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Original Article

Hydrogen mitigates acute lung injury through upregulation of M2 and downregulation of M1 macrophage phenotypes

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Abstract: Objectives: Acute lung injury (ALI) is still a leading cause of morbidity and mortality in critically ill patients. ALI can be induced by sepsis, ventilator, hyperoxia, and ischemia-reperfusion. In the management of ALI, studies have shown that low concentration hydrogen has a therapeutic effect on acute lung injury. The present study was designed to investigate the effects and corresponding mechanism of the inhalation of hydrogen on ALI. Methods: C57 male mice underwent intraperitoneal injection of lipopolysccharide (LPS) to induce sepsis. Then, the mice were given 2% hydrogen. The survival rate, lung injury, inflammatory factors and macrophage phenotypic changes were assessed in bronchoalveolar lavage fluid (BALF). In vitro experiment, we obtained primary mouse bone marrow-derived macrophages (BMDM) incubated by LPS to explore the anti-inflammatory effects and corresponding mechanism. Results: Treatment with 2% hydrogen showed a substantial attenuation of inflammations, adverse lung histopathological changes and decreased M1 macrophage phenotypes while increased M2 macrophage phenotypes. Importantly, the decreased M1 macrophage phenotypes while increased M2 macrophage phenotypes were observed in LPS-stimulated macrophages treated with 2% hydrogen. Further, we observed significantly low levels of TNFα in LPS-induced macrophages treated with 2% hydrogen. The effects were ascribed to an inhibition of phosphorylation of p38 MAPK. Conclusion: 2% hydrogen may reduce sepsis-induced inflammatory responses in animals and in macrophages, and the inhibition to the activation of the MAPK/TNF-α may contribute to this protection.

Keywords: Acute lung injury, molecular hydrogen, p38 MAPK, bone marrow-derived macrophages

Introduction

Acute lung injury (ALI) is a common clinical critical illness and the most common organ tissue damage induced by systemic inflammatory response syndrome, a severe systemic nonspecific response to a condition (as trauma, an infection, or a burn) that provokes an acute inflammatory reaction [1]. Although the treatment has made great progress, the mortality rate is as high as 35%~40% [2]. Even there is good approach in the pathophysiology of ALI; there is still no effective treatment for ALI. In spite of involvement of different mechanisms in the pathogenesis of ALI, inflammation is one of the leading causes. ALI is result of the extreme inflammatory process characterized by extensive neutrophil influx into the lungs, the expression of pro-inflammatory mediators, and lung epithelium and endothelium damages, which results in respiratory failure. Therefore, inhibiting the inflammatory cascades result in good therapeutic approach for ALI. In addition, ALI is associated with the continuous macrophage-associated production of reactive oxygen/nitrogen species (ROS/RNS), leading to lungs tissue damage. Molecular hydrogen is a good anti-inflammatory, antioxidant and antiapoptotic agent that reduces ROS level and suppresses lungs tissue damage.

LPS is a component of the cell wall of gram-negative bacteria, which can trigger a cascade of reactions by activating various effectors cells (such as neutrophils), cytokine networks and the expression of pro-inflammatory genes, lead-

ing to the occurrence of ALI. Upon activation of defense mechanism of body toward the foreign body (bacteria, virus), various defending cells (macrophage) release early response cytokines such as type I IFN, TNF-α, and IL-1β in an IRF- or NF-κB-dependent way. In ALI, TNF-alpha is considered to be the earliest and most important endogenous medium in inflammatory response. TNF-alpha can induce various inflammatory cells and epithelial cells in the lungs to produce multiple cytokines, mediate inflammatory cells infiltrating and produce lung tissue damage [3]. An inducible transcription factor p38 MAPK is a driving force in the initiation and progression of systemic inflammation and septic pathophysiology [4]. P38 MAPK is an important component of the MAPK family. Studies have shown that when LPS induced ALI, p38 MAPK was activated, causing the secretion of TNF-α [5]. Hence, the regulation of inflammatory response through the p38 MAPK signaling pathway has been a focus of studies.

