

# Hydrogen gas alleviates Lipopolysaccharide-induced Acute Lung Injury and inhibits inflammatory response

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## Research Article

**Keywords:** Lipopolysaccharide (LPS), acute lung injury (ALI), Hydrogen (H<sub>2</sub>), inflammation, Toll-like receptor 4 (TLR4), nuclear factor kappa-B (NF-κB)

**Posted Date:** March 23rd, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1456006/v1>

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# Abstract

**Background:** The two main causes of body inflammation caused by lipopolysaccharide (LPS) -induced acute lung injury (ALI) are chronic inflammation and oxidant / antioxidant imbalances. Hydrogen (H<sub>2</sub>), a gaseous molecule without known toxicity, could react with hydroxyl radical to remove the reactive oxygen species (ROS). Both in vivo and vitro studies support the protective effect of hydrogen on injuries caused by oxidative stress and inflammations.

**Methods:** In vivo study, mice were randomly divided into three groups: H<sub>2</sub> control group, LPS group and LPS+H<sub>2</sub> group. The mice were euthanized at the indicated time points and the specimens were collected. The 72h survival rates, cytokines contents, pathological changes, expression of Toll-like receptor 4(TLR4) and oxidative stress indicators were observed. In vitro study, according to different culture conditions, RAW 264.7 mouse macrophages were divided into the following groups: PBS group, LPS group and LPS+H<sub>2</sub> group. The cell Viability, intracellular ROS, cytokines and expression of TLR4 and nuclear factor kappa-B (NF-κB) were observed.

**Results:** In vivo, the 72h survival rate of hydrogen inhalation mice was increased to 80%, and H<sub>2</sub> significantly lighten LPS-induced lung damages and decreased inflammatory cytokine release. Besides, H<sub>2</sub> showed remarked anti-oxidative activity to reduce the MDA and NO contents in lung. In vitro, H<sub>2</sub> down-regulated the levels of ROS, NO, TNF-α, IL-6 and IL-1β in LPS-stimulated macrophages. Second, H<sub>2</sub> inhibited the expression of TLR4 and the activation of nuclear factor kappa-B (NF-κB). In summary, these findings supported that H<sub>2</sub> attenuates LPS-induced inflammatory responses, which may be mediated by TLR4-NF-κB pathway.

## 1. Introduction

Acute lung injury (ALI) is a severe disease syndrome, which pathogenesis is complicated with high morbidity and high mortality [1–3]. It is usually caused by inflammatory reactions, oxidative stress, apoptosis and autophagy, and the key to reducing the acute lung injury is unified to regulate the inflammatory response during lung injury [4]. Currently, the treatments such as glucocorticoids and surfactants stem cells have not significantly reduced lung damage and mortality [5–7]. Thus, novel therapeutic strategies and agents are of particular clinical significance.

Lipopolysaccharide (LPS) is an outer membrane of Gram-negative bacteria and a common inducing agent of ALI [8]. LPS activated the macrophages and inflammatory cells, which will result in the uncontrolled inflammatory cytokines release and activated / inhibited of multiple signal pathways [9]. A growing number of evidences has suggested that LPS-activated Toll-like receptors 4 (TLR4) which activated the NF-κB signaling pathway and resulted in the generation of inflammatory cytokines at last [10–13]. These cytokines play critical roles in the development of ALI by amplifying inflammatory responses [14]. Thus, targeted inhibition of TLR4 and downstream NF-κB signaling pathways, breaking the cascade of inflammation, may be a promising strategy for ALI.

It has been reported that inflammatory lung diseases are characterized by inflammation development and oxidant/antioxidant imbalance. The balance between the production and elimination of ROS maintained the functional integrity of redox-sensitive signaling [15]. Increasingly ROS activated redox-sensitive transcription factors, such as NF- $\kappa$ B, which regulated the chromatin remodeling and gene expression of pro-inflammatory mediators, thereby enhancing inflammatory responses and tissue injury [16–18]. In summary, we proposed that inhibition of the downstream NF- $\kappa$ B signaling pathway may be a useful strategy for scavenging ROS and treating ALI.

Hydrogen (H<sub>2</sub>) is a gaseous molecule without known toxicity, which could react with hydroxyl radical to remove the reactive oxygen species (ROS). Recent basic and clinical studies have revealed that hydrogen is an important physiological regulator that has antioxidant, anti-inflammatory and anti-apoptotic effects on cells and organs [19–22]. Research showed that, hydrogen in the human body will like other gaseous signaling molecules to act as a regulator of signal transduction, which has been proposed as ‘the fourth signal gas molecule’ [23, 24].

However, it is not clear that the H<sub>2</sub> improves the specific anti-inflammatory mechanism of LPS-induced ALI. The purpose of this study was to investigate the protective role of H<sub>2</sub> in LPS-induced lung injury and to explore its potential molecular mechanisms.

## **2. Material And Methods**

### **2.1 In vivo experimental design**

#### **2.1.1 Animals and Experimental design**

The total of 105 female C57BL/6 (6–8 weeks of age and weighing 19–22 g) mice were assessed in the present study. All mice were randomly divided into 3 groups (n = 35), namely the 42% hydrogen gas inhalation group (H<sub>2</sub> group), LPS-induced ALI group (LPS group) and ALI with 42% hydrogen inhalation group (LPS + H<sub>2</sub> group). The animals were kept in a humidity- and temperature controlled room under a 12-hour light/dark cycle with food and water ad libitum.

