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Original Article

Hydrogen sulfide protects lungs of rats with sepsis possibly by influencing thrombomodulin signaling pathways

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Abstract: The aim of this study was to investigate hydrogen sulfide (H_2S) in lungs of rats with sepsis. Seventy-five rats were randomly divided into a sham operation group, acute lung injury group, and H_2S low, middle, and high dose groups. Expression of inflammatory medium and oxidative stress in lung tissues was observed and compared. Lung tissue structure of rats in the sham operation group was normal, but a significantly thickened alveolar wall, congested blood vessels, damaged alveoli, and large amount of serous fibrinous exudation and neutrophil infiltration were observed in rats in the acute lung injury group. Compared with the sham operation group, levels of TNF-α and IL-1β in lung tissues in the acute lung injury group significantly increased ($P < 0.05$). Also, MDA content significantly increased ($P < 0.05$) and SOD content significantly decreased ($P < 0.05$). MDA content in the H_2S low, middle, and high dose groups significantly decreased ($P < 0.05$) and SOD content significantly increased in a dose-dependent manner ($P < 0.05$). Expression of TM and EPCR proteins and mRNA significantly increased ($P < 0.05$) in the acute lung injury group. Expression significantly decreased in the H_2S low, middle, and high dose groups ($P < 0.05$). Pretreatment with H_2S can significantly alleviate the inflammatory response in lung tissues of rats with sepsis and may be used as a preventative drug for sepsis.

Keywords: Hydrogen sulfide, acute lung injury, sepsis, inflammatory response

Introduction

When the body suffers severe trauma, burns, and infections or undergoes major surgical operations and other stress conditions, the body's defense system can trigger a systemic inflammatory response. This results in the generation of a large quantity of inflammatory mediators and lipid peroxide. These substances damage normal tissues and cells and induce sepsis [1, 2]. Acute lung injury (ALI) is a complication that appears early, with the highest incidence in sepsis [3]. ALI is characterized by the massive release of pro-inflammatory cytokines and excessive infiltration of neutrophils. These damage alveolar epithelial cells, resulting in increased permeability, decreased intercellular space, water-sodium transfer transport, and other activities, even penetration of large amounts of proteins and cellular components into lung tissue spaces. These events result in pulmonary interstitial edema, alveolar collapse, and formation of pulmonary interstitial fibrosis [4, 5].

Recent studies have found that protein C (PC) system features anti-thrombotic, fibrinolytic, anti-apoptotic, and anti-inflammatory effects. It also protects endothelial cell function and improves microcirculation. The PC system consists of PC, protein S (PS), thrombomodulin (TM), and endothelial protein C receptor (EPCR), in which TM and EPCR can activate PC. TM is a glycoprotein that is primarily synthesized by vascular endothelial cells. Studies [6, 7] have shown that inflammatory response can cause organ injury when sepsis occurs in the body. TM is released from vascular endothelial cells into the blood to form soluble TM segments delaying or inhibiting activation of TM to PC.

Hydrogen sulfide (H_2S) is a gas signaling molecule existing in tissues and organs outside the

central nervous system. Actions of H_2S include regulating inflammatory response and vasodilation, inhibiting proliferation of smooth muscle cells, and protecting myocardial and brain cells [8]. In addition, H_2S protects against kidney injury and cerebropathy caused by sepsis [9]. However, its protective effects in the lungs are less clear. Therefore, this study was undertaken to produce a rat sepsis model, observe the protective effects of H_2S on lungs of rats with sepsis, and preliminarily investigate mechanisms of action of H_2S.

Materials and methods

Laboratory animals

Seventy-five SFP male SD rats (aged 3-4 months, body mass 200±30 g) were purchased from Animal Experimental Center of the Medical College. Sodium hydrosulfide (NaHS) was purchased from Sigma, USA. Primers were designed and synthesized by Shanghai Sangon and an EUSA kit was provided by Wuhan Boster Company. Myeloperoxidase (MPO) kit was provided by Nanjing Jiancheng Biological Engineering Institute.