Macrophages, as a group of plasticity and pluripotent cells, show distinct functional differences in vivo and in vitro under different micro environments. Macrophages also maintain airway homeostasis, and play a pivotal role in the pathogenesis of ALI [6]. According to different functions, macrophages can be divided into M1 type macrophages that classically activated macrophages, and M2 type of alternatively activated macrophages [7]. The M1 program is associated with release of pro-inflammatory mediators such as iNOS-derived NO, TNF-α, IFN-γ, and IL-12 and critically contributes to pathogen elimination. In contrast, M2, which secrete anti-inflammatory cytokines like IL-1ra, IL-10, and TGF-β, downregulate IL-12, upregulate scavenger receptors, promote angiogenesis, and support wound healing and tissue remodeling. M2 macrophages prove beneficial in arresting the inflammatory cytokine response in sepsis [7].

Hydrogen is a kind of important physiological regulatory factors in the body and can selectively lower the hydroxyl free radicals and reactive oxygen species peroxynitrite anion [8], which has anti-inflammatory, antioxidant and antiapoptotic potential [9, 10]. Previous study showed that saturated hydrogen saline treatment improved the damage to pulmonary epithelial cells in rats with sepsis by increasing the expression of aquaporin-1 protein [11]. Furthermore, it showed that saturated hydrogen saline may downregulate expression of Beclin-1 transcription and inhibits autophagy through heme oxygenase-1 and p38 MAPK signaling [12].

In the present study, we applied lipopolysaccharide (LPS) intraperitoneal injection to establish the ALI model of mice and explored the effects of inhalation of low concentration of hydrogen on protection of ALI and the possible mechanism. The study will further provide theoretical basis for the hydrogen treatment of ALI.

Materials and methods

Animal models of ALI

The experimental animal protocols were approved by the Institutional Animal Care and Use Committee of the Second Affiliated Hospital of Harbin Medical University. All experiments were performed according to the experimental animal guidelines. Male C57 mice (20-25 g) were obtained from the Experimental Animal Center of the the Second Affiliated Hospital of Harbin Medical University. Endotoxin-induced ALI animal models were established by intraperitoneal injection of LPS (50 mg/kg, Sigma Chemical, St. Louis, MO, USA). The same volume of saline was given to animals in the control group.

Inhalation of hydrogen

Animals were placed in a sealed plexi glass chamber with an inflow and outflow hose. 2% Hydrogen with mixed air was delivered to the chamber through a tube, and carbon dioxide was removed from the chamber gases with baralyme. A gas analyzer (Bruel and Kjaer, Naerum, Denmark) was used to continuously monitor the concentration of hydrogen in the outflow hose of the chamber, which was maintained at a predetermined level during treatment. The animals were given two hours of inhalation of 2% hydrogen each time.

Cytokines in bronchoalveolar lavage fluid (BALF)

BALF was obtained by cannulating the trachea of mice and lavaging with 1 ml phosphate buffer saline (pH 7.4). Lavage samples were centrifuged 3000 rpm at 4°C for 10 minutes, and the supernatant was stored at -80°C. The cyto-

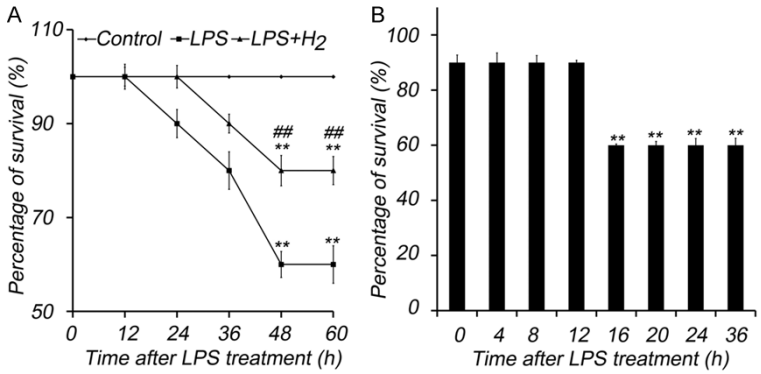

Figure 1. Two hours of inhalation of 2% hydrogen improved sepsis-induced lethality. Sepsis was induced by intraperitoneal injection of LPS. A. Protective effects of 2% at 4 h after LPS administration on LPS-induced sepsis in mice. $^{**}P < 0.01$ vs. Control and $^{\#\#}P < 0.0$ vs. LPS. B. Optimum time for protective effects of 2% hydrogen on LPS-induced sepsis in mice. 2% hydrogen was treated at different time point after LPS administration. $^{**}P < 0.01$ vs. time points 0, 4, 8 and 12 h.