Ten mice were randomly drawn from each group to determine the 72h survival statistics, which used to assess the protective effect of H<sub>2</sub>. The other 75 mice were used for collecting other samples. The two ALI groups were induced by intraperitoneal injection of 10 mg/kg LPS. Treatments with inhalation of 42% hydrogen gas for 72h was administered after the injection of LPS or saline. Hydrogen was produced by a hydrogen-oxygen nebulizer (license No: AMS-H-03, Shanghai Asclepius Meditec Co., Ltd., Shanghai, China), it generates 3 L/min hydrogen gas by water electrolysis. As measured by gas chromatography, the gas generated consisted of 67% hydrogen and 33% oxygen. To keep the oxygen content at 21%, a certain amount of nitrogen will be passed in. Thus, the hydrogen mixed gas contains 42% hydrogen, 21% oxygen and 37% nitrogen (endotoxin free, purity > 99.9%) in air.

All experimental procedures were approved by the Experimentation Ethics Committee of the East Hospital, Tongji University School of Medicine in Shanghai, China (permit numbers: SCXK (Shanghai) 2017-0005). All mice were given access to water and food freely and there were no other experiments performed on these mice.

## **2.1.2 Cytokine measurements**

To measure the cytokines in the serum of mice, blood samples were collected from the eyeball at 3, 6, 12, 24, 48 and 72h after LPS injection. The serum was separated by centrifugation at 3000g for 15min (4°C) and then stored at -80°C. The corresponding enzyme-linked immunosorbent kit (CUSABIO BioTECH., Ltd., China) was used to detect the concentrations of IL-1 $\beta$ , TNF- $\alpha$ , IL-6, IL-10. All samples were measured in triplicate.

## **2.1.3 Measurement of MDA and NO levels in lung tissues**

Malondialdehyde (MDA) and nitric oxide (NO) are commonly used to represent the local or systemic oxidative stress. The lung tissues were collected at 6, 12, 24h after LPS injection and 10% lung tissue homogenates were separated the supernatant by 3000 g centrifugation for 20 min at 4°C. Subsequently, MDA and NO content in the supernatants was measured using MDA assay kit and NO assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

## **2.1.4 Histological examinations**

Lung tissue were collected at 6, 12, 24, and 48 hours after LPS injection and fixed in 10% neutral buffered formalin for 24 hours, and then embedded in paraffin. All specimens were cut into 5 $\mu$ m thick sections and stained with hematoxylin and eosin (HE). Samples were photographed and examined immediately by Leica DM Microscopes (DM 2500B, Germany,  $\times$ 200).

## **2.1.5 TLR4 expressions in lung tissues**

The specimens of 12h and 24h were cut into 5 $\mu$ m thick sections, then immersed in 3% H<sub>2</sub>O<sub>2</sub> for 25 min and rinsed with PBS. They were blocked with 10% normal rabbit serum for 30 min at room temperature and stained with anti-TLR4 primary antibody (1:500; Abcam) overnight at 4°C, followed by horseradish peroxidase or fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G antibody (1:5000; zhongshan bio.) for 50 min at room temperature. Sections were observed and photographed under a microscope (Eclipse Ti-SR; Nikon, Japan,  $\times$ 200).

## **2.2 In vitro experimental design**

### **2.2.1 Cell Culture and groups**

The mouse RAW 264.7 macrophage cells was purchased from American Type Culture Collection [ATCC], USA. Cell were cultured in a humidified incubator containing 5% CO<sub>2</sub> with Dulbecco's modified Eagle's medium (DMEM) medium (Gibco, Grand Island, NY) at 37°C, containing 100-IU/mL penicillin G, 100-IU/mL streptomycin, and 10% heat-inactivated fetal bovine serum. Similarly, the hydrogen mixed gas

contains 60% hydrogen, 21% oxygen, 5% carbon dioxide and 14% nitrogen(endotoxin free, purity > 99.9%) in air. Hydrogen was produced by a hydrogen-oxygen nebulizer (license No: AMS-H-03, Shanghai Asclepius Meditec Co., Ltd., shanghai, China).

The experiment consists of three groups: PBS group, LPS group and LPS + H<sub>2</sub> group. The cell of PBS group were cultured with normal DMEM medium and stimulated with PBS; the LPS group was cultured with normal DMEM medium and stimulated with LPS; the LPS + H<sub>2</sub> group was cultured with normal DMEM medium + 60% H<sub>2</sub> and stimulated with LPS.

## 2.2.2 Nitric Oxide Measurement

1 × 10<sup>5</sup>cell/ml of RAW 264.7 mouse macrophages were plated in 96-well plates. Second day, LPS + H<sub>2</sub> group and LPS group were pretreated with H<sub>2</sub> (60% H<sub>2</sub> + normal cell medium) or vehicle (normal cell medium) for 24h respectively and then all of cell incubated with LPS (200ng/mL) for another 24h. The concentration of nitric oxide (NO) in media was determined using the NO assay kit (Jiancheng Bioengineering Institute, Nanjing, China).

## 2.2.3 Cell viability assay

Cell viability was assessed by the Cell Counting Kit (Beyotime, China). In 96-well culture dishes, 1 × 10<sup>5</sup> macrophages in each well and cell were pretreated with 60% H<sub>2</sub> for 24h, and then stimulated with LPS (200ng/ml) or PBS for another 24h. Added 20 μl CCK-8 solution into each well and incubated at 37°C for another 4h. The cell viability was measured by microplate spectrophotometer (Thermo, USA) at OD450 nm.

## 2.2.4 Detection of Intracellular ROS

Intracellular ROS production was monitored by ROS assay kit (Solarbio, China). The density of 1 × 10<sup>5</sup>cell/ml of macrophages were seeded into 6-well and cell were pretreated with 60% H<sub>2</sub> for 24h, and then stimulated with LPS (1 μg/ml) or PBS for another 24h. Then, cell were exposed to serum-free medium containing 10-μM 2', 7'-Dichlorodihydrofluorescein diacetate (DCFDA). After 20 min of incubation in darkness, the cells were washed three times by blank DMEM medium. At last, cell were observed under the fluorescence microscope( OLYMPUS, IX71 ).

