Original Article
Effects of inhaled hydrogen sulfide on smoke inhalation-induced acute lung injury in rats

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Abstract: The study aimed to observe the effects of inhaled hydrogen sulfide on smoke inhalation-induced acute lung injury in an animal model. In this study, male Sprague-Dawley (SD) rats were divided into intervention and control groups. The intervention group was divided into three subgroups: smoke group (n=6), smoke+H_2S 3 h group (n=6), and smoke+H_2S 6 h group (n=6). A control group (n=6) received treatment with fresh air only. After exposure to treatment conditions and blood gas analysis, intervention and control group rats were sacrificed and pathological changes in pulmonary tissue were observed under a light microscope. Enzyme-linked immunosorbent assay (ELISA) was used to analyze the levels of malondialdehyde (MDA), inducible nitric oxide synthase (iNOS), and the p65 subunit of nuclear factor kappaB (NF-κBp65) in homogenized lung tissue. The expression of iNOS mRNA in homogenized lung tissue was analyzed using fluorescence quantitative polymerase chain reaction (PCR). In the smoke+H_2S 3 h and smoke+H_2S 6 h groups, the sum integrated optical density of NF-kBp65 and MDA, the relative expression of iNOS mRNA, and the concentrations of NF-kBp65, iNOS, and nitrogen monoxide (NO) in lung tissue decreased significantly compared to the smoke-only group. No significant differences in lung tissue pathology, blood oxygen analysis, lung wet-dry weight ratio, MDA level, and other indicators were present between the smoke+H_2S 3 h group and smoke+H_2S 6 h group. Inhaled hydrogen sulfide shows a significant protective effect on cotton smoke inhalation-induced pulmonary injury, and it can ameliorate oxidative stress and tissue inflammation.

Keywords: Acute lung injury, smoke inhalation, cotton

Introduction

The pathogenesis of smoke inhalation injury is complicated by the complex composition of smoke [1-3]. It is thought that particles and toxic substances in smoke are the main causes of the pathological and physiological changes of smoke inhalation lung injury. The interaction between particles and toxic substances and lung parenchyma can stimulate the inflammatory cascade, which may lead to pulmonary edema, airway remodeling and obstruction, hypoxic pulmonary vasoconstriction dysfunctions, and disturbance of the ventilation-perfusion ratio.

Oxidative stress and inflammation response are two key points in smoke inhalation lung injury. Smoke inhalation can activate pulmonary macrophages, neutrophils, endothelial cells, and vascular smooth muscle cells, and cause the release of high levels of cytokines and chemokines. Cytokines can activate nuclear factor kappaB (NF-κB), which induces the synthesis of inducible nitric oxide synthase (iNOS) [4]. iNOS catalyzes the production of large amounts of nitrogen monoxide (NO), and it may also synthesize superoxide under conditions of substrate or cofactor limitation [5-9]. In addition, the reactive oxygen species (ROS) produced by smoke inhalation acute lung injury/acute respiratory distress syndrome (ALI/ARDS) can lead to an excess of NO synthesis. NO is an important oxygen-derived free radical that can cause vascular leakage and hypoxic pulmonary vasoconstriction dysfunction, thus aggravating ALI/ARDS.