kines in BALF were detected using specific IL1β, TNF-α, and IL-10 ELISA kits (R&D Systems Inc., Minneapolis, Minnesota, USA), according to manufacturer's instructions. All standards and samples were run in triplicate.

Lung myeloperoxidase (MPO) activity

MPO activity, an indicator of neutrophil infiltration in lung tissues, was detected in homogenated lung supernatants, as previously reported using an MPO Assay Kit (Nanjing Jian Cheng Bioengineering Institute, Nanjing, China), according to manufacturer's instructions.

Immunohistochemistry

Following PBS perfusion, the lung tissues were fixed for 1 hour by instillation of 10% PBS-buffered formalin through the trachea. Then the right lungs were taken out and fixed with 10% PBS-buffered formalin overnight at 4°C. After the paraffin embedding process, the tissues were sectioned in 5-μm-thick sections and the lung epithelial and endothelial biomarker E-cadherin (1:300, Sigma-Aldrich, St. Louis, MO, USA) antibodies at 4°C overnight. Then, the sections were incubated with biotinylated goat anti-mouse IgG for 1 h. The signal was detected with 3, 3'-diaminobenzidine.

Cell culture

Bone marrow derived mononuclear cells (BMDM) were isolated from the tibias and femurs of mice and cultured in Dulbecco's modified Eagle's medium along with 10% L929 cell conditioned with supplemented 10% heat-inactivated fetal calf serum (FBS)/1% penicillin and streptomycin. The incubator was set at 37°C with a humidified atmosphere containing 5% CO_2. After 7 days of culture, the cells were stained with CD11b and analyzed by flow cytometry analysis. The phase contrast microscopic image was taken to show the uniform morphology.

Western blotting

The protein from BMDM samples was directly extracted based on manufacturer's standard protocols (Beyotime Biotechnology, Shanghai, China). Primary rabbit antibodies for phosphor-p38 and total p38 (Cell Signaling Technology, Boston, USA) proteins were used to detect p38 protein expression. Then, the primary rabbit antibodies forglyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as control.

Immunofluorescence analysis

For immunofluorescence assays, cultured BMDM cells or lung tissue were rinsed once with PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes, then rinsed three times with PBS, permeabilized with 0.3% Triton X-100 (Sigma) in PBS for 5 minutes, washed twice with PBS. After processing with blocking solution for one hour at room temperature, cells were incubated with anti-CD68, and anti-Arginase 1 antibody overnight at 4°C. The slides were washed three times with PBS and incubated with fluorescein-conjugated secondary antibody for one hour at room temperature. DAPI staining was used for the counterstaining of the nucleus. Fluorescent images of the coverslips were obtained by either confocal and immunofluorescence microscope (Olympus, Tokyo, Japan).

Flow cytometer analysis

For flow cytometer phenotypic analysis, cells (1×10^6 cells/well) were initially incubated with