## 2.2.5 Real-time quantitative PCR

In 6-well culture dishes, 1 × 10<sup>5</sup> macrophages in each well and pretreated with 60% H<sub>2</sub> for 24h, and then stimulated with LPS (1 μg/ml) or PBS for another 24h. Total RNA was extracted by Trizol reagent (Invitrogen, USA), and reverse-transcribed by using Thermo Scientific RevertAid cDNA Synthesis Kit (Thermo, USA) to produce cDNA. The quantitative realtime PCR was performed with the ABI-7500 machine using SYBR Green PCR Kit (Thermo, USA).Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was used as an internal control. The quantitative PCR program used was as follows:

predenaturation (94°C, 10 min), denaturation (94°C, 20 sec), annealing (55°C, 20 sec), and extension (72°C, 20sec), using primers specific for GAPDH, IL-6, IL-1 $\beta$ , IL-10 and TNF- $\alpha$ .

Each sample was conducted in triplicate and the gene expression levels were calculated relative to the amount of GAPDH using the  $2^{-\Delta\Delta CT}$  method. The primer sequences for the tested genes are listed in Table 1.

## 2.2.6 Western Blot Analysis

All groups cell were collected and lysed in RIPA buffer (Beyotime, China) and the whole proteins, nucleoproteins, cytoplasmic proteins were extracted as required. All proteins were measured the concentrations by BCA protein assay kit (KeyGEN BioTECH, China).

Equal quantities of protein were separated on 12% SDS-PAGE, followed by electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, USA), then were blocked with 5% non-fat milk in TBST buffer for 2h at room temperature. The membranes were then incubated with the corresponding antibodies for overnight at 4°C. The corresponding antibodies contains anti-I $\kappa$ B, anti-pI $\kappa$ B, anti-TLR4 and anti-NF- $\kappa$ B(Abcam, Cambridge, UK).

On the other day, after washed three times, the membranes were incubated with the horseradish peroxidase-conjugated secondary anti-rabbit/mouse antibody for 1h at room temperature. The proteins were visualized using enhanced ECL detection kit (Dingguo changsheng biotechnology CO., Ltd., China) and scanned with a Clinx ChemiScope chemiluminescence imaging system (ChemiScope 5300 Pro). The relative optical densities of specific proteins were estimated utilizing a ChemiScope analysis program.

## 2.3 Statistical Analysis

Data was reported as the mean  $\pm$  SEM. All statistical analysis was performed using Prism 5.0 (GraphPad Software, USA). A significance level of 0.05 was considered to be significant for all calculations. #p or \*p represents p value < 0.05. ##p or \*\*p represents p value < 0.01. ###p or \*\*\*p represents p value < 0.001.

## 3. Results

### 3.1 Hydrogen inhalation improved the survival rate of ALI-mice

The 72h survival rates were recorded after experiments and analyzed by Log-rank (Mantel-Cox) test. As is shown in Fig. 1A, survival of H<sub>2</sub> group mice was 100%, the LPS group was 60% and the H<sub>2</sub> treatment group increased the survival rate to 80% (p < 0.05).

### 3.2 Hydrogen inhalation reduced the oxidative stress of ALI-mice

MDA and NO concentration was an index of lipid peroxidation. The levels of oxidative product (NO and MDA) in lung tissues were measured at 6, 12 and 24h after LPS administration. As shown in Fig. 2, NO and MDA of the H<sub>2</sub> treatment mice was decreased significantly (P < 0.01).

### **3.3 Hydrogen inhalation ameliorated inflammatory responses of ALI-mice**

Cytokines which promoted inflammation like TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and adjusted inflammation like IL-10 were chosen to represent the systematic inflammation level. Figure 3A, B and C represent TNF- $\alpha$ , IL-1 $\beta$  and IL-6 level in serum. The result showed the LPS induced TNF- $\alpha$ , IL-1 $\beta$  increase and the H<sub>2</sub> treatment group suppressed the increase markedly. However, the level of IL-6 gone near to the control group. Furthermore, Fig. 4D showed IL-10 level was significantly higher than the control group in a short time (< 24h).

### **3.4 Hydrogen inhalation attenuated the lung injury of ALI-mice**

Histological observation using H&E stain (Fig. 4). In the LPS mice, alveolar collapsed, alveolar septal thickened and amount of inflammatory cell infiltration also obviously in lung tissue. All of these histological examination showed LPS-induced oxidative stress and inflammations resulted in tissue injury, and consistent with the lipid peroxidation, cytokines results, whereas H<sub>2</sub> inhalation significantly prevented the histopathological changes caused by LPS.

### **3.5 Hydrogen inhalation reduced the expression of TLR4**

Immunohistochemistry was performed to explore the effects of H<sub>2</sub> on the expression of TLR4 in lung tissues. As shown in Fig. 5, H<sub>2</sub>-treated mice showed lower TLR4 expression.

### **3.6 Hydrogen reduced the cytokines release in LPS-stimulated Macrophages**

To assess the inhibitory role of H<sub>2</sub> on cytokine production, we used the quantitative real-time PCR assay to detect the TNF- $\alpha$ , IL-6, IL-1 $\beta$  and IL-10 mRNA expression in macrophages after LPS stimulation. Compared with the basal level without LPS stimulation, the contents of the four cytokines were significantly increased. At the same time, the TNF- $\alpha$ , IL-6 and IL-1 $\beta$  mRNA levels were all inhibited by H<sub>2</sub> (Fig. 6). These findings indicated that H<sub>2</sub> could neutralize the pro-inflammatory mediator effect of LPS, showing an anti-inflammatory effect.