Endogenous hydrogen sulfide (H_2S) plays a role in inflammation regulation, anti-oxidative stress vasodilatation, and anti-fibrosis; and it participates in endocrine regulation and reproductive function [10-12]. In a rat model in which ALI/ARDS was induced by various factors (including
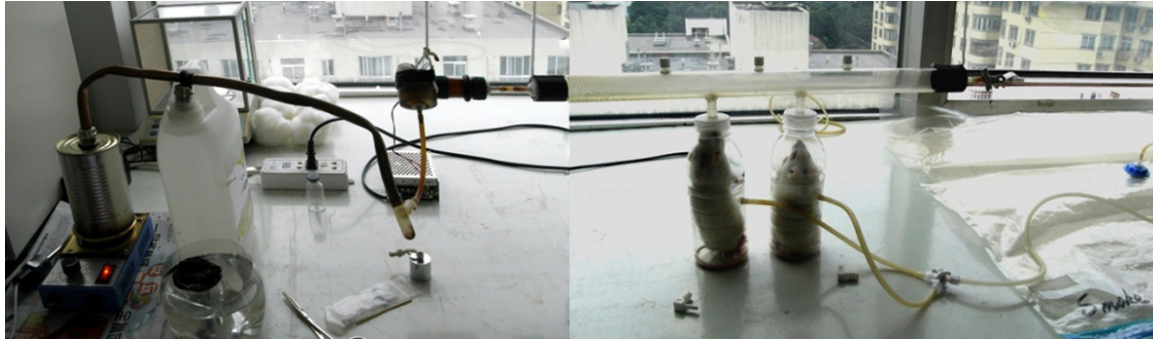
Figure 1. The smoke inhalation device in this study.

lipopolysaccharide injection, cabin explosion, lung or limb ischemia-reperfusion injury, and acute pancreatitis), exogenous H_2S can reduce injury to different degrees [13, 14]. Systemic inflammatory response can be inhibited by inhalation of H_2S gas in a rat model in which ALI/ARDS was induced by endotoxin, leading to improvement in survival time [15]. Lung inflammation and apoptosis of alveolar epithelial cells can also be inhibited by inhalation of H_2S gas, thus protecting the lungs of rats in which ALI/ARDS was induced by hyperventilation. Inhaling H_2S gas at a rate of 300 ppm after 60% trauma hemorrhage can improve the survival of rats, while inhalation of 100 ppm H_2S gas for 6 hours in rats injured by blunt trauma can reduce metabolic consumption and alleviate inflammatory response after trauma [16, 17]. In this study, we describe the intervention effect of H_2S gas (80 ppm) inhalation for 3 hours and 6 hours on smoke inhalation-induced acute lung injury in rats, and discuss the molecular mechanisms related to this process.

Materials and methods

Materials

The smoke inhalation device is showed in **Figure 1**. It consists of the following components: titanium solder pot with adjustable temperature (Fudi Brand HT-B, 150 W power, 50-mm inner diameter, 30-mm depth, made by Guangdong Hongtai Electronics Hardware Tools Factory); digital thermometer (Honghai Brand XMT-280, made by Beijing Honghai Yongchang Instrument Technology Development Center, measurement range: -50 to 500 degrees Celsius; smoke collecting chimney (self-made, 70 mm inner diameter, 110 mm height; the top of the chimney is connected to a cooling pipe, while the bottom stays opened; 3 holes with a diameter of 5 mm at one side wall of the chimney (5 mm from the bottom) are opened at equal distance for inhaling air during burning); cooling pipe (copper, 10 mm inner diameter, 1 mm wall thickness, 500 mm length, with interfaces at each side); fan (self-made, Nidec Brand GAMMA26, 24 V, 0.11 A, the air inlet and outlet are closed and then connected with the smoke guiding tube, as shown in **Figure 1**); AC to DC power supply (MS-50-24, 220 V to 24 V, made by Hongkong Mingwei Electronic Technology Co., Ltd); cotton (Sunshine Pure Cotton Brand, long-staple cotton from Xinjiang); electronic balance (JD100-3, made by Shenyang Longteng Electronic Co., Ltd); smoke distribution tube (self-made acrylic tubes, 30 mm diameter, 2 mm wall thickness, 500 mm length, joints with a 3 mm inner diameter are located at each side of the smoke distribution tube, and two 8 mm holes are opened at one side of the tube wall with equal distance to connect the joints); smoke collecting bottle (self-made round plastic bottle with 62 cm inner diameter, 180 mm height, the bottle is cut in the middle, and holes are opened in the top part to connect with the smoke distribution tube, while holes in the bottom part are connected by soft tubes with the waste gas bag); waste gas bag (self-made collecting bag, 80×90×40 cm, joints with 3 mm-inner diameter are placed at the opening of the bag).

