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Hydrogen inhalation ameliorates lipopolysaccharide-induced acute lung injury in mice

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ABSTRACT

Acute lung injury (ALI) is a serious illness, the incidence and mortality of which are very high. Free radicals, such as hydroxyl radicals (•OH) and peroxynitrite ($ONOO^-$), are considered to be the final causative molecules in the pathogenesis of ALI. Hydrogen, a new antioxidant, can selectively reduce •OH and $ONOO^-$. In the present study, we investigated the hypothesis that hydrogen inhalation could ameliorate ALI induced by intra-tracheal lipopolysaccharide (LPS, 5 mg/kg body weight). Mice were randomized into three groups: sham group (physiological saline + 2% hydrogen mixed gas), control group (LPS + normal air) and experiment group (LPS + 2% hydrogen mixed gas). Bronchoalveolar lavage fluid (BALF) was performed to determine the total protein concentrations and pro-inflammatory cytokines. Lung tissues were assayed for oxidative stress variables, wet/dry (W/D) ratio, histological, immunohistochemistry and Western blotting examinations. Our experiments exhibited that hydrogen improved the survival rate of mice and induced a decrease in lung W/D ratio. In addition, hydrogen decreased malonaldehyde and nitrotyrosine content, inhibited myeloperoxidase and maintained superoxide dismutase activity in lung tissues and associated with a decrease in the expression of TNF-α, IL-1β, IL-6 and total protein concentrations in the BALF. Hydrogen further attenuated histopathological alterations and mitigated lung cell apoptosis. Importantly, hydrogen inhibited the activation of P-JNK, and also reversed changes in Bax, Bcl-xl and caspase-3. In conclusion, our data demonstrated that hydrogen inhalation ameliorated LPS-induced ALI and it may be exerting its protective role by preventing the activation of ROS–JNK–caspase-3 pathway.

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1. Introduction

Acute lung injury (ALI), as manifested by acute respiratory distress syndrome (ARDS), is a serious illness, the incidence and mortality of which are very high [1–3]. The major pathological changes of ALI include impaired gas exchange, neutrophil accumulation, increased vascular permeability and parenchyma injury [4–6].

Endotoxin is thought to be the most important pathogen that leads to the development of ALI. Lipopolysaccharide (LPS) endotoxin, derived from the cell wall of gram-negative bacteria, is known to induce the release of free radicals, overproduction of inflammatory mediators, infiltration of inflammatory cells and tissue edema [4,7]. Of those molecules that mediate lung injury, free radicals are considered to be the final causative molecules in the pathogenesis of LPS-induced ALI. Increased production of free radicals combined with decreased antioxidant capacity of pulmonary vascular tissue contribute to the prognosis of LPS-induced ALI [2]. Free radicals, such as superoxide (O^{2-}), hydroxyl radicals (•OH) and peroxynitrite ($ONOO^-$), are all important mediators of LPS-induced ALI [2,8–11]. Treatment with antioxidants such as N-acetylcysteine, taurine, edaravone and N-methyl-D-aspartate receptor antagonist has been proved to be effective in ameliorating the LPS-induced ALI [12–15].

In 2007, Ohsawa et al. provided evidence that hydrogen could selectively reduce •OH and $ONOO^-$, both of which are important mediators of LPS-induced ALI [16]. Although hydrogen has been proved to be a novel therapeutic medical gas in several animal models of lung injury [17–19], to our knowledge, hydrogen gas has not been tested in the LPS-induced ALI. In the present study, we investigated the hypothesis that hydrogen inhalation could ameliorate LPS-induced ALI in a mice model.

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2. Materials and methods

2.1. Animals and reagents

Male C57BL/6 mice aged 8–10 weeks and weighing 20–25 g (Experimental Animal Center of the Second Military Medical University (SMMU), Shanghai, China) were housed in individual cages in a temperature-controlled room with a 12 h light/dark cycle and free access to food and water. All animal experiments were approved by the SMMU in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication No. 96-01).