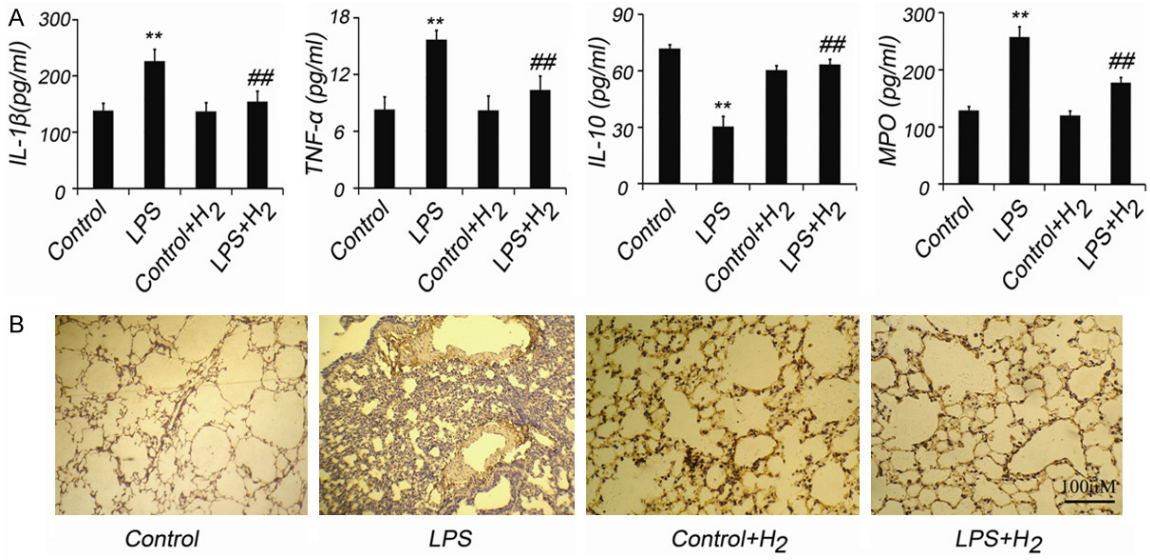

Figure 2. The 2% hydrogen at 4 h after LPS administration mitigated lung injury resulting from LPS-induced sepsis in mice. A: The production of IL1β, TNF-α, and IL-10 and MPO activity in BALF at 24 h after LPS administration (n=5). B: Morphological changes of lungs were assayed by immunohistochemistry. $^{**}P < 0.01$ vs. control group; $^{\#\#}P < 0.01$ vs. model group.

10% mouse serum for 20 minutes at 4°C. Subsequently, cells were incubated with the appropriately labeled primary antibodies for 1 hr. Anti-CD23 and anti-CD124 were used for quantifying M2 and anti-CD14 and anti-CD40 were used for quantifying M1 subtype. Then the cells were washed with washing buffer three times and incubated 20 minutes in an appropriate secondary antibody. All incubations were performed on ice. Appropriate isotype controls were used in all cases. Finally, the cells were washed three times with FACS buffer, resuspended in 0.5 ml PBS, and analyzed by flow cytometer (FACSCalibur, BD Biosciences) using Cell Quest software.

Statistical analysis

The measurement data are expressed as mean ± standard error of the mean (SEM). Intergroup differences in the levels of biochemical parameters, inflammatory cytokines, and the number of neutrophils were tested by one-way ANOVA, followed by the least significant difference test for multiple comparisons. Survival studies were analyzed using K-M analysis. Survival rates were expressed in percentage. The statistical analysis was performed using the SPSS 17.0 software (SPSS Inc, Chicago, USA). In all tests, $P < 0.05$ was considered statistically significant.

Results

The inhalation of 2% hydrogen protected against sepsis-induced lethality

LPS is the major component of the outer membrane of Gram-negative bacteria, which are considered as one of the predominant component that causes sepsis. We found all mice in the normal control group survived. In the model group, the mice died from 12 h and the lethality reached maximum at 48 h. A significant improvement of the survival rate was observed in mice treated with two hours of 2% hydrogen at 4h after LPS administration ($P < 0.01$) (**Figure 1A**). It was also observed that treatment with 2% hydrogen significantly increased the survival rates of animals with LPS-induced sepsis only when it was performed at 0, 4, 8 and 12 hours after LPS injection ($P < 0.05$) (**Figure 1A**). The results suggest that 2% hydrogen protects against lethality resulting from sepsis induced by LPS.