Animal model

All studies were conducted with the approval of the experimental animal center of Chinese Academy of Military Medical Sciences (SC-XK-(Army)-2012-0004). According to previous studies [18, 19], the experiments were conducted on 24 healthy and clean male Sprague-

Dawley rats (weighing 150-250 g each). This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of PLA Navy General Hospital. Rats were divided into four groups with 6 rats in each: control group, smoke group, smoke+H_2S 3 h group, and smoke+H_2S 6 h group. Then, a total of six rats were selected randomly from smoke group, the smoke+H_2S 3 h group, and the smoke+H_2S 6 h group (two rats from each group) respectively, and placed in the smoke collecting bottle. After the solder pot was heated to 300°C, 2 g of cotton was added to the pot and the smoke collecting chimney was immediately closed, and the fan opened. When the rats exhibited symptoms such as plantar skin turning cherry or purple red with dysphoria, ecphysesis, buccal respiration, slow or profound respiration, wheezing, or the two minute time limit had elapsed, smoke was stopped and the bottle opened so that the lungs of the rats could take in fresh air. The steps mentioned above were repeated 3-5 times, and the process ended when the rats remained unconscious after 7 min of taking in fresh air.

After smoke inhalation or smoke inhalation simulation, rats in the smoke+H_2S 6 h group were given continuous inhalation of 80 ppm H_2S and 30% oxygen for 6 h; rats in the smoke+H_2S 3 h group were given continuous inhalation of 80 ppm H_2S and 30% oxygen for 3 h, followed by 30% oxygen for 3 h; and rats in the control and smoke groups were given continuous inhalation of 30% oxygen for 6 h. During the experiment, rats could eat and drink freely.

Blood gas analysis

All rats were removed from the smoke environment, placed in a room for breathing fresh air (with normal diet), and given a hypodermic injection with 2 ml of normal saline. Rats in the smoke+H_2S 3 h and smoke+H_2S 6 h groups were given intraperitoneal injection with 50 mg/kg of pentobarbital sodium after inhaling H_2S for 3 h and 6 h, respectively. Arterial blood (1.5 mL) was extracted from the abdominal aorta after anesthesia, and after the blood PH

value, arterial partial pressure of oxygen (PaO$_2$), and partial pressure of carbon dioxide (PaCO$_2$) were tested, the oxygenation index (PaO$_2$/FiO$_2$=PaO$_2$/0.21) was calculated. Rats in the control and smoke groups did not inhale H_2S.

Analysis of bronchoalveolar lavage fluid (BALF) by ELISA

Rats were sacrificed after extracting arterial blood, and their tracheas were separated and occluded by thoracotomy in the midline. Entire lungs were completely removed without great vessels, and the right main bronchi were ligated. The left lungs were lavaged by a silicone tube (length: 50 mm, 22 g) with 2 ml of cold stroke-physiological saline solution. Then, 1.5-1.8 ml of stroke-physiological saline solution was retrieved and centrifuged (1500 r/min) for 10 min at 4°C, and the supernatant was obtained and tested by ELISA.

Management of lobi pulmonis

The lobes of the right lung were separated and the upper and inferior lobes frozen in cryovials. The middle lobe was infused in 4% polyformaldehyde solution. After 72 h, paraffin section and HE staining were performed. After being weighed, the lingular lobe in the right lung was put in a thermostatic drying box at 80°C for 48 h, after which it was weighed again to calculate wet/dry ratio of lung.