2% hydrogen mixed gas (endotoxin free, purity > 99.9%, 2% hydrogen, 21% oxygen and 77% nitrogen) in air was purchased from Weichuang Standard Gas Corporation (Shanghai, China). LPS (Sigma Chemical Company, St. Louis, MO, USA) was dissolved in physiological saline (Ps) and the concentration was adjusted to 5 mg/ml. Pentobarbital sodium (Sigma Chemical Company, St. Louis, MO, USA) was also dissolved in Ps and the concentration was adjusted to 10 mg/ml. Enzyme-linked immunosorbent assay (ELISA) kits of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 were obtained from R&D Corporation (Minneapolis, MN, USA). The kits of myeloperoxidase (MPO), malonaldehyde (MDA) and superoxide dismutase (SOD) were provided by Shanghai Gen Med Corporation (Shanghai, China). Total protein extraction kit was purchased from Cell Signaling Technology (Beverly, MA). The following primary antibodies were used: anti-Bax, anti-Bcl-xl, anti-cleaved caspase-3, anti-phospho-JNK (anti-p-JNK) (Cell Signaling Technology, Beverly, MA), anti-nitrotyrosine (Upstate, USA), and anti-β-actin (Sigma-Aldrich, St. Louis, MO). The anti-rabbit or anti-mouse secondary antibody was provided by the Sigma Chemical Company (St. Louis, MO, USA). The ECL chemiluminescence and the bicinchoninic acid (BCA) protein assay were purchased from Thermo Fisher Scientific Inc. (USA). Polyvinylidene fluoride (PVDF) membranes were provided by Millipore (Bedford, MA).

2.2. Acute lung injury model

Mice were anesthetized by intra-peritoneal injection of pentobarbital sodium (40 mg/kg body weight). Mice were placed in a supine position on a warming device and the trachea was surgically exposed by a cervical middle line incision in the skin, and LPS (5 mg/kg body weight) or the same volume of Ps was slowly injected into the trachea of each mouse [20].

2.3. Experimental design

Part 1 was designed for measuring the survival rate of mice. Thirty-six mice were randomized into three groups: the sham group (Ps + 2% hydrogen mixed gas, S group), the control group (LPS + normal air, Lc group) and the experiment group (LPS + 2% hydrogen mixed gas, Lh group). Survival of mice was monitored every 12 h for 96 h in each treated group.

Part 2 was designed for the molecule assays, and histological and immunohistochemistry examinations. In this part, thirty-six mice were also randomly divided into three groups: S group, Lc group and Lh group. Six mice were sacrificed at 12 h and 24 h post-LPS stimulation in all three groups.

At sampling, after the right lung was isolated and tied off with a micro clamp at the right bronchus, the left lung was used for bronchoalveolar lavage fluid (BALF). The right lower lobe was used for wet/dry (W/D) ratio measurement, the right middle lobe was fixed in 10% formalin and prepared for histological and immunohistochemistry examinations and the other portions of the right lung were immediately snap frozen in liquid nitrogen for Western blotting experiments.

2.4. Hydrogen inhalation

In the present experiment, the anesthetized mice were placed into a sample self-made suction device (Fig. 1) and allowed to spontaneous respiration for 2 h during the experiment.

2.5. W/D weight ratio

The right lower lobe was weighed immediately after collection and placed into a 55 °C oven to dry for 48 h. The dried tissue was also weighed to determine the W/D weight ratio. The W/D weight ratio was calculated to assess pulmonary vascular permeability.

2.6. BALF analysis

The left lung had slow intra-tracheal injection of three sequential 0.5 ml of ice cold Ps. A total of 1.2 ml of BALF was collected and centrifuged at 350 *g* for 10 min at 4 °C to pellet the cells. The cell pellets were resuspended in 1 ml Ps for total cell counts using a hemocytometer and supernatant was collected for detecting the total protein concentrations and pro-inflammatory cytokines. TNF-α, IL-1β and IL-6 in the BALF were measured by commercially available ELISA kits according to the manufacturer's protocol. Protein concentration in the BALF, an indicator of vascular permeability, was measured with the BCA method.

2.7. MDA, SOD and MPO assays in lung tissues

The levels of MPO, MDA and SOD contained in lung tissues were measured using commercially available assay kits following the manufacturer's recommendations. All samples were assayed in duplicate. MDA content was expressed as pg/mg. MPO activity in the lung was expressed as unit (U)/mg. SOD activity was presented as IC_{50} (μg/ml). The level of IC_{50} in lung tissues was inversely correlated with SOD activity.

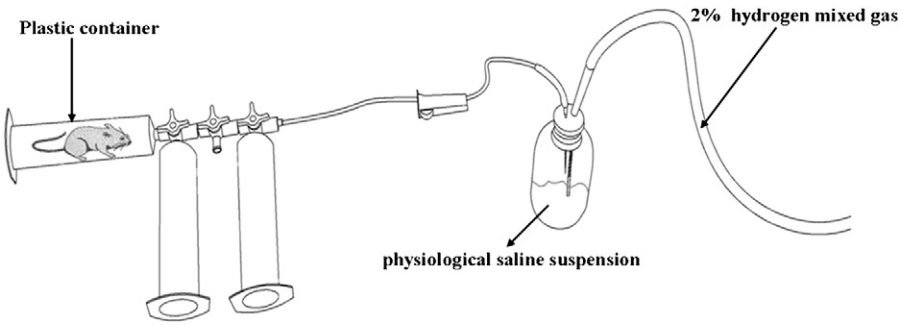

Fig. 1. Self-made experimental device for hydrogen gas delivery. Anesthetized mice can be placed in the plastic container and allowed to inhale the hydrogen by spontaneous breathing.