Animal grouping

Seventy-five rats were randomly divided into 5 groups, including a sham operation group, acute lung injury group, and H_2S low, middle, and high dose groups. There were 15 rats in each group. Rats in the control group underwent a sham operation. This consisted of opening the abdominal cavity, finding the cecum but not ligating it, and reincorporation the cecum back into the abdominal cavity. The remaining groups underwent surgery using the cecal ligation and puncture (CLP) method to produce the sepsis animal model. Additionally, rats in the H_2S low, middle, and high dose groups were intraperitoneally injected with NaHS at doses of 0.78, 1.56, and 3.12 mg/kg, respectively, 30 minutes before cecal ligation.

Model preparation

The rat sepsis model was prepared using the CLP method, as reported in the literature [10]. Rats were anesthetized by intraperitoneal injections of 0.3% pentobarbital sodium at a dose of 1 mL/100 g, after being fasted for 24 hours. After complete anesthesia, rats were immobilized on the operating table in a supine position and the skin was disinfected and prepared. Next, a medioventral line incision of approximately 1-1.5 cm was made and the abdominal cavity was explored to find the cecum. The cecum was then removed from the abdominal cavity and ligated at approximately half the distance from the root segment with No. 4-0 silk thread, cut through, and punctured three times with a No. 18 needle. The cecum was reincorporated into the abdominal cavity after the overflowing of a little content was observed. The abdominal incision was sutured layer by layer. Immediately, 10 mL of normal saline was intraperitoneally injected to prevent shock. Rats were then placed back into the cages and allowed food and drink.

Lung coefficient and lung wet/dry weight

Survival was monitored for 7 days and the rats were sacrificed with diethyl ether on postoperative day 7. Chests were opened and lung tissues were removed and weighed. In addition, inferior lobe tissues of the right lungs were isolated and wet weights were measured. Tissues were then dried in the oven at 75°C and weighed to calculate lung coefficient (lung mass/body mass × 100%) and lung wet/dry weight ratio (wet weight/dry weight).

Lung histomorphological observation

Upper-middle lobe tissues of the right lungs were fixed with 10% paraformaldehyde for 24 hours, embedded with paraffin, and stained with H&E. Histopathological changes were observed under a light microscope. In addition, 10 visual fields were selected under 200× magnified visual fields to calculate the ratio of alveolar injury (Index of quantitative assessment of histological lung injury, IQA, %) and ratio of the number of injured alveoli containing 2 or more erythrocytes and (or) neutrophils to the total number of injured alveoli. Extent of lung injury was quantitatively evaluated.

Expression of inflammatory medium in lung tissues

One hundred milligrams of right lung tissues were used to produce 5% tissue homogenate on ice. These were used to evaluate changes in TNF-α and IL-1β levels in lung tissues using enzyme linked immunosorbent assay.

Table 1. Comparison of lung coefficient, lung wet/dry weight ratio, and IQA of rats in each group

Groups	Lung coefficient	Lung wet/dry weight ratio	IQA (%)
Sham operation group	0.36±0.06	4.23±0.29	14.81±2.16
Acute lung injury group	0.75±0.12※	5.39±0.79※	48.98±4.85※
Hydrogen sulfide low dose group	0.59±0.07※,△	5.01±0.46※,△	29.51±6.33※,△
Hydrogen sulfide middle dose group	0.51±0.07※,△	4.78±0.32※,△	27.01±3.46※,△
Hydrogen sulfide high dose group	0.47±0.07※,△	4.31±0.16※,△	21.57±2.16※,△

Note: Compared with sham operation group, ※$P < 0.05$; Compared with acute lung injury group, △$P < 0.05$.

Oxidative stress of lung tissues

One hundred milligrams of right lung tissues were used to produce 5% tissue homogenate on ice and determine MPO and SOD levels, according to manufacturer instructions.

