Hydrogen-Rich Saline Protects Against Acute Lung Injury Induced by Extensive Burn in Rat Model

Yong Fang, MD, PhD,*† Xiu-Jun Fu, MD,*† Chuan Gu, MD,*† Peng Xu, MD,*† Ying Wang, MD,*† Wei-Rong Yu, MD, PhD,*† Qiang Sun, MD,‡ Xue-Jun Sun, MD, PhD,*†‡ Min Yao, MD, PhD*†§

Hydrogen has been reported to selectively quench detrimental reactive oxygen species, particularly hydroxyl radical, and to prevent myocardial or hepatic ischemia/reperfusion injury in multiple models. The aim of this study is to investigate whether hydrogen protects against severe burn-induced acute lung injury in rats. Rats were divided into four groups: sham plus normal saline, burn injury plus normal saline, burn injury plus hydrogen-rich saline, and burn injury plus edaravone. Animals were given full-thickness burn wounds (30% TBSA) using boiling water, except the sham group that was treated with room temperature water. The rats in hydrogen group received 5 ml/kg of hydrogen-rich saline, sham and burn controls obtained the same amount of saline, and the edaravone group was treated with 9 mg/kg of edaravone in saline. Lactated Ringer's solution was given at 6 hours postburn. The lungs were harvested 12 hours postburn for laboratory investigations. Severe burns with delayed resuscitation rapidly caused lung edema and impaired oxygenation in rats. These dysfunctions were ameliorated by administration of hydrogen-rich saline or edaravone. When compared with the burn injury plus normal saline group, hydrogen-rich saline or edaravone group significantly attenuated the pulmonary oxidative products, such as malondialdehyde, carbonyl, and 8-hydroxy-2′-deoxyguanosine. Furthermore, administration of hydrogen-rich saline or edaravone dramatically reduced the pulmonary levels of pulmonary inflammation mediators and myeloperoxidase. Intraperitoneal administration of hydrogen-rich saline improves pulmonary function by reducing oxidative stress and inflammatory response in severe burn-induced acute lung injury. (J Burn Care Res 2011;32:e82–e91)

Extensive burn-induced acute lung injury (ALI) and its extreme manifestation, acute respiratory distress syndrome, are associated with high morbidity and mortality.[1–4] Because the underlying pathophysiological processes remain elusive, there are no effective therapies at the moment. In recent years, numerous studies have shown that oxidative stress and systemic inflammatory response play critical roles in the pathogenesis of ALI, as burn-induced ALI is mainly considered to be a secondary attack to the lung (indirect injury).[1,2,5–7]

The excessive generation of reactive oxygen species (ROS) by severe burn injury is a potential mediator in ALI. It has been shown that many of the detrimental effects associated with ALI are related to the presence of superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), or peroxynitrate ($ONOO^-$) after burn damage. Hydroxyl radical and peroxynitrate are especially problematic because they

*From the *Department of Burns and Plastic Surgery, Number 3 People's Hospital, Institute of Traumatic Medicine; †School of Medicine, Shanghai Jiao Tong University; and ‡Department of Diving Medicine, Second Military Medical University, Shanghai, People's Republic of China; and §Department of Dermatology, Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston*

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The first two authors contributed equally to this article.

Address correspondence to Min Yao, MD, PhD and Xue-Jun Sun, MD, PhD, Department of Burns and Plastic Surgery, No.3 People's Hospital, School of Medicine, Shanghai Jiao Tong University, 280 Mohe Road, Shanghai 201900, People's Republic of China.

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have much higher oxidative energy levels than superoxide anion and hydrogen peroxide.[8,9] ROS attack lipid, protein, and DNA, causing irreversible oxidative stress in cells, which leads to lung damage.[2] Furthermore, ROS can cause additional acute injury to the lungs by inducing the production of inflammatory mediators through activation of the nuclear factor-κB signaling pathway.[10,11] Therefore, therapies that inhibit or reduce oxidative stress may favorably impact the treatment of severe burn-induced ALI.[12–16]

Hydrogen has been documented as a novel antioxidative stress substance capable of selectively reducing ROS. Molecular hydrogen is originally used to prevent decompression sickness during deep diving.[17] Recently, Ohsawa et al[18] demonstrated that H_2 could serve as a potent free radical scavenger by exclusively quenching ROS, particularly hydroxyl radical and peroxynitrate, in cultured cells and in brain tissue. Although the underlying mechanism remains unclear, similar results were obtained by other researches using models of hepatic ischemia/reperfusion (I/R) injury,[19] myocardial I/R injury,[20] neonatal hypoxia–ischemia,[21] and intestinal graft with oxidative injury.[22]

Given the potential antioxidative stress effect of hydrogen and the key roles of oxidative stress and inflammatory response in the pathogenesis of burn-induced ALI, we hypothesized that hydrogen may be used to protect against extensive burn-induced ALI. In this study, we showed that severe burns with delayed resuscitation rapidly caused lung edema and impaired oxygenation in rats. In addition, the injury increased the oxidative damage products of lipid, protein, and DNA; released inflammatory mediators; and activated inflammatory cells. Intraperitoneal (IP) administration of hydrogen-rich saline ameliorated the lung edema and improved pulmonary function in severely burn-injured rats, by significantly reducing oxidative stress and attenuating inflammatory response.