Inhalation of 2% hydrogen inhibited inflammatory responses

ALI, which is typically observed in individuals with sepsis, is an inflammatory disease. Dysregulated inflammatory response in lungs, as well as the altered permeability of alveolar

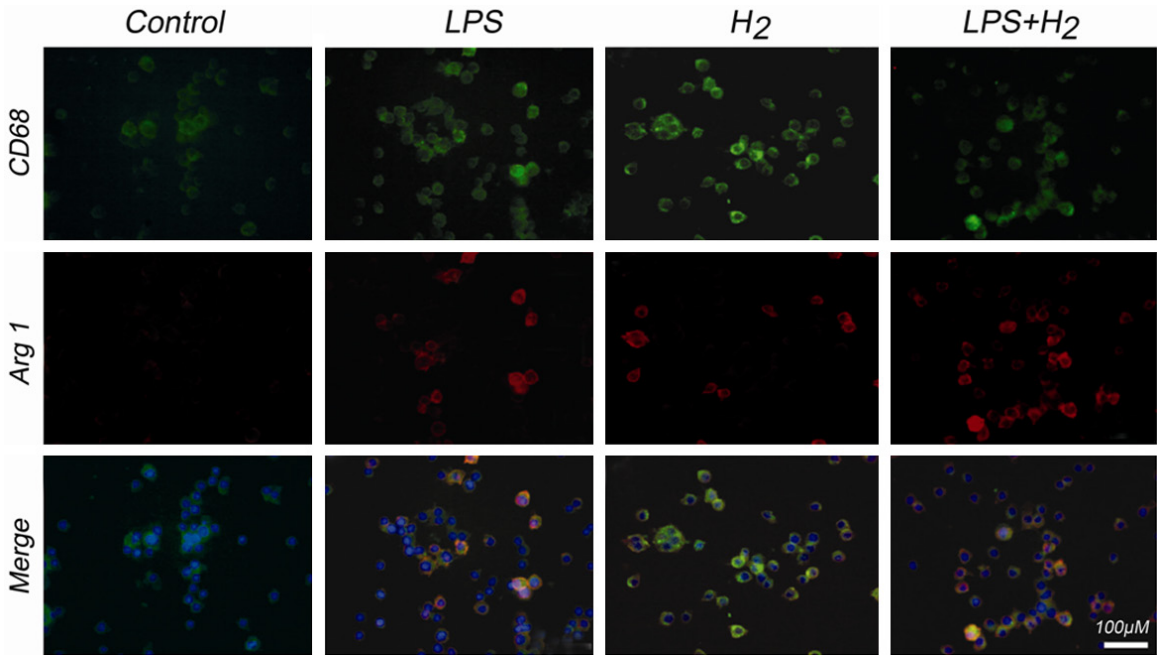

Figure 3. Treatment with 2% hydrogen showed increased M2 macrophages in ALI mice lung tissues.

endothelial and epithelial barriers, remains as central pathophysiologic concepts in ALI and acute respiratory distress syndrome (ARDS) [13]. In this study, we found, in LPS-challenged mice, the treatment of 2% hydrogen at 4 h after LPS administration significantly attenuated lung MPO activity ($P < 0.01$, **Figure 2A**), and reduced the production of TNF-α ($P < 0.01$, **Figure 2A**) and IL1β in BALF ($P < 0.05$, **Figure 2A**), but promoted the production of IL-10 ($P < 0.01$, **Figure 2A**). Furthermore, it was also observed that 2% hydrogen s at 4 h after LPS administration significantly decreased the level of TNF-α, IL1β and promoted IL-10 in serum ($P < 0.05$, **Figure 2A**). Additionally, it was also found that LPS induced infiltration of inflammatory cells in bronchial lumen, alveolar cavity and pulmonary interstitial and the treatment of 2% hydrogen at 4 h after LPS administration alleviated LPS-induced lung inflammation (**Figure 2B**). The above results demonstrate that treatments with 2% hydrogen attenuate lung injury resulting from LPS-induced sepsis by inhibiting lung inflammation, and protecting alveolar endothelial and epithelial barriers.

2% hydrogen increased M2 macrophages in lung tissues and in BMDM after LPS stimulation

Immunofluorescence staining was performed to determine the immune-plasticity of the LPS-challenged ALI mice after treatment with 2% hydrogen. Immunofluorescence staining of macrophage markers CD68 (pan marker, green) and Arg1 (M2 specific marker-red) showed consistently increased LPS-induced co-localization of M2 macrophages (yellow fluorescence in the merged image) in ALI mice (**Figure 3**) with 2% hydrogen treatment.