Evaluation of expression of TM and EPCR using fluorescent quantitative PCR

One hundred milligrams of lung tissues were preserved and total RNA was extracted from lung tissues using the TRIzol method. cDNA was synthesized, according to manufacturer instructions, after the purity was tested. Next, samples were loaded to perform amplification using fluorescent quantitative PCR. Reaction conditions were as follows: pre-degeneration at 95°C for 2 minutes, degeneration at 95°C for 30 seconds, tempering at 58°C for 30 seconds, extension at 72°C for 45 seconds, and extension at 72°C for 10 minutes after 30 circulations. Primer sequence: TM, upstream 5' TCC CTG TTC TGG AGG ACT CAG-3', downstream 5'-GCC ACC TTG GTC TCT GGA GTA-3', size 309 bp; EPCR, 5'-CGACGT GGT CTT TCC TCT GAC-3', 5'-TCA GGA TAC CCA GGA CCA GTG-3', size 341 bp; β-actin: upstream 5'-CGTTGACATCCGTAAAGACCTC-3', downstream 5'-TAGGAGCCAGGGCAGTAATCT-3', size 110 bp. After amplification, 2% agarose gel electrophoresis (AGE) was used to calculate relative expression of genes with the ratio of target genes to gray value of β-actin.

Evaluation of expression of TM and EPCR in lung tissues using western blotting

Lung tissues of the rats were preserved, tissue lysate was added, and total protein was extracted and purified. Total protein was quantified using the BCA method. Briefly, 200 μg of sample was injected and SDS-polyacrylamide gel electrophoresis was used to transfer the membrane of the target band. The membrane was sealed with skim milk powder at 4°C overnight and anti-TM and anti-EPCR antibody was added at a proportion of 1:1000. Next, the membrane was incubated for 2 hours at room temperature. A corresponding secondary antibody was added after the membrane was cleaned. ECL developing agent was used after incubation to analyze expression of target proteins using an image analysis system.

Statistical analysis

SPSS19.0 was used to analyze data. Measurement data are represented by ($\bar{x} \pm s$) and analyzed using one-way variance. *t*-test was used for comparison among two groups. Values of $p < 0.05$ are considered statistically significant.

Results

Comparison of lung coefficient, lung wet/dry weight ratio, and IQA in each group of rats

Compared with the sham operation group, lung coefficient, lung wet/dry weight ratio, and IQA were significantly higher in the acute lung injury group ($P < 0.05$). These were significantly and dose-dependently less in the H_2S low, middle, and high dose groups ($P < 0.05$; **Table 1**).

Lung histopathological observation

Lung tissue structure of rats in the sham operation group was normal. The alveolar wall was complete and continuous. However, a significantly thickened alveolar wall, congested blood vessels, damaged alveoli, and a large amount of serous fibrinous exudation and neutrophil infiltration were observed in rats in the acute lung injury group. In comparison, alveolar septum of rats in all H_2S dose groups was found to be narrowed, congestion of blood

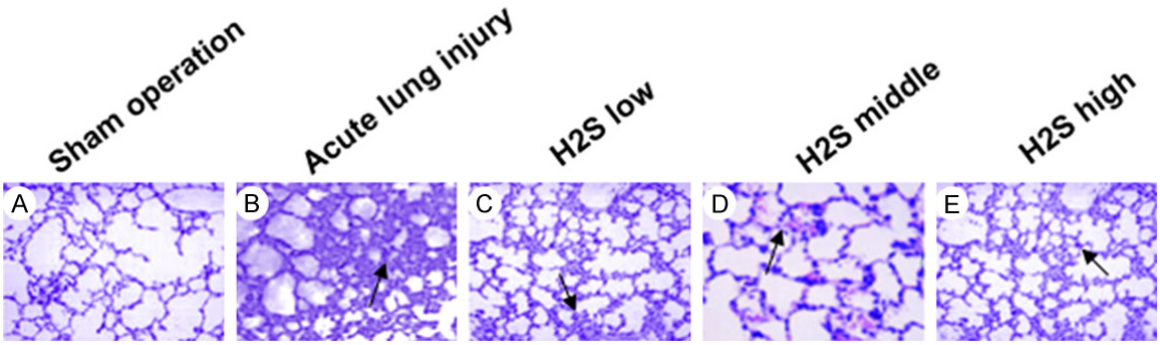

Figure 1. Lung histopathological observation in each group of rats (HE, 200×).