METHODS

Preparation of Hydrogen-Rich Saline

The hydrogen-rich saline was prepared as described previously.[23] Briefly, hydrogen gas was dissolved in normal saline for 6 hours under high pressure (0.4 MPa) by using a custom-designed, hydrogen-rich, water-producing apparatus. An aluminum bag was used to store the saturated hydrogen saline under atmospheric pressure at 4°C, and this saturated hydrogen saline was sterilized using gamma-ray radiation. To maintain the concentration of hydrogen above 0.6 mmol/L, hydrogen-rich saline was freshly prepared every week. Gas chromatography was used to verify the content of hydrogen in hydrogen-rich saline as described by Ohsawa et al.[18]

Study Animals

The procedures used in this study and the handling of study animals were performed in adherence to the National Institutes of Health guidelines on the use of experimental animals. The experimental protocol was approved by the Committee on Research Animal Use of Shanghai Jiao Tong University. Male Sprague–Dawley rats weighing 200 to 220 g were purchased from the Experimental Animal Center at Shanghai Jiao Tong University and housed at constant temperature with 12-hour periods of light–dark exposure, respectively. Animals were allowed to access standard rat chow and water ad libitum and to acclimatize for at least 1 week before the experiment.

Burn Injury and Delayed Resuscitation

Rats were fasted overnight and had free access to water for 16 to 18 hours before injury. All animals spontaneously breathed room air throughout the experiment. The rats were anesthetized with 3% pentobarbital sodium (30 mg/kg; Sinopharm Chemical Reagent Co. Ltd., China) by IP injection. The dorsal hair of the animals was shaved to allow direct skin contact to hot water. We developed a device for creating a burn injury on the back of rats. It consists of a wooden plate with a circular opening that exposes 30% TBSA. After anesthesia, the animals were placed on the device in the supine position. Each rat, along with the device, was immersed into hot water (98°C) for 12 seconds, creating a full-thickness skin burn injury. Sham animals were subjected to identical procedure and resuscitation but were immersed into water at room temperature. The depth of the burn wound was confirmed histologically. All rats were then resuscitated with an IP injection of 4 ml/kg/TBSA of Lactated Ringer's solution (Baxter Healthcare Co. Ltd., Shanghai, China) at 6 hours postburn. Rats were given 0.25 mg/kg of buprenorphine subcutaneously and housed in individual cages.

Experimental Design

The rats were randomly divided into four groups: 1) sham plus normal saline-treated group (S + N, n = 8); 2) burn plus normal saline-treated group (B + N, n = 8); 3) burn plus hydrogen-rich saline-treated group (B + H, n = 8); and 4) burn plus edaravone saline-treated group (B + E, n = 8). For B + N group, the rats were given normal saline by IP (5 ml/kg; Baxter Healthcare Co. Ltd). Animals in B +

H group received hydrogen-rich saline (5 ml/kg, IP, this dosage was shown to be effective in a previous study[24]). B + E–treated animals were given edaravone saline by IP (5 ml/kg) with edaravone at a dose of 9 mg/kg (Simcere Pharmaceutical Co. Ltd., Nanjing, China). The treatments were performed 10 minutes before liquid resuscitation. All animals were killed at 12 hours postburn to harvest tissue samples.

Measurement of Hydrogen Concentration in Blood

Five milliliters of arterial blood was collected from the abdominal aorta at two time points (5 and 30 minutes) after injection (groups of 12 rats received each of the 4 treatments; 6 rats were used for blood collection after 5 minutes and the remaining 6 rats used after 30 minutes). Heparin was used to avoid clotting during the blood collection process. The blood samples were injected immediately into sealed aluminum bags that contained 20 ml of air. The aluminum bags were oscillated to release the hydrogen gas from blood into air at room temperature. One hundred microliters of the rebalanced gas per bag was loaded into a gas chromatograph (Shimaduz GC-14BPF, Shimaduz, Japan) for measuring the hydrogen content. The conditions for measuring hydrogen were 120°C measuring temperature, 65 mA electric current, using a thermal conductivity detector with a 1.5-m long TDX-02 column. Standard hydrogen gas purchased from KeRui Technology Co. (Dalian, China) was used to construct a standard curve. The hydrogen concentration in each blood sample was calculated using this hydrogen gas standard curve.