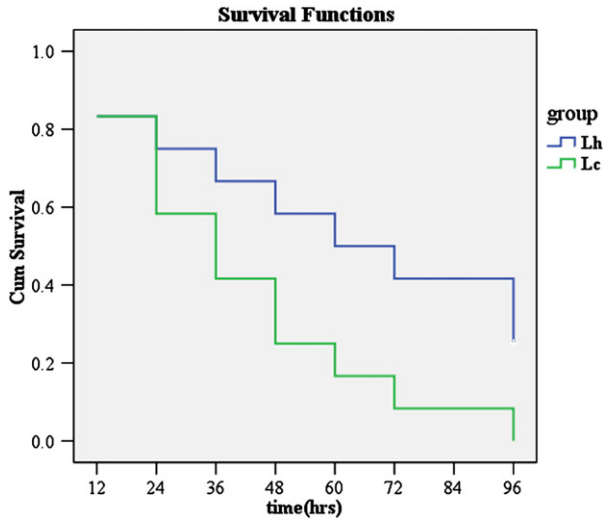

Fig. 2. Effects of hydrogen on the survival rate of mice with LPS stimulation. Survival was observed for 12, 24, 36, 48, 60, 72, 84 and 96 h after LPS challenge. Results are expressed as percent survival, n = 12. The survival rate was estimated by the Kaplan–Meier method. Hydrogen significantly delayed LPS-induced mice death. $^{\#}P<0.05$ vs. Lc group.

2.8. Histological examination of the lung tissues

The right middle lobes of the lungs were fixed in 10% formalin, embedded in paraffin, sectioned to 6 μm, in thickness, and stained with hematoxylin and eosin (HE). Lung injury was observed in a blinded fashion [21].

2.9. Detection of apoptosis in lung tissues

Apoptosis test in the lung tissues was performed by TACSTM TdT Kit. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells were counted under a light microscope (400×). At least three high power fields were scored per mouse, and six mice were used to generate each data point. The average number of TUNEL-positive cells was calculated.

2.10. Western blot analysis for Bcl-xl, Bax, cleaved caspase-3, P-JNK, nitrotyrosine and β-actin

The right lung tissues were homogenized and analyzed for Bcl-xl, Bax, cleaved caspase-3, P-JNK, nitrotyrosine and β-actin. Protein concentrations were determined by the method of BCA. Samples were electroblotted onto PVDF membranes and probed with primary antibodies against P-JNK (1:1000), Bcl-xl (1:1000), Bax (1:1000), cleaved caspase-3(1:1000), nitrotyrosine (1:500) and β-actin (1:1000),