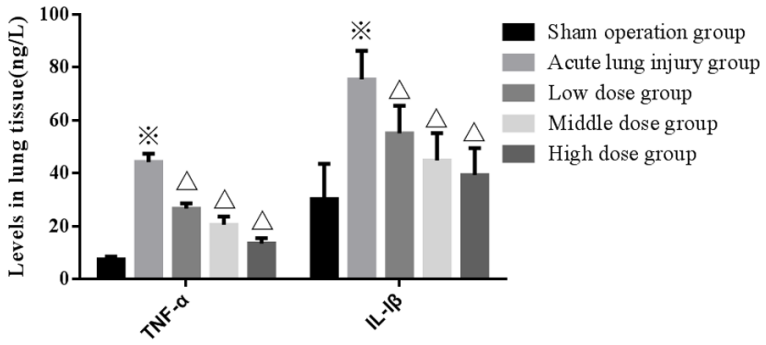

Figure 2. Changes in TNF-α and IL-1β levels in lung tissues of rats in each group. Compared with the sham operation group, ※$P < 0.05$; Compared with the acute lung injury group, △$P < 0.05$.

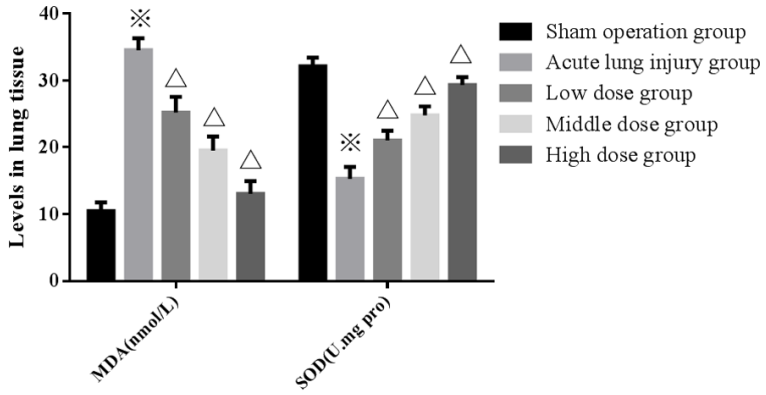

Figure 3. Comparison of MDA and SOD content in lung tissues of rats in each group. Compared with the sham operation group, ※$P < 0.05$; Compared with the acute lung injury group, △$P < 0.05$.

vessels was not obvious, and inflammatory exudation and neutrophil infiltration were found to have been alleviated (**Figure 1**).

Changes of TNF-α and IL-1β levels in lung tissues in each group of rats

Compared to the sham operation group, TNF-α and IL-1β levels in lung tissues of the acute lung injury group significantly increased ($P < 0.05$). These were significantly and dose-dependently decreased in the H_2S low, middle, and high dose groups ($P < 0.05$; **Figure 2**).

Oxidative stress conditions of lung tissues in each group of rats

Compared to the sham operation group, MDA content in lung tissue of the acute lung injury group was significantly higher ($P < 0.05$) and SOD content was significantly less ($P < 0.05$). MDA content in lung tissues of the H_2S low, middle, and high dose groups was significantly less ($P < 0.05$) and SOD content was significantly and dose-dependently higher ($P < 0.05$; **Figure 3**).

TM and EPCR protein and mRNA expression in each group of rats

Compared to the sham operation group, TM and EPCR protein and mRNA expression in lung tissues of the acute lung injury group were significantly higher ($P < 0.05$), whereas these were significantly less in the H_2S low, middle, and high dose groups ($P < 0.05$; **Figures 4**-**6**; **Table 2**).