Measurement of Lung Tissue Dry:Wet Weight Ratio

Lung tissue from each group was cleansed of blood with absorbent paper and weighed. One hundred milligrams of tissue per sample was placed in a 75°C thermostatic baking oven for 72 hours, and the dry lung weight of each sample was measured. The dry: wet lung weight ratio was calculated for every sample.

Histological Analysis

The lung specimens were fixed in 10% formaldehyde for 24 hours, embedded in paraffin wax, and cut into 5-μm sections. The sections were stained with hematoxylin and eosin and viewed under light microscope (Olympus IX51, Olympus, Tokyo, Japan). Images were randomly chosen and captured by a digital camera (Olympus DP 70).

Analysis of Blood-Gas

Blood samples from the abdominal aorta were collected for gas analysis using a clinical blood-gas analysis device (GEM Premier3000; Instrumentation Laboratory, The Netherlands). Partial pressure of oxygen in artery (PaO_2), partial pressure of carbon dioxide in artery ($PaCO_2$), and arterial oxygen saturation (SaO2) were measured to evaluate pulmonary oxygenation function.

Detections of MDA, Carbonyl, and 8-OH-dG Levels

Malondialdehyde (MDA) and carbonyl were used as indicators of lipid and protein oxidation, respectively. Pulmonary MDA and carbonyl concentrations were determined using the MDA and carbonyl chemical method kits (Nanjing Jiancheng Biochemistry Co., Nanjing, China), according to the manufacturer's instructions. Briefly, fresh lung tissue (100 mg) was homogenized in 900 μl of cold normal saline. After centrifugation at 3000g for 10 min at 4°C, the supernatants were used for determining MDA and carbonyl levels by the corresponding kits. Absorbance at 586 nm was measured using a spectrophotometer (SmartSpec Plus, BIO-RAD, Hercules, CA). The average and standard deviation were calculated from eight animal samples per group. The levels of lipid or protein peroxides were expressed as nanomoles of MDA or carbonyl per milligram protein, respectively.

The level of 8-hydroxy-2′-deoxyguanosine (8-OH-dG) was used for quantitative detection of the oxidative DNA. Fresh lung tissue was harvested and assayed using an ELISA kit (Rapidbio, CA). According to the manufacturer's instructions, the level of 8-OH-dG was detected with monoclonal antibody specific for 8-OH-dG, and the absorbance was measured using Model 680 Microplate Reader (BIO-RAD). The results were calculated from eight animals per group and expressed as nanograms per gram protein.

Measurements of IL-1β, IL-6, and TNF-α Protein Levels in the Lung

Lungs were collected and washed in normal saline and then homogenized immediately in 1 ml normal saline on ice (4°C). The homogenates were centrifuged at 3000g at 4°C for 15 min. The levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α were measured with corresponding ELISA kits following the instructions of the manufacturer (R&D Systems, MN). The absorbance was measured using a microplate reader (Model 680 Microplate Reader; BIO-RAD), and the concentrations were cal-

culated according to the standard curve. The results were calculated from eight animals per group and expressed as micrograms per milligram protein.

Measurement of Myeloperoxidase

Myeloperoxidase (MPO) expression and protein level in lung tissue were measured by immunohistochemistry and ELISA, respectively. Half of the lung specimens were fixed in 10% formaldehyde and embedded in paraffin for sectioning. The sections were treated with 1% H_2O_2 and preincubated with bovine serum to block nonspecific binding and then incubated with anti-MPO primary antibody (Beijing Boisynthesis Biotechnology Co., Beijing, China) overnight at 4°C. Biotinylated secondary antibody (ZSGB-Bio, Beijing, China) was applied to the specimens for 1 hour. ABC kit (Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine (DAB) were used for color development. Images were captured using an Olympus IX51 microscope (Olympus). MPO levels in protein extracted from the remaining lung specimens were measured by ELISA (R&D Systems). The results were expressed as micrograms per milligram protein.

Statistical Analysis

Results are expressed as the mean ± SD. We used KaleidaGraph software (Synergy Software, PA) for statistical analysis. For single comparisons, we used an unpaired two-tailed Student's *t*-test; for multiple comparisons, we carried out an analysis of variance followed by a post hoc Tukey test. For ELISA, we performed the experiments in triplicate for each sample, and the data in each group were taken from eight samples. A P value less than .05 was considered statistically significant.

RESULTS

Systemic Hydrogen Level After Hydrogen-Rich Saline Injection

Systemic hydrogen levels were undetectable in both sham plus normal saline-treated and burn plus normal saline-treated groups. The hydrogen level in blood from burn plus hydrogen-rich saline-treated animals was 0.03 ± 0.002 mmol/L at 5 minutes after IP injection, but 30 minutes after injection of hydrogen-rich saline, hydrogen levels became undetectable in arterial blood.