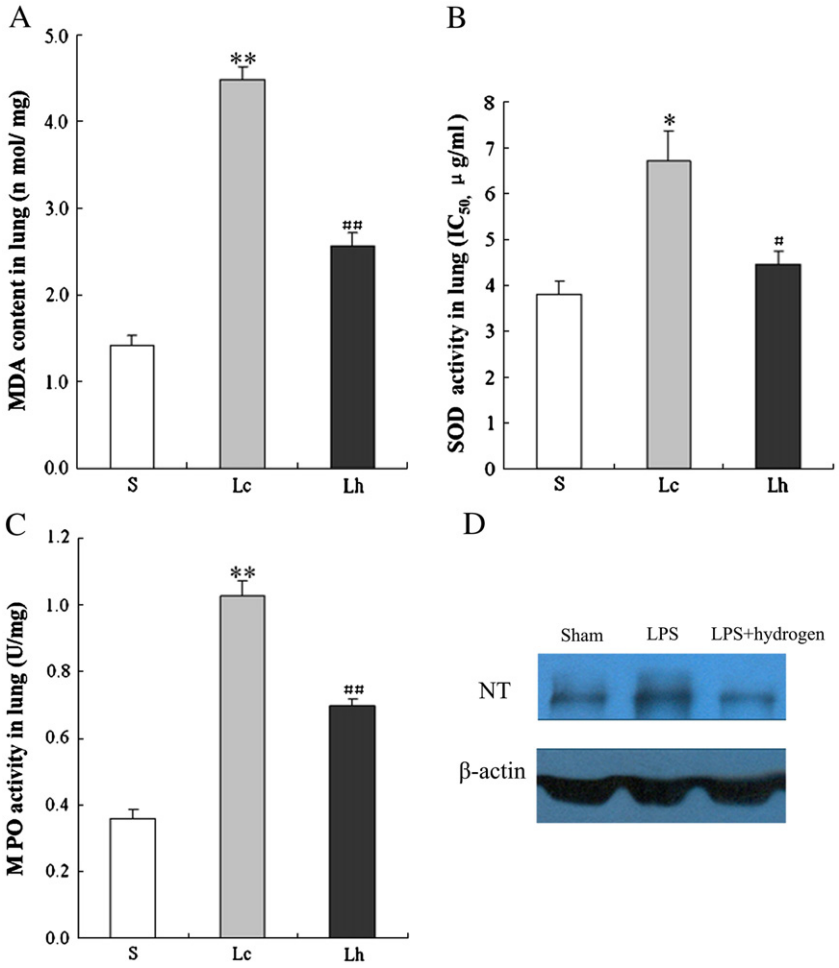

Fig. 3. Effects of hydrogen on MDA levels, SOD and MPO activity and nitrotyrosine content in lungs of mice after LPS injection. MDA content (A), SOD activity (B), MPO activity (C), and nitrotyrosine content (D) in lung tissues were determined at 12 h after LPS stimulation. Data are presented as the mean ± SEM (n = 6 in each group). $^{*}P<0.05$, $^{**}P<0.01$ vs. S group, $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. Lc group.

respectively. Membranes were then incubated with the horseradish peroxidase-tagged secondary antibody (1:5000) and visualized with the enhanced chemiluminescence reagent followed by autoradiography [18].

2.11. Statistical analysis

Statistical description was performed using SPSS 16.0 (SPSS Inc., Chicago, IL) for windows. Analysis of variance and Student's *t* test were used for statistical analysis. Kaplan–Meier analysis was used for assessment of survival data. All values were expressed as mean ± SEM. Significance was accepted when $P<0.05$.

3. Results

3.1. Hydrogen inhalation improved the survival rate of mice challenged with LPS

To evaluate the protective effect of hydrogen on mice challenged with LPS, 2% hydrogen mixed gas was inhaled for 2 h at 30 min after LPS stimulation as well as subsequently every day until they were sacrificed. As shown in Fig. 2, hydrogen remarkably improved the survival rate of mice challenged with LPS ($P<0.05$).

3.2. Hydrogen inhalation reduced LPS-induced lung oxidative stress

MDA concentration was an index of lipid peroxidation. The results of MDA content in the lung tissues after LPS stimulation were shown in Fig. 3A. LPS stimulation increased the lung MDA concentration and hydrogen gas inhalation prevented this increase ($P<0.05$).

SOD is a group of endogenous antioxidants that inhibit or delay the oxidative processes by being oxidized themselves. Fig. 3B showed SOD activity in lung tissues associated with LPS stimulation. Hydrogen inhalation significantly maintained SOD activity ($P<0.05$).

$ONOO^-$, mainly derived from the interaction of nitric oxide (NO) with O^{2-}, is a highly toxic RNS and can initiate cell injury via oxidation of protein moieties such as tyrosine [10,22]. In the present study, the formation of nitrotyrosine, an indirect marker of $ONOO^-$ generation, was observed in the lungs of LPS-exposed mice by Western blotting. Our results showed that hydrogen administering inhibited the nitrotyrosine formation (Fig. 3C).

3.3. Hydrogen inhalation inhibited LPS-induced neutrophil accumulation

Neutrophils are considered to be central to the pathogenesis of most forms of ALI [6]. Lung MPO activity is an indicator of neutrophil accumulation. LPS caused a significant up-regulation of MPO activity