Discussion

H_2S is a gaseous signal molecule and scholars have differing opinions regarding the effects

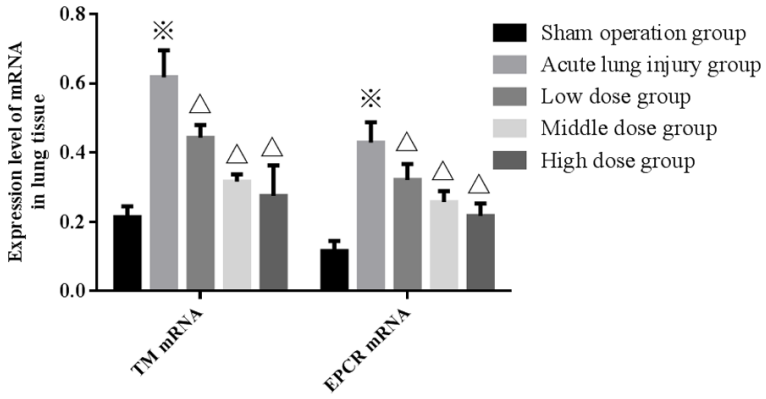

Figure 4. TM and EPCR mRNA expression in each group of rats. Compared with the sham operation group, ※$P < 0.05$; Compared with the acute lung injury group, △$P < 0.05$.

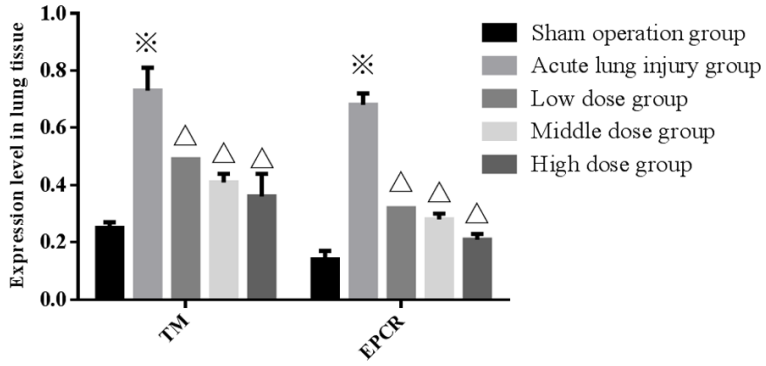

Figure 5. TM and EPCR protein expression in each group of rats. Compared with the sham operation group, ※$P < 0.05$; Compared with the acute lung injury group, △$P < 0.05$.

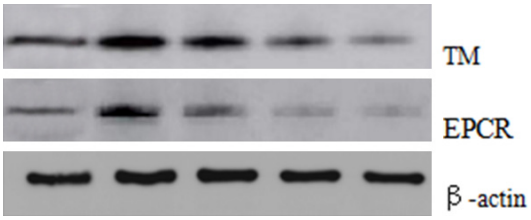

Figure 6. TM and EPCR protein expression in each group of rats.

of H_2S in sepsis. One study [11] indicated that endogenous H_2S significantly increased infectious or endotoxic shock, damaging the body's hemodynamics and aggravating inflammatory response during sepsis. However, administration of PAG, an inhibitor of endogenous H_2S, reduced endogenous H_2S, alleviated inflammatory response, and decreased death rate. Other studies [9, 12] have indicated that administration of exogenous NaHS significantly improves chemotaxis of leukocytes caused by sepsis, reducing elevated blood pressure, inhibiting inflammatory injury of lung tissues, and enhancing antioxidation. These indicate that exogenous H_2S has good anti-inflammatory effects. However, specific mechanisms remain unknown.

In the present study, exogenous NaHS was used to pretreat rats with sepsis to evaluate protective effects on the lungs of rats. Results indicated that lung coefficient, lung wet/dry weight ratio, and IQA of rats in the acute lung injury group were significantly higher than those of the rats in the sham operation group. Typically, lung injuries can be observed histopathologically. Lung coefficient, lung wet/dry weight ratio, and IQA of rats in the H_2S low, middle, and high groups were significantly and dose-dependently lower. In addition, lung injury was significantly reduced as indicated by histopathological observation, indicating that pretreatment with exogenous H_2S protected against secondary lung tissue injury in rats with sepsis. Compared to the sham operation group, TNF-α, IL-1β, and MDA levels in lung tissues of the acute lung injury group significantly increased and SOD content significantly decreased. TNF-α, IL-1β, and MDA levels in lung tissues of the H_2S low, middle, and high groups significantly decreased and MDA content significantly decreased in a dose-independent manner. This indicated that administration of exogenous H_2S significantly decreases secretion of inflammatory mediators in lung tissues of rats with sepsis and inhibits inflammatory injury caused by oxidative stress response, thereby playing an important role in lung protection.