Hydrogen-Rich Saline Reduces Pulmonary Edema in Severely Burned Rats

To determine whether hydrogen-rich saline alleviates ALI induced by severe burn, we evaluated the effect of hydrogen on pulmonary edema in the different treatment groups. The lung dry:wet ratio was used for assessing lung edema. At 12 hours postburn, the lung dry:wet ratios in S + N, B + N, B + H, and B +E groups were 0.252 (±0.011), 0.228 (±0.013), 0.245 (±0.013), and 0.238 (±0.014), respectively (Figure 1A). The lung dry:wet ratio in burn plus normal saline group was significantly lower than that in the sham controls ($P < .01$). Compared with the burn plus normal saline treatment rats, the lung dry:wet ratios in the hydrogen-rich saline- and edaravone-treated rats were markedly increased ($P < .05$). There was no significant difference in the lung dry:wet ratio between the B + H and B + E groups ($P = .31$).

To verify this difference in lung edema, pulmonary histological evaluation was carried out using hematoxylin and eosin staining. At 12 hours postburn, pulmonary histology in burn plus normal saline animals presented an excessive interstitial edema, thickened alveolar walls, and numerous infiltrations of inflammatory cells (Figure 1C). The histological image in burn plus hydrogen-rich saline and plus edaravone-treated rats (Figure 1D, E) showed moderate interstitial edema in the alveolar walls and moderate infiltrations of inflammatory cells into the alveoli when compared with that in sham controls (Figure 1B). These results indicate that hydrogen-rich saline reduced the pulmonary edema in severe burn-induced ALI.

Hydrogen-Rich Saline Improves Pulmonary Oxygenation Function in Severe Burn-Injured Rats

To ascertain whether hydrogen-rich saline has an effect on pulmonary oxygenation function, arterial blood-gas analysis was conducted using a clinical device. As shown in Table 1, the PaO_2 level in burn plus normal saline-treated animals was substantially lower compared with the sham controls (B + N vs S + N, $P < .05$) at 12 hours postburn. PaO_2 levels in the B + H and B + E groups were significantly higher than that in the B + N group ($P < .05$), whereas there was no significant difference between the B + H and B + E groups. $PaCO_2$ and SaO_2 were also measured in the four treatment groups, but there were no significant differences in $PaCO_2$ or SaO_2 among the four groups ($P > .05$).

Hydrogen-Rich Saline Attenuates Pulmonary Oxidative Stress in Severe Burn-Injured Rats

To determine the effects of hydrogen-rich saline on pulmonary oxidative stress in ALI-induced by burn injury, the levels of MDA and carbonyl in the lungs

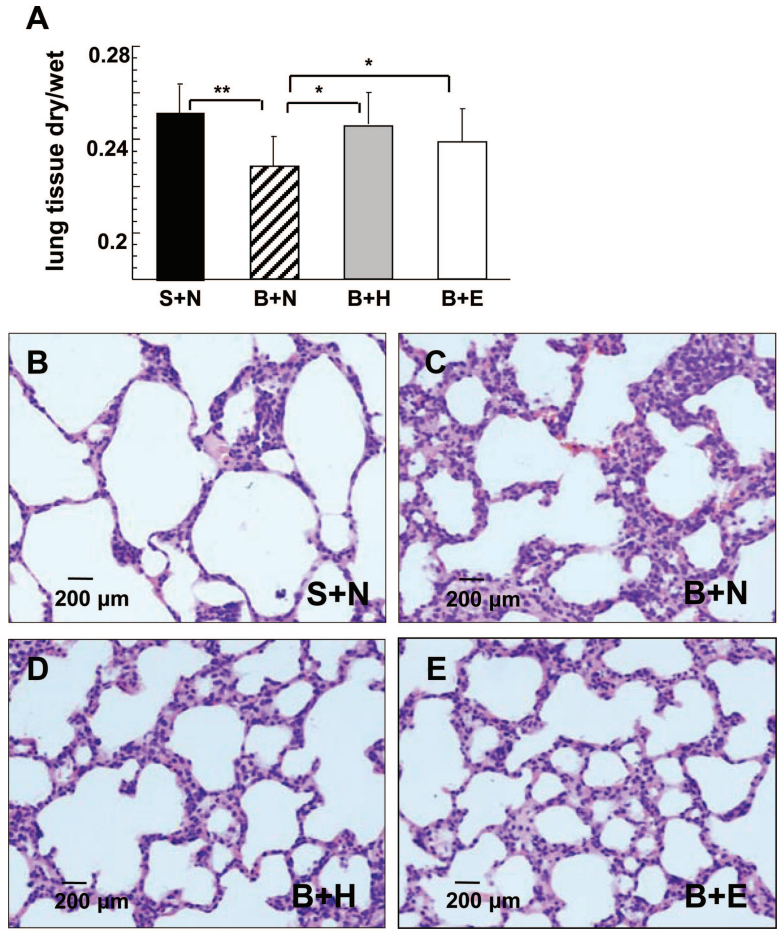

Figure 1. Effects of hydrogen-rich saline on lung edema in extensive burn-injured rats. A, Lungs were harvested from sham controls, burn plus normal saline, burn plus hydrogen-rich saline, and burn plus edaravone rats (n = 8 per group) at 12 hours after injury. The ratio of dry:wet lung weight was determined (mean ± SD, *$P < .05$, **$P < .01$). Pulmonary histological images in the sham (B), burn plus normal saline (C), burn plus hydrogen-rich saline (D), and burn plus edaravone (E) groups were evaluated (hematoxylin and eosin).