Next, BMDM were isolated from the tibias and femurs of mice and treated with LPS (1 μg/ml) for 1 hour. M1 macrophage phenotypes CD-14 CD40 double positive cells decreased while M2 macrophage phenotypes CD23 CD124 double positive cells were observed more in BMDM that treated with 2% hydrogen than LPS-treated BMDM (**Figure 4**). In vivo and vitro, these data indicated that the 2% hydrogen treatment modulates the lung macrophages towards anti-inflammatory M2 subtype.

2% hydrogen inhibited the activation of the p38 pathway in BMDM after LPS stimulation

P38 MAPK is a key regulator of inducible gene expression in the immune system, and is crucial to the signaling networks involved in sepsis. Previous studies have suggested that increasing P38 MAPK activity is associated with the development and mortality of sepsis [14]. Treatment was given on cells by 2% hydrogen starting at the addition of LPS. A significant

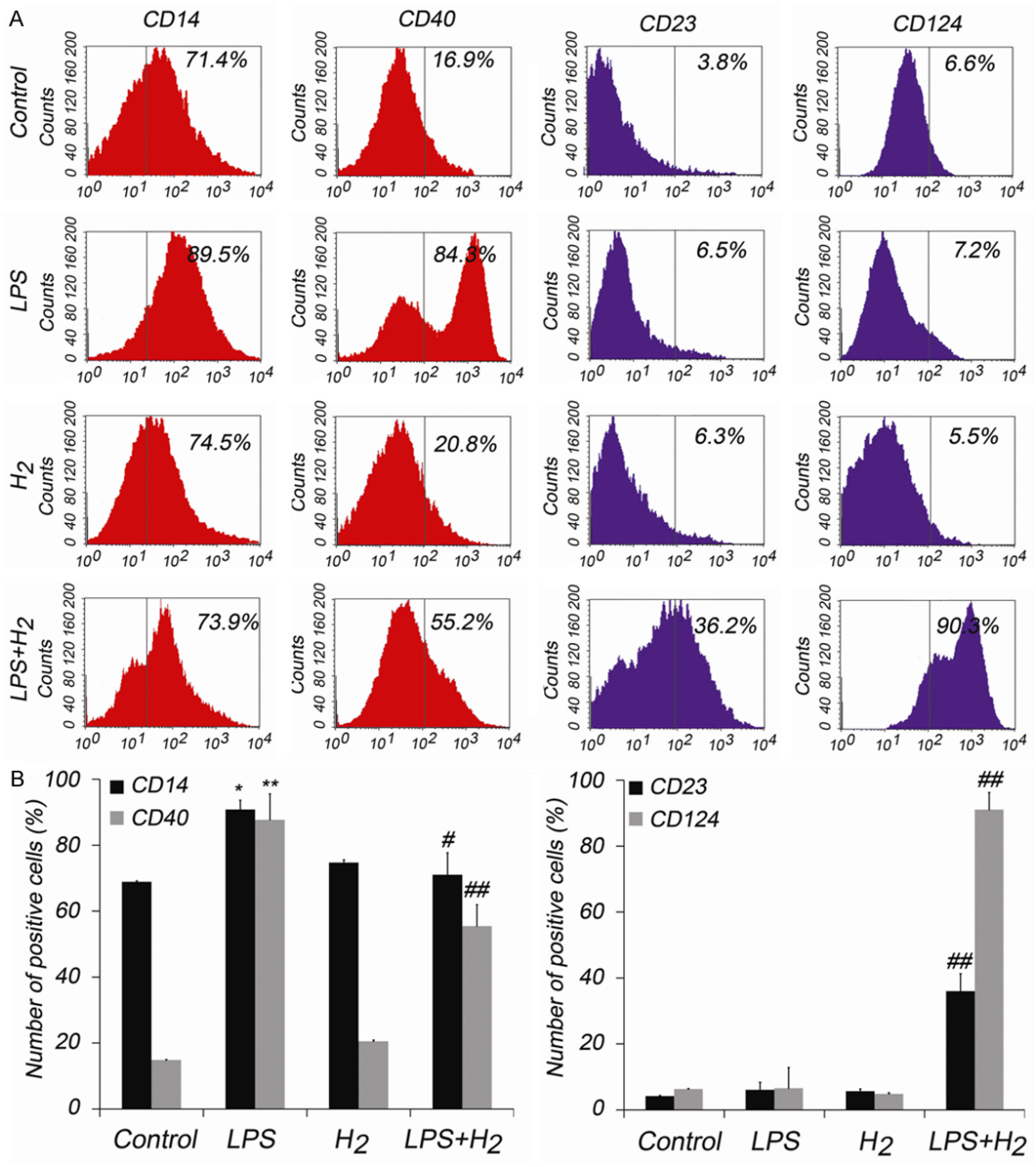

Figure 4. Treatment with 2% hydrogen showed increased M2 macrophages and decreased M1 macrophages in BMDM. A: Representative images; B: Positive cells rates of M1 and M2 macrophages. M1 