Table 1  
The primer sequences of mouse GAPDH, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-10

Gene	Sequences
GAPDH-F	AGGTCGGTGTGAACGGATTTC
GAPDH-R	TGTAGACCATGTAGTTGAGGTCA
TNF- $\alpha$ -F	GAGTGACAAGCCTGTAGCC
TNF- $\alpha$ -R	CTCCTGGTATGAGATAGCAAA
IL-1 $\beta$ -F	GATCCACACTCTCCAGCTGCA
IL-1 $\beta$ -R	CAACCAACAAGTGATATTCTCCAT
IL-6-F	AGTCCGGAGAGGAGACTTCA
IL-6-R	ATTTCCACGATTTCCAGAG
IL-10-F	AGCCGGGAAGACAATAACTG
IL-10-R	CATTTCCGATAAGGCTTGG

### 3.7 Hydrogen increased the Cell viability and reduced the NO Level in RAW 264.7 Cells

After LPS stimulation, the cell status of LPS group became worse and the cell proliferation became slower compared with the PBS group (Fig. 7). When H<sub>2</sub> treatment was performed before LPS stimulation, the degree of cell damage was palliation, and the cell state was better than that of LPS group (Fig. 7A).

The RAW 264.7 + PBS group had good cell status and low NO content in the cell (Fig. 7B). After LPS stimulation, the cell status deteriorated and the NO content increased significantly ( $116.632 \pm 7.729$ ,  $p = 5.14E-14$ ,  $n = 9$ ). The amount of NO in the cells was significantly reduced after hydrogen treatment before stimulation ( $48.999 \pm 8.661$  vs  $116.632 \pm 7.729$ ,  $p = 7.56E-12$ ,  $n = 9$ ).

### 3.8 Hydrogen reduced the ROS production in LPS-Stimulated RAW 264.7 Cells

The RAW 264.7 + PBS group had good cell status and low intracellular ROS levels. After LPS stimulation, the cell status deteriorated and ROS levels increased significantly. When H<sub>2</sub> treatment was performed before LPS stimulation, intracellular ROS levels were significantly reduced (Fig. 8).

## 3.9 Hydrogen reduced the TLR4 expression and suppressed NF-κB activation in RAW 264.7 Cells caused by LPS

Compared with the control group, the expression level of IκB was significantly decreased after stimulation with LPS at 1 μg/ml, while the expression levels of phosphorylated IκB, TLR4 and NF-κB (cytoplasm and nucleus) were significantly increased (Fig. 9). After 60% H<sub>2</sub> treatment, the expression level of IκB was significantly increased, while the phosphorylated IκB, TLR4 and NF-κB (cytoplasmic and nuclear) expression levels were significantly reduced compared to the LPS stimulation group.

## 4. Discussion

Acute lung injury (ALI), is a serious inflammatory disease [1–3], and LPS is a key mediator of organ dysfunction and death associated with severe gram-negative infections [8], which is the most important pathogen causing ALI to occur [25, 26]. Intraperitoneal injection is a widespread method to rodents for administering drugs [27], and we established the ALI model caused by LPS (i.p., 10 mg/kg).

Recently, basic and clinical researches have shown that Hydrogen gas (H<sub>2</sub>) is an important physiological regulatory factor with antioxidant, anti-inflammatory and anti-apoptotic properties [28]. In the present study, we investigated the protective effects of H<sub>2</sub> treatment in LPS-induced ALI mice and the protective effect might be related to its ability of ameliorating the extent of oxidative stress and preventing the release of pro-inflammatory molecules [15–18]. H<sub>2</sub> inhalation can attenuate many kinds of lung injuries caused by ventilator, transplantation, hyperoxia, irradiation, and sepsis [29–32]. The histopathologic evaluation shows that, lung in the LPS group showed a thickened alveolar wall, edema and hemorrhage, less alveolar space and inflammatory cell infiltration after LPS stimulation. H<sub>2</sub> inhalation significantly prevented these histopathological changes caused by LPS. The H<sub>2</sub> treatment ameliorates LPS-induced lung neutrophil infiltration and inflammation.

To verify the success of the ALI model, we observed the pathological and inflammatory changes of different time points of mice after LPS injection intraperitoneally. The critical feature of ALI is the lung parenchyma injury and acute inflammatory process, including the release of inflammatory mediators such as TNF-α, IL-1β and IL-6 [33]. Proinflammatory cytokines appear in the early stages of inflammation, indicating the severity of ALI in a certain sense [34]. In this study, the inflammatory factors gradually increased at 6 h after LPS administration, which was significantly higher than that in H<sub>2</sub> groups at 24 h. These results suggest that the ALI model was successfully established after intraperitoneal injections of LPS, with the lung injury being obvious at 24h point and the H<sub>2</sub> markedly reduced the inflammation of ALI mice.

In the vivo study, we found that (1) hydrogen gas inhalation protected mice against ALI lethality (Fig. 1); (2) H<sub>2</sub> markedly reduced the inflammation and oxidative damages in ALI mice (Figs. 2 and 3); (3) H<sub>2</sub>

improved the lung injury caused by LPS stimulation significantly (Fig. 4); and (4) H<sub>2</sub> inhibited the TLR4 expression in lung tissues (Fig. 5). In conclusion, these results shows that molecular hydrogen alleviates LPS-induced ALI by reducing lung inflammation and oxidative damages, it may be associated with decreased NF-κB activity.

As well known that LPS causes the decrements of neutrophilic inflammation and pulmonary function, which act via Toll-like receptor 4 to induce the expression of inflammatory cytokines and chemokines [35, 36]. The mechanism of ALI injury may be due to bacterial endotoxin (LPS) activation, promoting the interaction of TLR4 and resulting in activation of NF-κB and release of TNF-α and IL-6 [37], all of these factors act a pivotal part in lung inflammatory damages. A lot of experiments have proved that inhibiting the activity of NF-κB in different models can alleviated the tissue damage and down-regulated release of cytokines [38, 39]. Therefore, inhibition of NF-κB activation will be an effective choice for protecting ALI.