Recent studies [13-15] have indicated that internal environment disorders caused by inflammation, coagulation and fibrinolytic imbal-

Table 2. mRNA expression of TM and EPCR in each group of rats

Group	TM mRNA	EPCR mRNA
Sham operation group	0.214±0.031	0.116±0.029
Acute lung injury group	0.618±0.078※	0.429±0.059※
Hydrogen sulfide low dose group	0.443±0.037※,△	0.321±0.046※,△
Hydrogen sulfide high dose group	0.316±0.021※,△	0.257±0.032※,△
Hydrogen sulfide high dose group	0.0275±0.088※,△	0.217±0.036※,△

Note: Compared with sham operation group, ※P < 0.05; Compared with acute lung injury group, △P < 0.05.

ance, and endothelial cell injuries are fundamental factors in sepsis progression. The PC system plays an anti-inflammatory, anti-coagulation, fibrinolytic promotion, and endothelial cell regulation role, directly affecting inflammatory response and coagulation disorders during sepsis [16, 17]. TM and EPCR are key components of the PC system. TM is a transmembrane glycoprotein primarily expressed in endothelial cells that binds with high affinity to thrombin, thereby promoting activation of the PC system. Studies [18, 19] have shown that TM has both anti-coagulation and anti-fibrinolytic effects, directly affecting the coagulation process, and expression of TNF-α, IL-1β, and other inflammatory mediators. Expression of inflammatory mediators increases the onset of sepsis, downregulating expression of TM and resulting in coagulation disorders. In addition, TM also has a direct anti-inflammatory effects which ensure cell function and integrity of connection among cells, inhibit adhesion and migration of inflammatory cells, activate NF-κB and MAPK signaling pathways, reduce generation of inflammatory mediators, alleviate vascular endothelial cell injury, and decrease sensitivity of the body to endotoxins [20].

EPCR is an important component of the PC system, playing a role in activating PC [21, 22]. EPCR includes conjunction-type EPCR and free EPCR (sEPCR), both of which have a high affinity for PC/activated PC (APC). However, these have different biological effects, namely sEPCR inhibits activation of PC, which inhibits the anticoagulation activity of APC. Conjunction-type EPCR enhances the activity of PC and promotes anticoagulation. Conjunction-type EPCR starts the activation of PC by binding with T-Tm compound and improves the activity of PC synergistically with TM. Studies [23, 24] have shown that anticoagulation activity of the PC system increases by approximately 10-fold under the mediation of EPCR. In addition, EPCR combines with APC to play anti-inflammatory and anti-apoptotic roles. Therefore, internal environment disorders caused by the combined action of EPCR and TM accelerate the deterioration of sepsis.

Results of this study indicated that, compared with the sham operation group, TM and EPCR protein and mRNA expression in lung tissues in the acute lung injury group significantly increased ($P < 0.05$). These significantly decreased in the H_2S low, middle, and high dose groups ($P < 0.05$). Results indicated that H_2S significantly decreased expression of TM and EPCR in lung tissues of rats with sepsis and alleviated inflammatory injuries of membrane-bound TM and EPCR to lung tissues. This may be a mechanism by which H_2S prevents and cures acute lung injury during sepsis.

In conclusion, pretreatment with H_2S significantly alleviates inflammatory response in lung tissues of rats with sepsis, possibly mitigating inflammatory damage to lung tissues by regulating the coagulation-inflammation network. Therefore, H_2S may be useful as a preventive drug for acute lung injuries in sepsis. However, because this study was of short duration, specific action pathways of the coagulation-inflammation network were not evaluated in depth, but should be further investigated in the future.

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Disclosure of conflict of interest

None.

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