (E): the lung tissues revealed severe edema, alveolar hemorrhage, and inflammatory cell infiltration; H2 (D): moderate edema, hemorrhage and inflammatory cell infiltration. Routine hematoxylin and eosin stained (×200).
reduction of SOD, MDA, and 8OHdG levels ($P = \text{NS}$ versus CON group). There was no significant difference of SOD, MDA, and 8OHdG levels between SHAM group and NORM group ($P = \text{NS}$).

**Effect of Hydrogen-Rich Saline on TNF-α, IL-1β, and MPO Levels**

ELISA detection showed that the levels of TNF-α, IL-1β, 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-α, IL-1β, and MPO in the lung tissues (Fig. 3). Nitrogen-rich saline showed no significant reduction of TNF-α, IL-1β, and MPO levels ($P = \text{NS}$ versus CON). Intraperitoneal injection of hydrogen-rich saline alone in NORM group showed no significant changing of TNF-α, IL-1β, and MPO levels compared with SHAM group ($P = \text{NS}$).

**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 O₂ 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 ($P = \text{NS}$ versus CON group). NORM group showed no significant increasing number of TUNEL-positive cells compared with SHAM group ($P = \text{NS}$).

**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-1β and TNF-α.

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. 4. Detection of apoptotic cell death by TUNEL staining in the SHAM, NORM, CON, H2, and N2 groups 60 h after O2 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 H2 group. (E) TUNEL staining of lung sections in N2 group. Relative to the CON, hydrogen-rich saline significantly reduced the number of TUNEL-positive cells (brown staining). Values are mean ± 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 \( \text{H}_2\text{O}_2 \), 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 chemot- tractants 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-1\( \beta \) 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.

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REFERENCES