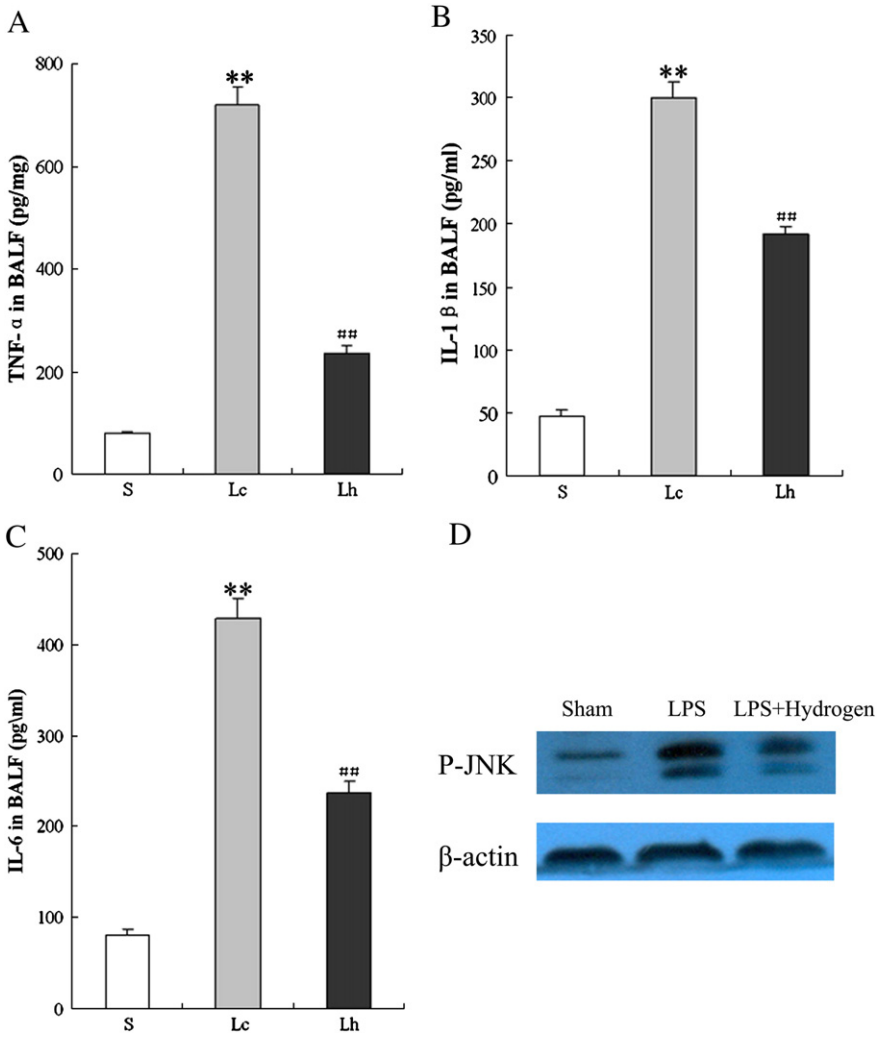

Fig. 4. Effects of hydrogen on the TNF-α, IL-1β and IL-6 levels in the BALF and P-JNK activity in the lung tissues of mice after LPS stimulation. TNF-α (A), IL-1β (B) and IL-6 (C) levels in the BALF and expression of P-JNK were determined by Western blot analysis (D) in the lung tissues at 12 h after LPS stimulation. Data are presented as the mean ± SEM (n = 6 in each group). **P<0.01 vs. S group, ##P<0.01 vs. Lc group.

in the lung tissues. However, hydrogen significantly abrogated the changes in the activity of MPO (Fig. 3C, $P<0.01$).

3.4. Hydrogen inhalation ameliorated inflammatory responses associated with LPS

To determine the pro-inflammatory chemokine levels in response to LPS stimulation, TNF-α, IL-1β and IL-6 levels were measured in the BLAF. As Fig. 4A, B and C exhibited those excessive amounts of TNF-α, IL-1β and IL-6 were found in the Lc and Lh groups. Hydrogen inhalation prevented the release of TNF-α, IL-1β and IL-6 associated with LPS stimulation ($P<0.01$).

The stress-activated protein JNK is potently and preferentially activated by a variety of environmental stresses including γ-radiation and inflammatory cytokines. Administration of LPS induced inflammatory response, therefore, activating the JNK. Our Western blotting results showed that hydrogen also attenuated P-JNK levels in the lungs of mice administered with LPS intratracheally at 12 h post-injury.

3.5. Hydrogen inhalation reduced LPS-induced high lung vascular permeability

LPS challenge produced a significant increase in capillary leakage [23]. The lung W/D ratio (Fig. 5A) and total protein concentrations in the BLAF (Fig. 5B), both of which were a measurement of pulmonary vascular permeability, were all markedly increased after LPS stimulation. The present study results showed that hydrogen inhalation significantly inhibited the increase of lung W/D ratio and total protein concentrations in BALF.