To explore the beneficial effect of H<sub>2</sub> inhalation on LPS-induced ALI by inhibiting NF-κB activation, we raise the question: how can H<sub>2</sub> inhibit NF-κB activation? First, it is reported that H<sub>2</sub> can directly activate the NF-κB signaling through inhibiting the phosphorylation of IκB-α [40]. Second, research shows that H<sub>2</sub> can inhibit the activation of the NF-κB signaling pathway by scavenging the oxygen radicals [41]. Hydrogen gas, a potential antioxidant with rapid gaseous diffusion, is very effective in reducing cytotoxic free radical, such as reactive oxygen species(ROS) and mild enough to prevent interference with metabolic redox reactions or disrupt cell signaling. In our vitro experimental, (1) the elevations of TNF-α, IL-6 and IL-1β mRNA levels were suppressed by H<sub>2</sub> (Fig. 6); (2) H<sub>2</sub> alleviates the cell damages induced by LPS and reduces the ROS, NO level of cell (Fig. 7); (3) the intracellular ROS levels were significantly reduced in LPS + 60%H<sub>2</sub> group (Fig. 8); (4) H<sub>2</sub> inhibits TLR4 expression and NF-κB activation in macrophages caused by LPS(Fig. 9).

In this study, LPS administration induced a massive inflammatory cells and the release of cytokines in the serum, which were attenuated by H<sub>2</sub> inhalation treatment. Moreover, we assessed the lung oxidative damages by measuring the level of MDA and NO, the result shows that hydrogen gas decreased the lung oxidative damages caused by LPS. On the other hand, we found that H<sub>2</sub> dramatically inhibited the release of pro-inflammatory cytokine (TNF-α and IL-1β) of LPS-challenged mice and significantly up-regulated anti-inflammatory cytokines (IL-10). Previous studies reported that H<sub>2</sub> inhalation could inhibit the release of pro-inflammatory cytokines [42], however, there are no studies about its effects on anti-inflammatory cytokines.

## Conclusions

In conclusion, the vivo and vitro study demonstrate that inhalation of H<sub>2</sub> relieves LPS-induced ALI and downregulates TLR4-mediated NF-κB signaling pathway to inhibit inflammation.

## Declarations

## **Ethics approval and consent to participate**

All experimental procedures were approved by the Experimentation Ethics Committee of the East Hospital, Tongji University School of Medicine in Shanghai, China (permit numbers: SCXK (Shanghai) 2017-0005).

## **Consent for publication**

All authors read and approved the final manuscript. All authors consent to publication.

## **Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

## **Competing interests**

All the authors declare that they have no conflicts of interest.

## **Funding**

No funding was received.

## **Authors' contribution**

HY and YW contributed to the design and writing of this article. HY, YD and YF performed the experiments and acquired all data. SM and ZG conducted literature collection. All the authors participated in analysis and interpretation of data, critical revision and final approval of this article.

## **Acknowledgments**

This work was supported by the Shanghai Asclepius Meditec Co., Ltd, the project number is MR002-18-006.

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## Figures

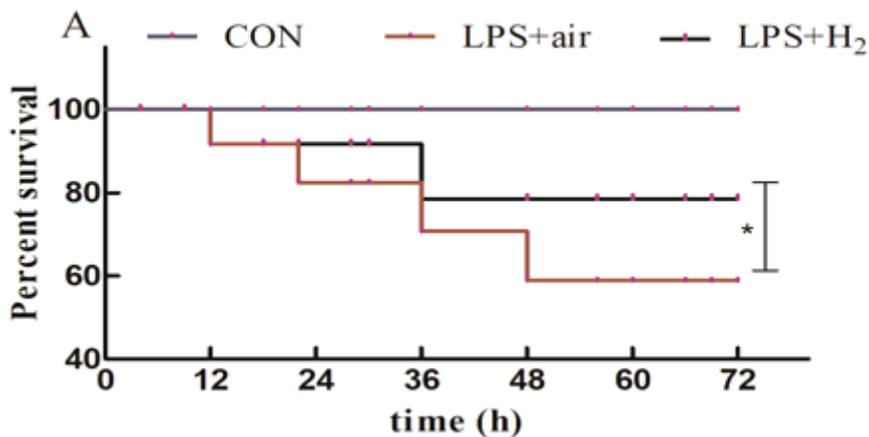
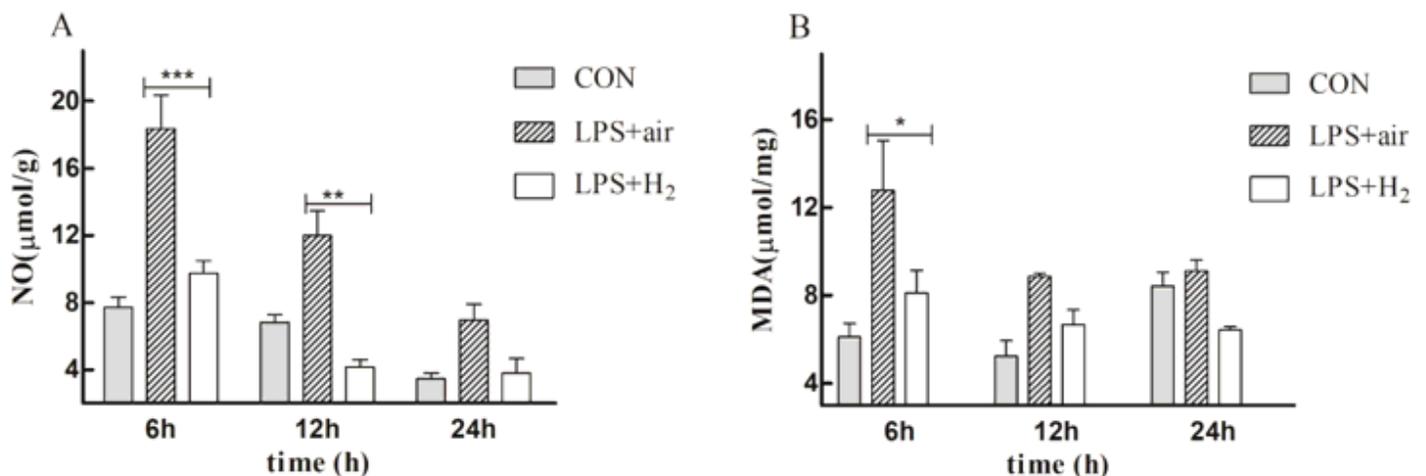