were used to assess oxidative damage to lipid and protein, respectively. As shown in Figure 2A, the pulmonary content of MDA in the burn plus normal saline group was significantly higher than that in the sham controls ($P < .01$) at 12 hours postburn. When compared with the burn plus normal saline treatment, the pulmonary MDA levels in the burn plus hydrogen-rich saline or plus edaravone groups were dramatically lower ($P < .01$). Likewise, pulmonary level of carbonyl (Figure 2B) in burn plus normal saline-treated rats was significantly higher than that in sham rats (B + H vs S + N, $P < .01$), and the carbonyl levels in the burn plus hydrogen-rich saline or plus edaravone groups were remarkably lower than that in the burn plus normal saline group ($P < .01$). There were no significant differences in the levels of either MDA or carbonyl between the B + H and B + E groups ($P > .05$).

8-OH-dG content, as a marker for DNA damage caused by oxidative stress, was measured in the lungs of rats from the four different treatment groups at 12 hours postburn (Figure 2C). Pulmonary level of 8-OH-dG in the sham group was dramatically lower than that in the B + N group (S + N vs B + N, $P <$

Table 1. Results of arterial blood gas analysis

Treatment	S + N	B + N	B + H	B + E
PaO_2 (mm Hg)	98.3 ± 3.7	73.5 ± 11.0*	86.0 ± 6.1†	87.0 ± 6.9†
$PaCO_2$ (mm Hg)	45.8 ± 5.0	38.2 ± 6.2	42.5 ± 5.5	41.7 ± 7.5
SaO_2 (%)	96.3 ± 2.7	91.7 ± 4.3	94.2 ± 3.3	94.7 ± 2.3

Data are expressed as mean ± SD (n = 8 per group).
* $P < .05$ vs sham plus normal saline-treated group.
† $P < .05$ vs burn plus normal saline-treated group.
S + N, sham plus normal saline-treated group; *B + N*, burn plus normal saline-treated group; *B + H*, burn plus hydrogen-rich saline-treated group; *B + E*, burn plus edaravone saline-treated group.

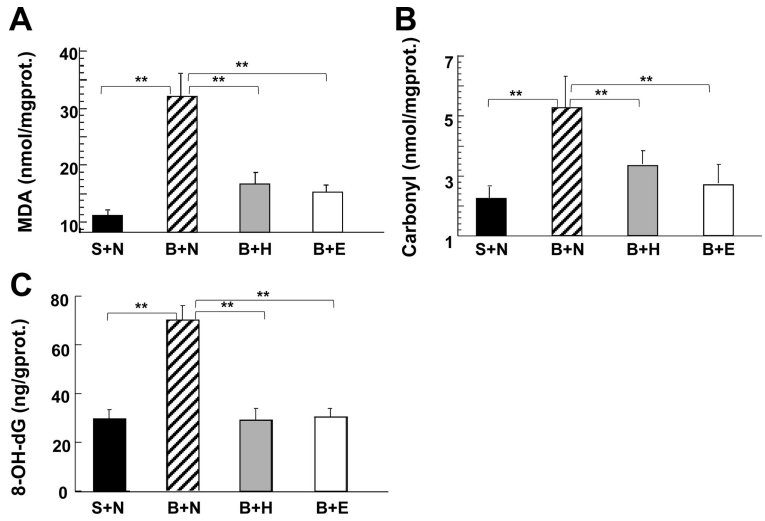

Figure 2. Hydrogen-rich saline attenuates oxidative stress induced by severe burn. Lungs were harvested from sham controls, burn plus normal saline, burn plus hydrogen-rich saline, and burn plus edaravone rats (n = 8 per group) at 12 hours after injury. A, The products of oxidized lipid (malondialdehyde), (B) oxidized protein (carbonyl), and (C) oxidized DNA (8-OH-dG) in the lung were determined using chemical reaction kits or ELISA kit (mean ± SD, $^{**}P < .01$).

.05). The levels of 8-OH-dG in the B + H and B + E groups were also significantly lower, compared with that in the B + N group ($P < .05$).