macrophage phenotypes showed CD14 and CD40 double positive cells and M2 macrophage phenotypes showed CD23 and CD124 double positive cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. control; $^{\#}P<0.05$ and $^{\#\#}P < 0.01$ vs. LPS.

increase in TNF-α concentration in the cell culture supernatant at three hours after LPS stimulation ($P < 0.05$) was inhibited by 2% hydrogen starting at one hour after the addition of LPS ($P < 0.01$, **Figure 5**). Furthermore, the LPS-induced phosphorylation. of the P38 MAPK subunit was prevented by 2% hydrogen, as shown in **Figure 5**. The above findings suggest that 2% hydrogen inhibit the activation of P38 MAPK signaling in macrophages induced by LPS stimulation, contributing to the improvement of sepsis.

Discussion

Severe sepsis is sepsis plus either failure of an essential system in the body or inadequate blood flow to parts of the body due to an infec-

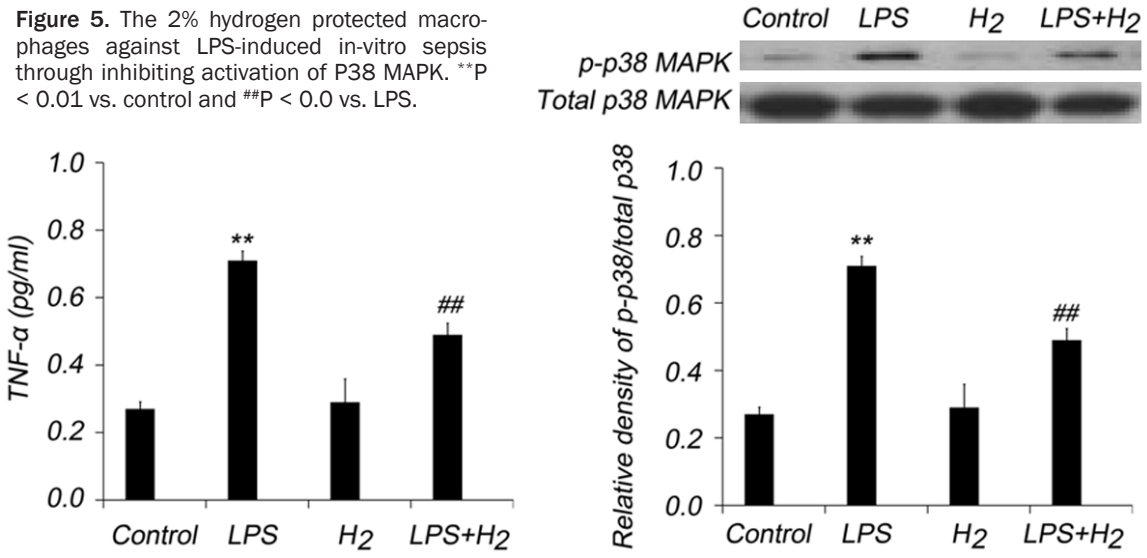

Figure 5. The 2% hydrogen protected macrophages against LPS-induced in-vitro sepsis through inhibiting activation of P38 MAPK. $^{**}P < 0.01$ vs. control and $^{\#\#}P < 0.0$ vs. LPS.