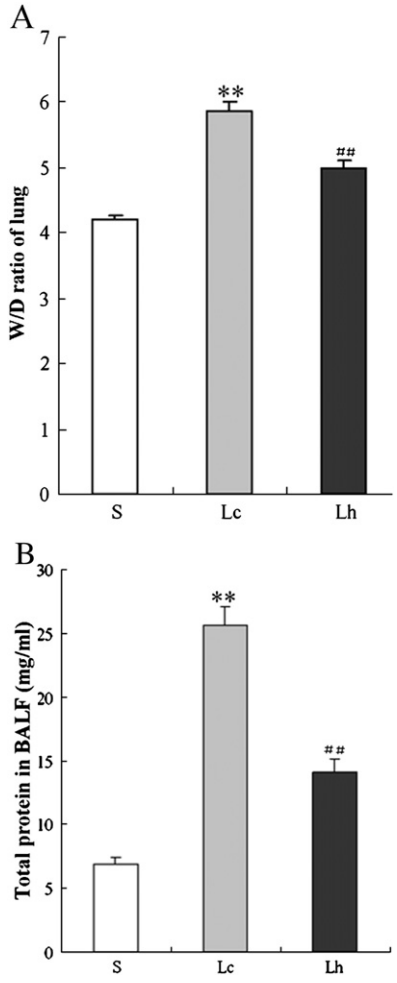

Fig. 5. Effects of hydrogen on the lung W/D ratio and total protein concentration in the BALF of LPS-induced ALI mice. The lung W/D ratio (A) and total protein concentration in the BALF (B) were determined at 12 h after LPS challenge. Data are presented as the mean ± SEM (n = 6 in each group). **P<0.01 vs. S group, ##P<0.01 vs. Lc group.

3.6. Hydrogen inhalation attenuated the LPS-induced lung injury

Mice in the Lc group had a pattern of accumulation of a large number of neutrophils in the intra- and inter-alveolar space, a thickened alveolar wall, less alveolar space, interstitial congestion and edema whereas hydrogen inhalation markedly attenuated the LPS-induced lung injury (Fig. 6A).

3.7. Hydrogen inhalation mitigated LPS-induced lung cell apoptosis

As shown in Fig. 6B, LPS stimulation resulted in an obvious increase of TUNEL-positive cells in lung tissues. However, hydrogen inhalation significantly decreased the TUNEL-positive cells relative to the Lc group.

Overexpression of Bcl-xl ameliorates lung injury by inhibiting apoptotic pathways. LPS resulted in down-regulation of Bcl-xl protein and hydrogen prevented this decrease. The pro-apoptotic protein, Bax, can be induced within alveolar epithelial cells by oxidative stress. LPS resulted in an up-regulation of Bax protein and hydrogen inhalation inhibited over-activity of Bax protein. Sequential activation of caspases plays a central role in the process of cellular apoptosis. We investigated one caspase family, caspase-3, by Western blot analysis. Activated caspase-3 protein levels increased in mice treated with LPS and hydrogen inhalation reduced the activated caspase-3 level in the 12 h post-injury [18] (Fig. 6C).

4. Discussion

To our knowledge, this was the first study that demonstrated that hydrogen inhalation markedly protected the lung from LPS-induced injury. This protective effect might be related to its ability of ameliorating the extent of oxidative stress and preventing the release of pro-inflammatory molecules as well as inhibiting lung cell apoptosis.

LPS is the most important pathogen that leads to the development of ALI and intra-tracheal instillation of LPS has been commonly used to induce an animal model of ALI [24,25], so we established an ALI mice model through intra-tracheal injection of LPS (5 mg/kg) in our present study. The increased production of free radicals combined with decreased antioxidant capacity of pulmonary vascular tissue contributes to the prognosis of LPS-induced ALI. In addition, hydrogen is a new and highly effective antioxidant. Moreover, inhaled therapeutic medical gas is a promising, easily delivered and straightforward therapeutic option for lung injury. In our previous work, based on pilot studies, we used 2% hydrogen mixed gas inhalation to improve imbalance of oxidation and reduction, therefore protecting the lung against LPS-induced injury [16–18,26,27].

Hydrogen is a novel energy source and recent attention has been focused on it as an energy storage medium with less air pollution when burned. In the field of biological medicine, hydrogen has been considered as a physiologically inert gas whose current application was limited in diving medicine. In 2007, Ohsawa et al. discovered hydrogen's ability of selectively reducing •OH and $ONOO^-$. Since then, hydrogen rapidly arouses attraction in the field of medical research and has been proved to be an effective treatment measure for many animal disease models in the past 4 years and the protective mechanisms involve antioxidant, anti-inflammatory and, ultimately, anti-apoptosis. [16,28–31]. Compared with other antioxidants, hydrogen has several advantages. Firstly, it is contained in the human body and reacts with •OH to produce water, so it is nontoxic. Secondly, it is mild enough not to disturb metabolic oxidation–reduction reactions or to disrupt