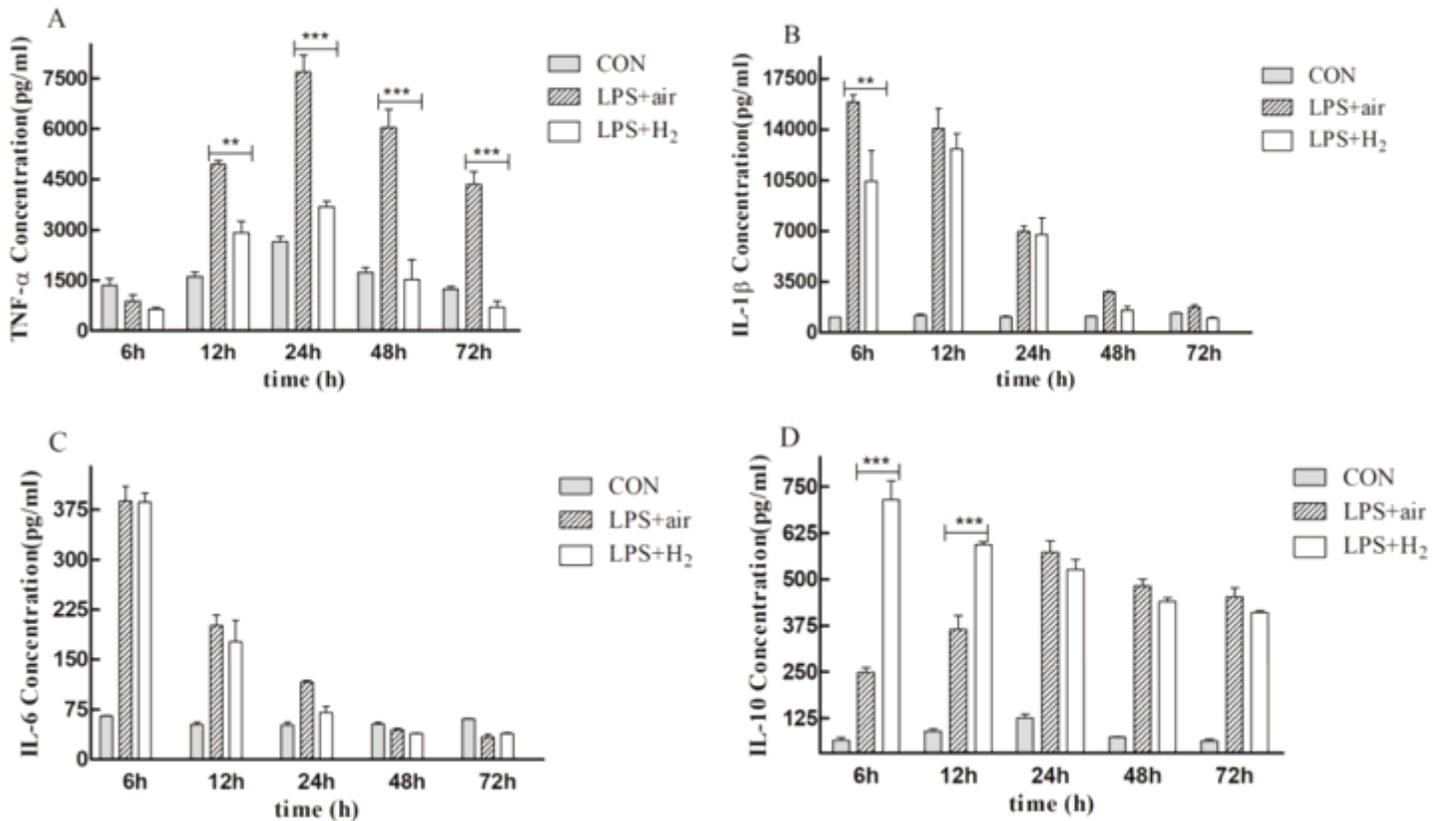
Figure 1

Effects of H<sub>2</sub> on the survival rate of mice. Results are expressed as percent survival, n=10. \*P < 0.05 values of LPS mice compared with the LPS+H<sub>2</sub> mice.



**Figure 2**

Effects of H<sub>2</sub> on MDA levels and NO content. H<sub>2</sub> treatment significantly attenuated the increased NO and MDA levels caused by LPS. (A) NO levels; (B) MDA levels. \*P < 0.05 and \*\*P < 0.01 values of LPS mice compared with the LPS +H<sub>2</sub> mice.