Hydrogen-Rich Saline Decreases the Protein Levels of Inflammatory Mediators in the Lungs of Severely Burned Rats

Inflammatory mediators play a key role in the pathogenesis of burn-induced ALI. Protein concentrations of IL-1β, IL-6, and TNF-α in lung tissues were determined using ELISA. Compared with those in the sham-burned controls, the levels of IL-1β, IL-6, and TNF-α in lung tissues of the burn plus normal saline-treated rats were markedly increased by 3.13-, 4.94-, and 4.10-folds, respectively (Figure 3A–C) at 12 hours after injury. Administrations of hydrogen-rich saline and edaravone significantly suppressed the burn-induced

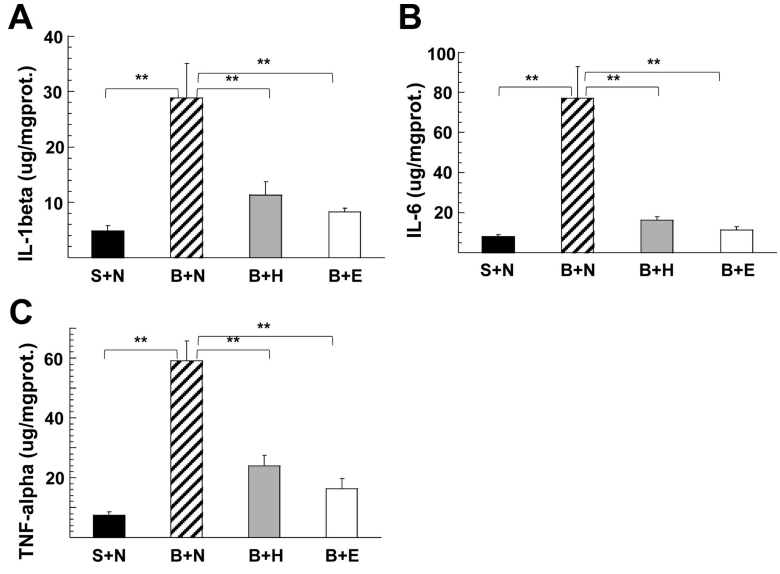

Figure 3. Hydrogen-rich saline reduces the protein levels of inflammatory mediators in burn-injured rats. Lungs were harvested from sham controls, burn plus normal saline, burn plus hydrogen-rich saline, and burn plus edaravone rats (n = 8 per group) at 12 hours postburn. Pulmonary proteins were subjected to ELISA analysis with anti-interleukin (IL)-1β (A), anti-IL-6 (B), anti-tumor necrosis factor (TNF)-α (C) antibodies (mean ± SD, $^{**}P < .01$).