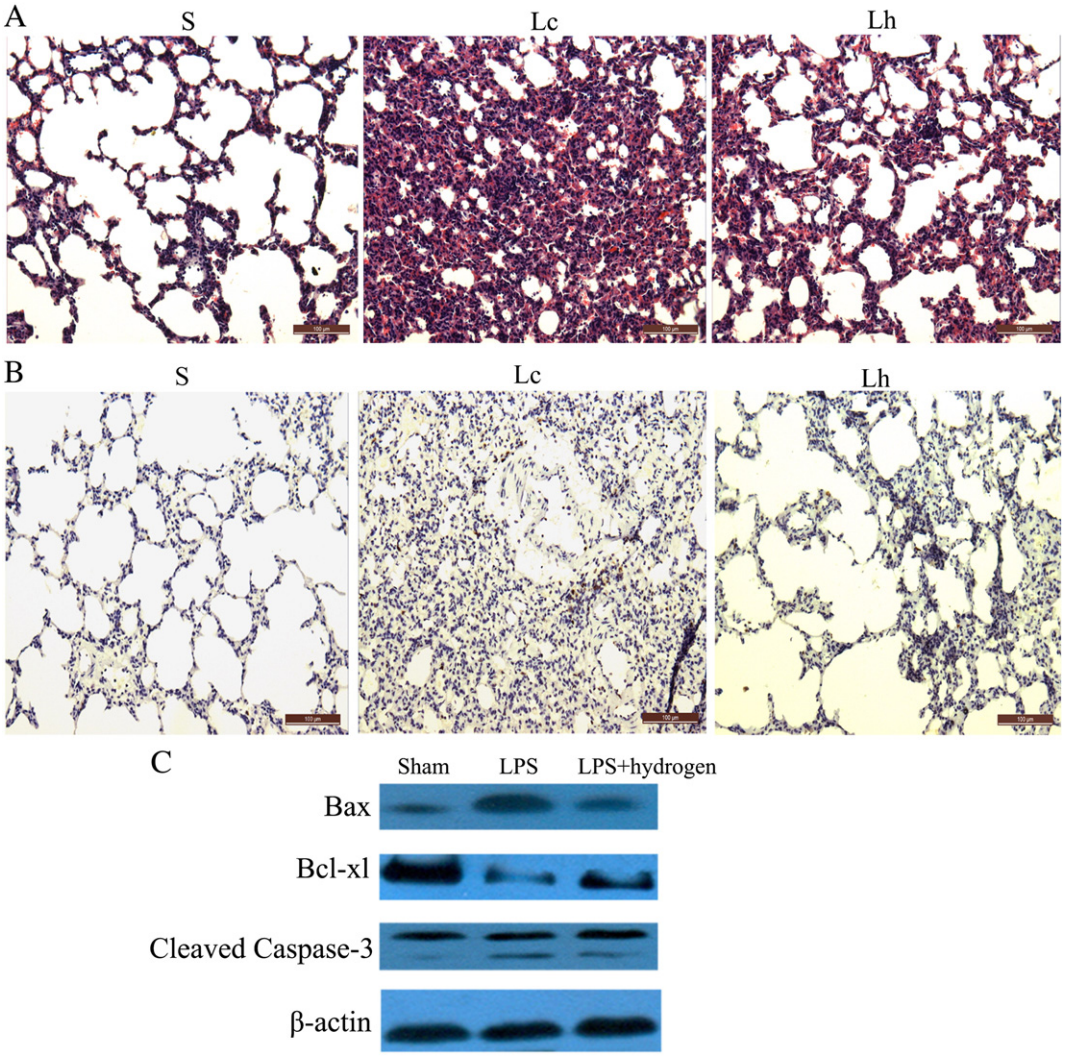

Fig. 6. Effect of hydrogen on histopathological changes and cell apoptosis in lung tissues of LPS-induced ALI mice (×200). (A) Comparison of histopathological changes using HE staining: after LPS stimulation, lung in the Lc group showed a thickened alveolar wall, edema and hemorrhage, less alveolar space and obvious inflammatory cell infiltration. Hydrogen inhalation significantly prevented the histopathological changes caused by LPS. (B) Representative micrographs of TUNEL staining: there was obviously increased lung cell apoptosis after LPS stimulation. However, a lower level of apoptosis was found in Lh group compared with that in Lc group. (C) Expression of Bcl-xl, Bax and cleaved caspase-3 at 12 h post-injury: there was a significant up-regulation of Bax and caspase-3 and a down-regulation of Bcl-xl activity in mice treatment with LPS. However, hydrogen markedly reversed these changes.