**Figure 3**

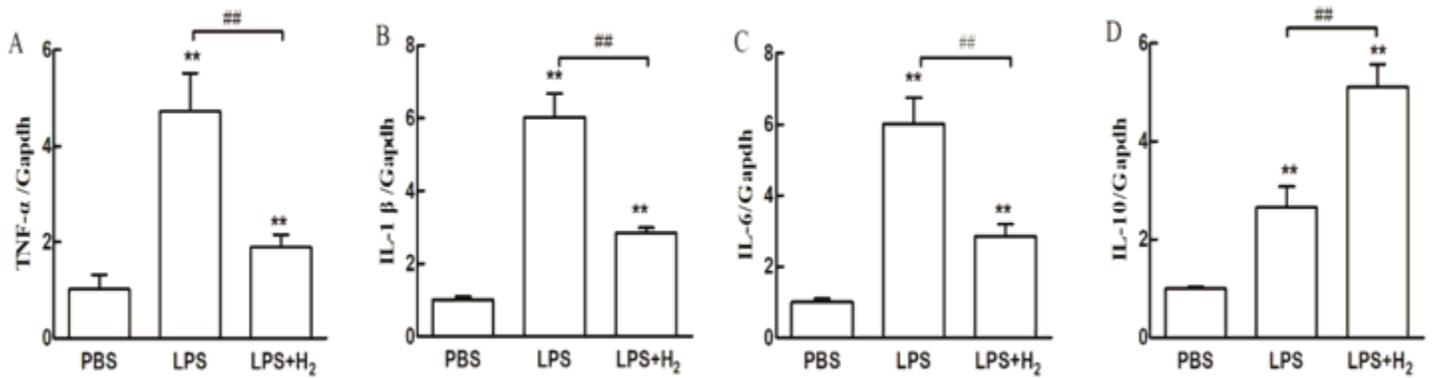
Effect of H<sub>2</sub> on inflammatory Cytokines in the serum of mice after LPS stimulation. (A) TNF-a; (B) IL-1β; (C) IL-6; (D) IL-10. \*P < 0.05, \*\*P < 0.01 and \*\*\*P < 0.001 values of LPS mice compared with the LPS +H<sub>2</sub> mice.

**Figure 4**

Effects of H<sub>2</sub> treatment on histological changes in lung of LPS-induced ALI mice (x200).

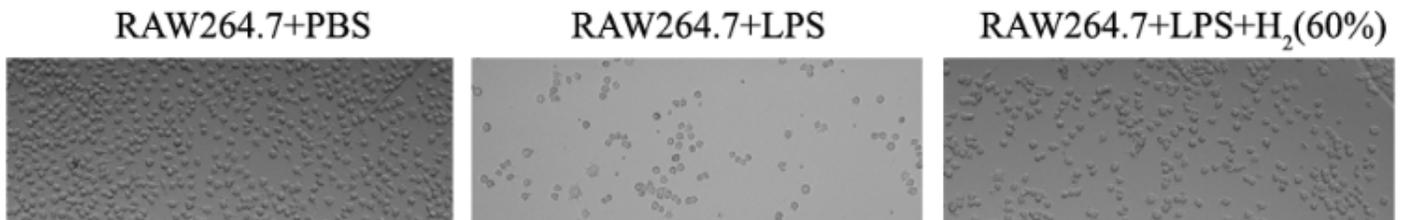
**Figure 5**

The expression of TLR4 in mice lung tissues analyzed by immunohistochemical staining in LPS and LPS+H<sub>2</sub> group (x200).