Detection of inflammatory mediators and cytokines in lung homogenate of the right inferior lobe

Double antibody ABC-ELISA was employed to analyze nitrogen monoxide (NO), iNOS, and NF-κBp65 levels in BALF. Colorimetric analysis was used to test the concentration of malondialdehyde (MDA) in homogenates of the right inferior lobe of the lung.

iNOS mRNA quantification in lung tissue

Changes in the transcription level of iNOS mRNA in lung tissue of rats were tested by fluorescence quantitative PCR. The PCR primer sequence of iNOS (target gene) was designed by Jiamay Biotech Co. Ltd. The forward and reverse primers of iNOS were 5'-ACACCGATT-CCACTCAACTA-3' and 5'-ACCACCTGTTAGTTCA-

Table 1. Weight, wet/dry ratio of lung and blood gas results of rats in each group ($\bar{x}$±s, n=6)

Groups	Weight (g)	W/D	PaO$_2$ (mmHg)	Oxygenation index (mmHg)
Control group	176.22±31.07	4.58±0.41[*]	105.85±13.38[*]	504.05±63.72[*]
Smoke group	181.35±22.39	6.45±0.58[#]	54.10±6.83[#]	257.62±32.51[#]
Smoke+H$_2$S 3 h group	181.18±21.96	5.31±0.45[*,#]	78.35±4.47[*,#]	373.10±21.27[*,#]
Smoke+H$_2$S 6 h group	161.80±19.52	5.62±0.61[*,#]	76.78±4.06[*,#]	365.63±19.35[*,#]
F value	0.458	12.588	34.515	34.515
P value	0.804	<0.001	<0.001	<0.001

Note: [*]vs. smoke group, P<0.05; [#]vs. control group, P<0.05.

Table 2. Test results of MDA, NO, iNOS, iNOS mRNA and NF-κBp65 in the lung tissue of rats in each group ($\bar{x}$±s, n=6)

Groups	MDA (nmol/ml)	NO (μM)	Inos (pg/ml)	iNOS mRNA	NF-κBp65 (pg/ml)
Control group	161.24±15.68[*]	85.25±10.07[*]	320.11±30.91[*]	0.07±0.03[*]	7636.77±535.48[*]
Smoke group	332.00±52.23[#]	179.00±16.04[#]	603.44±50.67[#]	2.20±0.21[#]	13803.19±2196.37[#]
Smoke+H$_2$S 3 h group	272.77±24.30[*,#]	100.40±5.42[*,#]	579.36±36.80[#]	1.13±0.15[*,#]	9747.71±1005.39[*,#]
Smoke+H$_2$S 6 h group	240.38±24.26[*,#]	93.09±5.33[*]	406.33±52.45[*,#]	1.04±0.24[*,#]	8123.51±2095.33[*,#]
F value	31.306	91.492	47.646	177.725	15.001
P value	<0.001	<0.001	<0.001	<0.001	<0.001

Note: [*]vs. smoke group, P<0.05; [#]vs. control group, P<0.05.

AGCC-3', respectively, yielding an amplification product length of 159 bp. β-actin (CWbio. Co. Ltd, Cat# CW0918) was used as the internal control. Total RNA was extracted from tissue samples using an ultrapure RNA kit (CWbio. Co. Ltd, Cat# CW0581), and 5 μL of RNA was subjected to 1% agarose gel electrophoresis. HiFi-MMLV First Strand cDNA Synthesis Kit (CWbio. Co. Ltd, Cat#CW0744) was used for reverse transcription and Ultra SYBR Mixture (with Rox) (CWbio. Co. Ltd, Cat# CW0956) was used for PCR amplification. The conditions were as follows: 10 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 60 s. Fluorescence ratio PCR (Light Cycler-480II, Roche Diagnostics, Basel, Switzerland) was used to analyze data using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Statistical analysis was performed using SPSS (version 18.0, SPSS, Chicago, IL, USA). The data and the experimental results were described as mean ± standard deviation, and a *t*-test was used to estimate the difference between two groups. One-way ANOVA was used to evaluate the quantitative data, and the LSD method was used to compare the results between groups. A difference of P<0.05 was considered significant.