ROS involved in cell signaling—unlike some antioxidant supplements with strong reductive reactivity, which increase mortality, possibly by affecting essential defensive mechanisms. Thirdly, it also can easily penetrate biomembranes and diffuse into the cytosol, mitochondria and nucleus. Its rapid gaseous diffusion might make it highly effective for reducing cytotoxic radicals. Fourthly, dissolving hydrogen gas in Ps is easy to apply and safe. It is also safe for humans to inhale hydrogen at a relatively low concentration (<4%, poses no risk of explosion in air and oxygen). Therefore, it has great potential for clinical use [16,28,29,32].

In the present study, we found that hydrogen gas inhalation significantly protected mice against ALI lethality. In addition, histopathologic results showed that hydrogen inhalation improved the lung injury caused by LPS stimulation. Moreover, hydrogen inhalation also reduced W/D ratio of lung and total protein concentrations in the BALF, both of which were markedly increased because LPS induced high lung vascular permeability, as well as inhibited the lung neutrophil recruitment. All of these results showed that 2% hydrogen mix gas inhalation had truly a protective effect against LPS-induced lung injury.

To explore the underlying mechanisms of the protective effect of hydrogen, we first investigated its influence on production of free radicals which contributes to the prognosis of LPS-induced ALI. LPS injection resulted in an occurrence of increased oxidative stress and a reduced anti-oxidative status. In the present study, we were not able to detect free radical time course changes. However, the MDA level significantly increased and SOD activity markedly decreased at 24 h after LPS injection and these parameters were obviously improved by hydrogen inhalation. The enhanced production of both NO and O^{2-} during acute lung inflammation favors the formation of $ONOO^{-}$ and it may be involved in the pathogenesis of LPS-induced lung injury [10]. In our present study, we also measured lung tissue nitrotyrosine as an index of formation of RNS, such as $ONOO^{-}$. Direct administration of hydrogen gas, compared with normal gas, significantly decreased nitrotyrosine in lung tissue. In the present study, we were not able to detect RNS and ROS time course changes; however MPO, MDA, and nitrotyrosine significantly increased at 24 h in the control group which were all significantly reduced by hydrogen treatment. These results strongly suggested that LPS stimulation caused free radicals to appear in the lung tissue and hydrogen might have a strong

ability as an antioxidant which might be its basic protection mechanism. We referred to this new cytoprotective approach of using extrinsic hydrogen treatment to improve imbalance of oxidation and reduction as "hydrogen resuscitation" [29].

Previous studies have showed that hydrogen has a potential anti-inflammatory effect [30,33]. TNF-α, IL-1β and IL-6 are potent pro-inflammatory cytokines that play a role in the initiation and amplification of inflammatory responses [34]. Inhibiting the over-production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 showed the lessening of pulmonary injury in LPS induced ALI model [5,23,34,35]. In the present study, we investigated hydrogen's effect on LPS-induced inflammatory response. Our preliminary experiments showed that the concentrations of TNF-α, IL-1β and IL-6 in BALF significantly increased following LPS injection and treatment with hydrogen markedly reduced the cytokine secretion in BALF. In addition, we also investigated JNK which could be activated by LPS-induced inflammatory response. In a previous study, scientists have proved that hydrogen had the ability of inhibiting the overexpression of JNK [36,37]. We proved that, in the present study, phosphorylation, and consequent activation of the pro-apoptotic kinase JNK, were blocked by hydrogen inhalation.

To determine whether inhibition of apoptosis was involved in the hydrogen's protective effect on LPS-induced ALI, we examined lung cell apoptosis by TUNEL staining. Results showed that hydrogen gas inhalation markedly inhibited lung cell apoptosis. In addition, we investigated several proteins such as Bcl-xl, Bax and caspase-3 which were related with the LPS-induced lung cell apoptosis. We demonstrated, in the present study, that hydrogen gas inhalation prevented LPS-induced apoptosis through down-regulation of apoptotic genes, including Bax and caspase-3 and upregulation of antiapoptotic gene, Bcl-xl. Although our findings did not explain all of the mechanisms underlying the protective effects of hydrogen, we postulated that the Bcl-2/Bcl-xl/caspase-3 pathway might be one of the key mechanisms.

5. Conclusion

In conclusion, we have provided the first evidence that hydrogen inhalation significantly attenuated pulmonary inflammation and improved survival rate in mice with LPS-induced ALI, and that the potential mechanism of this action is through ameliorating the extent of oxidative stress and preventing the release of pro-inflammatory molecules with the inhibition of lung cell apoptosis. Hydrogen gas may be exerting its protective role by preventing the activation of the ROS–JNK–caspase-3 pathway. Although the exact mechanisms involved in the protective role of hydrogen in LPS-induced ALI need to be further investigated, hydrogen may be considered potential therapeutic molecules.

Competing interests

There are no competing interests in our work.

Acknowledgments

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