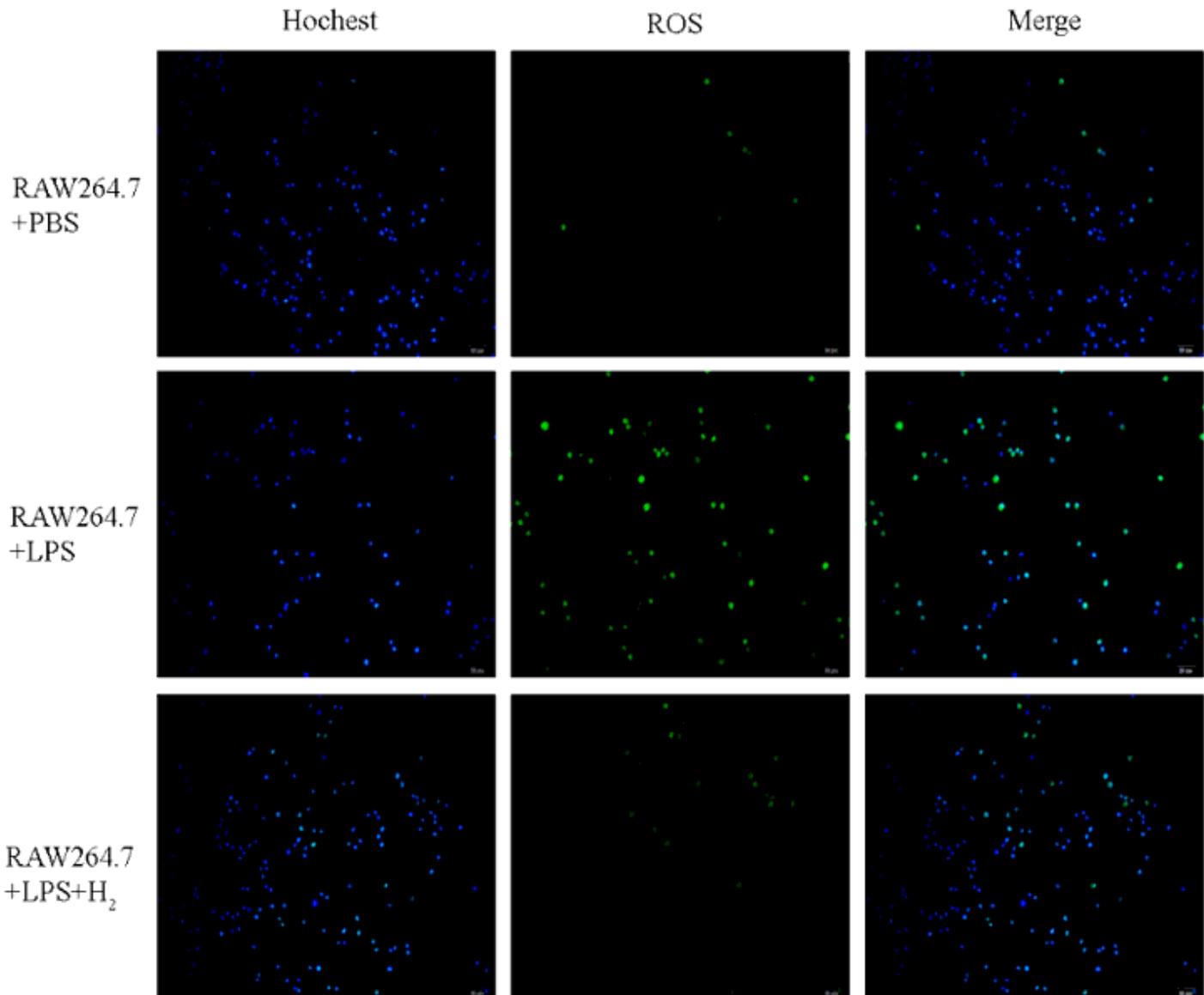
**Figure 6**

Effect of H<sub>2</sub> on inflammatory cytokines. (A) TNF-α; (B) IL-1β; (C) IL-6; (D) IL-10. \*\*P < 0.001 values of LPS +H<sub>2</sub> and LPS group compared with the PBS group. ##P < 0.001 values of LPS +H<sub>2</sub> group compared with the LPS group.



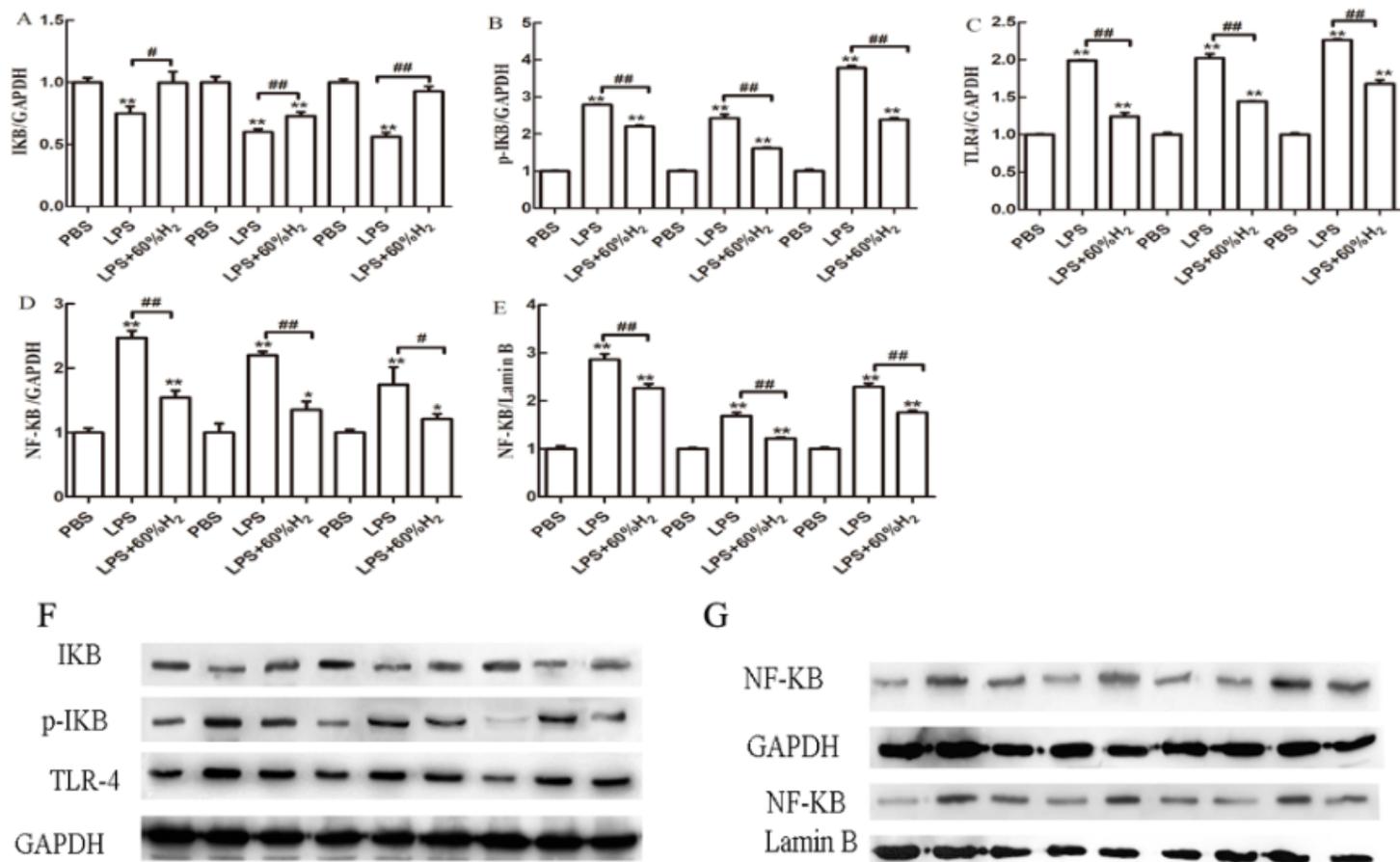
**Figure 7**

Cell status and the cell viability, NO level of different groups. (A) Cell viability; (B) NO levels. \*\*P < 0.001 values of LPS +H<sub>2</sub> and LPS group compared with the PBS group. ##P < 0.001 values of LPS +H<sub>2</sub> group compared with the LPS group.



**Figure 8**

The ROS level of RAW 264.7 +PBS group, RAW 264.7 +LPS group and RAW 264.7 +LPS+H<sub>2</sub> group.



**Figure 9**

$H_2$  inhibits the TLR4 expression and NF- $\kappa$ B activation. Expression of GAPDH (Proteintech) and Laminin B (Abcam) were internal controls. The values presented are the means  $\pm$  SEM of three independent experiments. ## $p$  < 0.01 compared to PBS group; \* $p$  < 0.05 and \*\*\* $p$  < 0.01 compared to LPS group.

## Supplementary Files

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