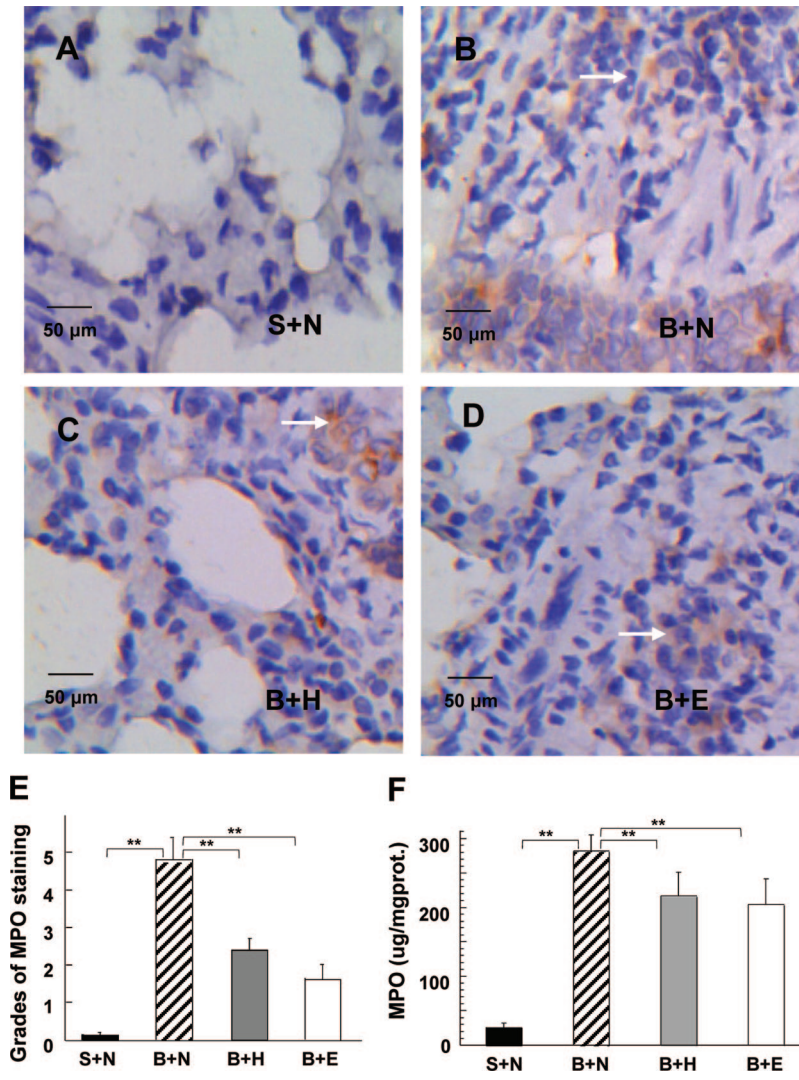

Figure 4. The activation of myeloperoxidase (MPO) in the lung tissue of burn-injured rats was inhibited by hydrogen-rich saline. Lungs were harvested from sham controls (A), burn plus normal saline (B), burn plus hydrogen-rich saline (C), and burn plus edaravone (D) rats (n = 8 per group) at 12 hours after injury. The tissue sections were analyzed by immunohistochemistry with anti-MPO. Brown color indicates positive staining of MPO (arrows indicate positive staining in alveolus). (E) A semi-quantitative analysis of MPO staining intensity. (F) Pulmonary protein was subjected to ELISA for quantitative analysis of MPO (mean ± SD, $^{**}P < .01$).

increase of IL-1β, IL-6, and TNF-α levels in lung tissue (B + H vs B + N, $P < .01$). Treatment with hydrogen-rich saline led to a similar suppression of these three inflammatory mediators compared with edaravone.

Hydrogen-Rich Saline Suppresses the Level of MPO in the Lungs of Severe Burn-Injured Rats

MPO is an enzyme secreted predominantly by neutrophils. Thus, pulmonary MPO levels were analyzed using immunohistochemistry to evaluate the infiltration of neutrophils into the lungs. At 12 hours postburn, there was almost no MPO staining in the lungs harvested from the sham controls (Figure 4A). An increased staining for MPO was found in the alveoli obtained from burn plus normal saline-treated rats (Figure 4B). Administration of hydrogen-rich saline or edaravone markedly reduced MPO in the lung, compared with that in burn plus normal saline-treated animals (Figure 4C, D). The degrees of staining intensity were scored and quantified as Figure 4E. To confirm the immunohistochemistry data, we used ELISA to measure pulmonary MPO protein levels. The changes in pulmonary MPO protein levels were

consistent with the immunohistochemistry results (Figure 4F).

DISCUSSION

This is the first study to demonstrate that IP administration of hydrogen-rich saline ameliorated lung edema and pulmonary oxygenation function in a rat model with extensive burn injury. The pulmonary protective effect of hydrogen-rich saline is achieved by reducing oxidative stress (damages) and suppressing the inflammatory response. The antioxidant and antiinflammation properties of hydrogen in burn-injured rats are supported by the following: 1) treatment with hydrogen-rich saline decreased the levels of MDA, carbonyl, and 8-OH-dG in the lung after severe burn injury, indicating that hydrogen can reduce the oxidative damages in membrane lipid, protein, and DNA by scavenging ROS; 2) hydrogen-rich saline reduced the pulmonary levels of IL-1β, IL-6, and TNF-α; 3) hydrogen-rich saline suppressed the level of MPO in the lung, indicating that hydrogen can inhibit the infiltration of neutrophils; and 4) the pulmonary protective effect by hydrogen-rich saline was similar to that by edaravone, a topical and clinical drug used as a ROS scavenger.

There are many medicinal chemical agents (N-acetylcysteine [NAC], vitamin C and E, leupeptin, several superoxide dismutase mimics, and verapamil) targeting oxidant pathology that have been studied in humans or animals with various tissue injury models including ALI. In general, the effectiveness of antioxidant treatments in tissue injury models has been found to be moderate to low. The low effectiveness of these antioxidants may be due to several reasons. Most of these antioxidant agents are nonspecific scavengers for ROS because the lifetime of activated ROS is too short to be targeted, and inactivated ROS (which have a longer lifetime) are much less toxic to tissue cells. In addition, some of the chemical compounds are too large to get into intracellular space, which affects the efficacy of treatment. Some of the antioxidant agents are further limited by causing undesirable side effects. Many publications have reported that the use of molecular hydrogen in I/R injury ameliorates myocardial, intestinal barrier, and neuron pathologies.[20–22] Ohsawa et al[18] have shown that hydrogen treatment protects against brain injury and suppresses the progression of damage through its ability to selectively neutralize ·OH and $ONOO^-$. Moreover, as reported by Sato et al,[25] hydrogen inhibits superoxide formation in brain slices from vitamin C-depleted SMP30/GNL knockout mice. In this study, we showed that hydrogen treatment caused a quick increase in blood hydrogen level and markedly attenuated pulmonary edema and improved oxygenation function in the lung after severe burn injury. Our findings demonstrate that hydrogen treatment effectively ameliorates ALI induced by burn injury. Hydrogen can easily enter cells because of its lower molecular weight and possesses a potent ability for selectively reducing hydroxy radical and peroxynitrate, which are the most active and toxic radicals. Hydrogen-rich saline is much cheaper than other antioxidative drugs and has no known side effects. Clinical application of hydrogen gas inhalation has associated risks of flammability and explosion at/over a concentration of 4.7% in air. Hydrogen dissolved in saline, however, could be easily delivered intravascularly and is much safer for clinical use.