Results

Arterial blood gas analysis in rats

No statistical differences were present in the weight of rats (P>0.5) (**Table 1**), which suggests that weight had no effect on the results of this experiment. The oxygenation index of rats in the smoke group was 257.62±32.51 mmHg, which met the blood gas diagnostic criterion for acute lung injury. Compared with the control group, the arterial partial pressure of oxygen in rats in the smoke group was significantly decreased (54.10±6.83 mmHg, P<0.001), while the wet/dry ratio of lung was significantly increased (P<0.001). Compared with the smoke group, the arterial partial pressure of oxygen and oxygenation index of rats in the smoke+H$_2$S 3 h and smoke+H$_2$S 6 h groups were significantly increased (P<0.001), while the wet/dry ratio of lung was significantly decreased (P<0.01). There were no significant differences in the factors described above between the smoke+H$_2$S 3 h and smoke+H$_2$S 6 h groups (P>0.1).

Analysis of the lung homogenates of rats

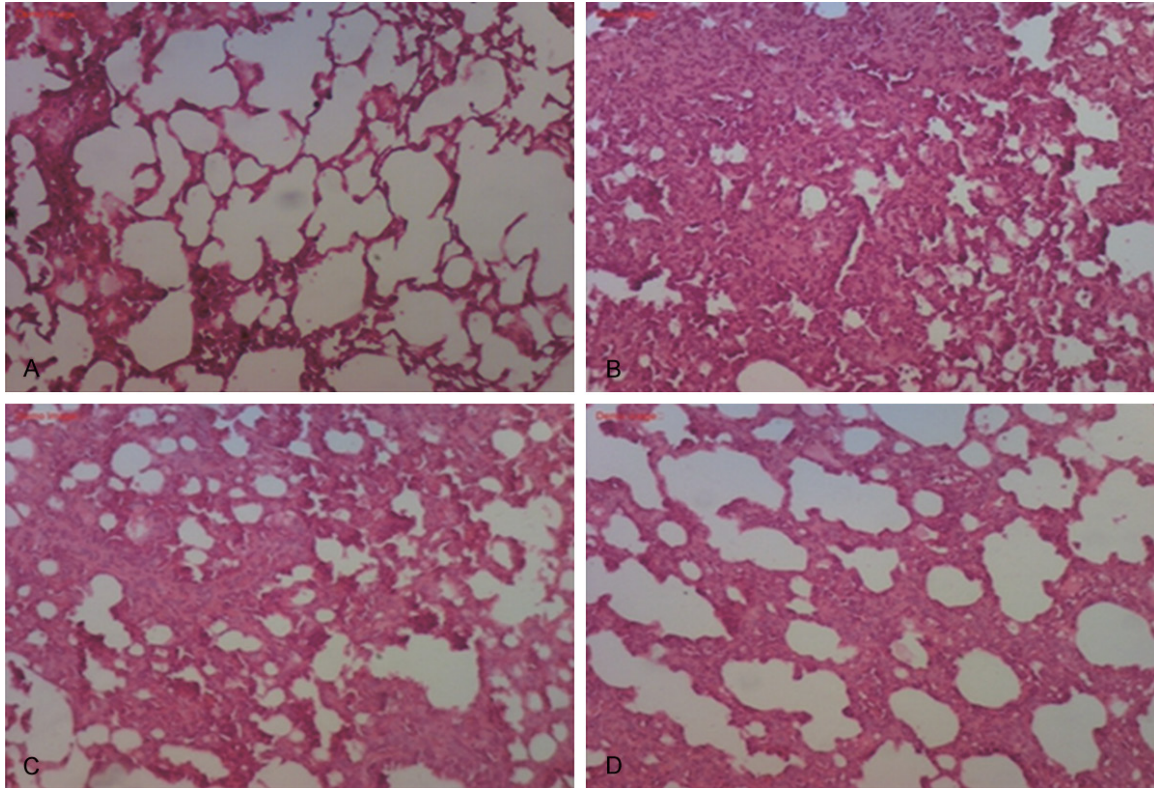As shown in **Table 2**, the concentrations of MDA, NO, iNOS, NF-κBp65 and the mRNA rela-