tion, which often results in significantly poor outcomes and mortality. Sepsis occurs when toxins produced by certain bacteria cause cells in the body to release substances that trigger inflammation. Acute lung injury (ALI) secondary to sepsis is a complex syndrome associated with high morbidity and mortality. Nearly 50% of patients with severe sepsis will develop ALI, and in its more severe form, acute respiratory distress syndrome. The respiratory system is the most frequently affected organ system. Diffuse inflammation of lung parenchyma and severe lung dysfunctions are the first steps in the development of multiple organ failure and one of the leading causes of death in sepsis. In the present study, ALI occurred to animals with LPS-induced sepsis characterized by increased lung inflammation and impaired alveolar endothelial and epithelial barriers. These were significantly improved with the treatment of 2% hydrogen. In addition, the inhalation of 2% hydrogen improved the survival rate in animals with sepsis. The above statements further support the protective action against sepsis by 2% hydrogen.

Inflammation is a beneficial response of the body for effective host defense. However, excessive inflammatory responses can cause tissue damage, fibrosis and eventual organ failure. To control the excessive inflammation, nature has developed different control mechanism in organisms, such as anti-inflammatory cytokines and antioxidant agents. Based on literature, a dysregulated, excessive proinflammatory cytokine expression contributes to the pathogenesis of sepsis [15]. In the present study, cytokine expression in BALF (IL-1β and TNF-α) were abnormally increased to animals with LPS-induced sepsis; which were inhibited by 2% hydrogen. In addition, 2% hydrogen promoted the increase of IL-10 in BALF from animals with LPS-induced sepsis, which was considered to be an anti-inflammatory factor [16]. These results suggest that 2% hydrogen protect sepsis and its consequent injury may be through the reduction of inflammatory response in sepsis, thus, improving sepsis and sepsis-induced lung injury.

To understand the molecular changes that occur during hydrogen treatment, we evaluated the M1 and M2 macrophages in ALI animals and LPS-induced BMDMs. Increased M2 macrophages was noticed with treatment with 2% hydrogen in ALI mice lung tissues. Moreover, treatment with 2% hydrogen showed significant reduction in the expression of M1 macrophage phenotypes (CD14 and CD40 double positive cells) and significant increment in the expression of M2 macrophage phenotypes (CD23 and CD124 double positive cells). These findings further support the previous observations of a distinct epigenetic reprogramming being switched on in favor of an anti-inflammatory subtype of Macrophages.

P38 MAPK is activated by a variety of cellular stresses including osmotic shock, inflammatory cytokines, LPS, Ultraviolet light, and growth

factors. P38 MAPK is a key regulator of inducible gene expression in the immune system, and is crucial to the signaling networks involved in sepsis. Our results also revealed that treatments of 2% hydrogen protected LPS-induced in vitro sepsis by inhibiting p38 activation and expression, which plays a key role in regulating immune response to infection. Previous studies have suggested that increasing P38 MAPK activity is associated with the development and mortality of sepsis [14]. The inhibition to p38 activation can reduce injury to organs resulting from sepsis, and improve survival for critically ill patients. These results indicate that the suppression of the hyperinflammatory phase in sepsis via the inhibition of the p38 signaling pathway contributes to the protective effects on sepsis through 2% hydrogen.

In summary, the administration of 2% hydrogen effectively protects against sepsis, demonstrating that 2% hydrogen is a safer, novel therapy for sepsis. Hence, our data provides a potential therapy for sepsis in clinic. We suggest that this protective mechanism was the inhibition of inflammation via the p38 MAPK/TNF signaling pathway. Our study contributes to a new therapeutic approach to improve sepsis and its relevant ALI, especially for the clinical treatment of sepsis.

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Disclosure of conflict of interest

None.

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