Although in this study the administration of hydrogen-rich saline only increased systemic hydrogen levels for a short time (<30 min), it is conceivable that this transient increase in hydrogen was sufficient to quench toxic ROS, leading to the reduction in pulmonary damage that we observed 12 hours postburn. Understanding the mechanism by which hydrogen treatment mediates ROS and inflammatory mediators in the presence of burn-induced ALI is important, as increased generation of ROS and cytokines is believed to contribute to the pathophysiology of this injury. Studies have shown that extensive burn with delayed resuscitation frequently results in multiple organ dysfunction syndromes, including ALI, by triggering massive release of inflammatory molecules including ROS and cytokines.[7,26,27] It is known that lipid peroxidation caused by ROS generates lipid radicals, which subsequently attack other lipid molecules and propagate as a chain reaction that alter the function of plasma membranes.[28] Under oxidative stress, all amino acid residues could be oxidized by ROS through carbonylation or nitrosylation, resulting in irreversible alteration of proteins. In addition, oxidized proteins and lipid peroxides or glycation products can form aggregations of bulky protein complexes, which may inactivate both 26S and 20S proteosomes, leading to the accumulation of damaged proteins and cell death.[29] Furthermore, nucleophilic centers N and O contained in purine and pyrimidine bases and the sugar backbone are susceptible to ROS attack, especially by the hydroxyl radicals, which may result in single-strand or double-strand breaks.[30]

MDA, carbonyl, and 8-OH-dG are common indicators of oxidative damage. Several studies have demonstrated that I/R injury and burn injury are associated with increased levels of MDA, carbonyl, or 8-OH-dG in different tissues. Our results show that

the levels of MDA, carbonyl, and 8-OH-dG were significantly increased in pulmonary tissue after burn injury, and IP administration of hydrogen-rich saline dramatically decreased the levels of these metabolites. These results are consistent with a previous report that hydrogen was effective in reducing cytotoxic radicals in vivo.[18] Our findings suggest that hydrogen serves as a free radical scavenger to protect against oxidative damage, and this protective effect of hydrogen depends on its ROS scavenger effect.

Oxidative stress is known to induce inflammatory cascades mediated through TNF-α and IL-1β, leading to further augmentation of inflammatory mediator production and exacerbation of ALI.[31–33] Ballinger et al[34] has reported that peroxynitrite mediates mitochondrial DNA damage and alters gene expression in vascular endothelial and smooth muscle cells. A recent study has demonstrated that the effect of peroxynitrite causes increased expression of vascular endothelial growth factor in vascular endothelial cells by activation of two transcription factors during hypoxia.[35] In addition, a number of studies have shown that hydroxyl radicals damage mitochondria and initiate a cascade of cytokines that causes the pathological changes in organs.[36,37] In this study, a significant decrease in inflammatory mediators (IL-1β, IL-6, and TNF-α) occurred in the lung tissue of hydrogen-treated rats after burn injury, demonstrating the antiinflammatory property of hydrogen in burn-induced inflammation.

It is well known that inflammatory mediators, such as IL-1β and IL-6, recruit polymorphonuclear leukocytes into the microvascular wall[38] and increase the permeability of endothelium,[39,40] which are associated with pulmonary microvascular leakage and edema in ALI. Our data reveal that IP administration of hydrogen-rich saline attenuated the level of MPO in the lungs of the rats induced with severe burn injury and delayed resuscitation. These results suggest that hydrogen not only prevents the production of inflammatory mediators but also inhibits the infiltration of neutrophils into pulmonary tissues. However, in this study, we did not obtain enough data to elucidate whether the inhibition of inflammatory mediators is because of the reduction in ROS caused by hydrogen, this question will be addressed in our future study.

In conclusion, we have demonstrated that hydrogen-rich saline reduces pulmonary oxidative damage to lipid, protein, and DNA, and attenuates the inflammatory response, thereby alleviating lung edema and enhancing the recovery of pulmonary oxygenation function after severe burn injury. Thus, hydrogen treatment may serve as a promising strategy to alleviate pulmonary injury in severe burn patients care. However, this was a study of single time point and single dose, and further studies are needed to reveal the underlying mechanisms of the signaling pathways in hydrogen-mediated reduction of oxidative stress and the efficacy of hydrogen treatment in burn-induced ALI.

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