Figure 2. Histopathological observation of the lung tissues (HE ×100). A: Control group; B: Smoke group; C: Smoke+H$_2$S 3 h group; D: Smoke+H$_2$S 6 h group.

tive expression of iNOS in the homogenate of lungs of rats in the smoke group were significantly increased compared to the control group (P<0.001), while they were significantly decreased in the smoke+H$_2$S 3 h and smoke+H$_2$S 6 h groups (P<0.001). The concentration of iNOS was significantly higher in the smoke+H$_2$S 3 h group compared to the smoke+H$_2$S 6 h group (P<0.001), while the concentrations of MDA, NO, NF-κBp65 and the mRNA relative expression of iNOS showed no statistical differences between these groups (P>0.05).

Histopathological analysis

As shown in **Figure 2**, observation of lung tissue sections with light microscopy indicated that the alveoli of rats in the control group were clear, integrated, and uniform with no exudation in lung cavities and no swelling in the alveolar septum. In contrast, the alveoli of rats in the smoke group showed diffuse alveolar damage with hyaline membranes and leakage of red blood cells into lung cavities, thickened alveolar septum, and evident inflammatory cell

infiltration. The symptoms of rats in the smoke+H$_2$S 3 h and smoke+H$_2$S 6 h groups were alleviated significantly compared to the control group.

Discussion

In this study, our results demonstrated that rats in the smoke group showed typical symptoms of acute lung injury after cotton smoke inhalation for 6 h, including the significantly decreased arterial oxygen saturation and oxygenation index, while the wet/dry ratio of lung increased significantly. Rats in the smoke group showed diffuse alveolar damage under the light microscope, with hyaline membranes and leakage of red blood cells into lung cavities, thickened alveolar septum, and evident inflammatory cell infiltration. MDA, generated by the reactions between free radicals and polyunsaturated fatty acids in the biomembrane, is a kind of peroxide. MDA levels were increased in the smoke group, suggesting that lung tissue injury was aggravated with exacerbated oxidative stress and enhanced lipid peroxidation.

After smoke inhalation, NF-kB was activated, and the concentration of NF-kBp65 increased and entered the cell nucleus, thus promoting the mRNA transcription of iNOS and NO synthesis. The increase in mRNA relative expression of iNOS, and in concentrations of NF-kBp65, iNOS, and NO is completely consistent with the symptoms of oxidative stress after smoke inhalation.

Inhaling H_2S immediately after smoke inhalation decreased the exudation in lung cavities and the water content in lung tissue, alleviated the inflammatory cell infiltration and lipid peroxidation damage to membranes, lowered the wet/dry ratio of lung, and increased the arterial partial pressure of oxygen of rats, which suggests that inhalation of H_2S can alleviate acute lung injury of rats caused by smoke inhalation. Inhaling H_2S immediately after smoke inhalation also decreased the relative expression of iNOS mRNA and the concentrations of NF-kBp65, iNOS, and NO, and thus lowering the level of MDA. This suggests that in the rat model of smoke inhalation-induced lung injury, inhalation of H_2S may inhibit the activation of NF-kBp65, decrease the mRNA transcription and expression of iNOS and NO synthesis, and thus alleviate oxidative stress and acute lung injury in rats.

This study only examined lung damage after smoke inhalation, and after giving H_2S to rats 3 h or 6 h following smoke inhalation. We found that inhalation of H_2S [20] can have a repair effect on lung damage, but we observed no differences in repair due to variation in inhalation length. It is not clear how longer inhalation of H_2S would affect repair, and whether the longer inhalation would cause lung damage.

Acknowledgements

Disclosure of conflict of interest

None.

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