In vivo Study of the Effect of Exogenous Hydrogen Sulfide on Lung Mitochondria in Acute Lung Injury Rats

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Abstract

Background: Acute lung injury (ALI) is a common critical disease in ICU. Mitochondria plays an important role in the pathogenesis of ALI. There have been previous studies showed that the function of mitochondria can be affected by Hydrogen sulfide (H\textsubscript{2}S). In this study, we examined the possible role of exogenous H\textsubscript{2}S in regulation of the lung mitochondria in Lipopolysaccharide (LPS)-induced ALI in rats.

Methods: The rat model of ALI was induced by intra-tongue vein injection of LPS. Sodium hydrosulphide (NaHS) was used as the H\textsubscript{2}S donor. Forty Sprague-Dawley rats were randomly divided into five groups: control group, LPS injury group, LPS + low-dose NaHS (0.78 mg•kg\textsuperscript{-1}) group, LPS + middle-dose NaHS (1.56 mg•kg\textsuperscript{-1}) group and LPS + high-dose NaHS (3.12 mg•kg\textsuperscript{-1}) group. Rats were killed 3 h after administration of NaHS. The activities of ATPase, GSH-PX, and SOD, the content of malondialdehyde (MDA) in lung mitochondria, the swelling and activity of lung mitochondria were determined. Lung mitochondria and cytosol Cyt-c protein expression were analysed using Western blotting. The ultrastructures of lung mitochondria were observed with electron microscope.

Results: For the comparison of LPS injury group vs control group. activities of mitochondrion, ATPase, SOD, GSH-Px, the Cyt-c protein expression of mitochondrion were significantly decreased, whereas the content of MDA, the swelling of mitochondrion and the Cyt-c protein expression of cytosol were
increased. The mitochondrial ultrastructure was damaged in LPS injury group; for the comparison of LPS + low, middle and high-dose NaHS groups vs LPS injury group, activities of mitochondria, ATPase, SOD, GSH-Px, the Cyt-c protein expression of mitochondria were significantly increased, the content of MDA, the swelling of mitochondria and the Cyt-c protein expression of cytosol were significantly decreased ($P<0.05$ or $P<0.01$). The mitochondrial ultrastructure damage lighter in LPS + NaHS middle and high-dose groups.

**Conclusion:** The exogenous H$_2$S provided a protective effect against ALI via decreasing the mitochondrial lipid peroxidation level and protecting the cell structure in the LPS-Induced rat models. Its regulatory effect on the lung mitochondria is positively correlated with the dose.

**Key words:** acute lung injury, mitochondria, Lipopolysaccharide, exogenous hydrogen sulfide, mitochondrial lipid peroxidation, ultrastructure

**Background**

Acute lung injury (ALI) and its most severe manifestation, acute respiratory distress syndrome (ARDS), is a clinical syndrome characterized by acute hypoxemic respiratory failure, bilateral pulmonary infiltrates on frontal chest radiograph consistent with edema, and normal cardiac filling pressures [1]. The resulting lung damage can evoke lung failure and multiple organ dysfunctions associated with increased mortality [2]. A variety of stimuli can initiate ALI, such as mechanical ventilation, hyperoxia, ischemia/reperfusion, transfusion, or polytrauma, etc. Sepsis (the presence of pus-forming bacteria or their toxins in the blood or tissues) reflects one of the most important causes of ALI/ARDS [3]. Lipopolysaccharide (LPS), which is the major component of gram-negative bacillary endotoxin, plays an important role in initiating inflammatory response and causing systemic inflammatory response syndrome (SIRS) and sepsis. Lung is one of the target organs primarily impaired in endotoxin infection and sepsis. ALI induced by LPS is an acute pulmonary inflammation response in the lung, in which the accumulation and activation of polymophonuclear neutrophil (PMN) and the release of oxygen free radical are the key
link [4]. Injection of LPS in vivo is a classic way to manufacture sepsis-induced animal model of ALI [5]. Inflammatory cell activation and increased oxidative stress have been implicated in this pathogenesis[6] (Malondialdehyde [MDA], one of the end-products of the peroxidation of membrane lipids, the adenosine triphosphatase [ATPase], anti-oxidants superoxide dismutase[SOD] and glutathione peroxidase [GPx] are currently considered to be the basic markers of oxidative stress). LPS damage mitochondrial structure and ATP enzyme and oxidative phosphorylation coupling process, so that the energy metabolism disorders. LPS changes immune function, direct damages to the lysosomal membrane mononuclear phagocyte system cells, resulting in cell damage, and makes a series of pathological changes in the body.

LPS-induced ALI can cause abnormal mitochondrial structure and function: mitochondrial abnormalities tend to cause a change in the other cell organelles and the whole cell, thereby increasing the degree of ALI [7]. Studies have shown that oxidant-induced death and dysfunction of pulmonary vascular cells play important roles in the evolution of ALI, and oxygen radicals damage DNA in their mitochondria play an important role in the process of ALI [8]. Mitochondria as a cell's energy sub-units, is an important place for biological energy metabolism. It plays an important role of the maintenance of cell function and metabolism. When subjected to outside influence, its dysfunction may be an important part of lung injury. Mitochondrial dysfunction plays an important role in ALI in improving mitochondrial function may be an important means of ALI treatment.

Gaseous transmitters, such as nitric oxide (NO) and carbon monoxide (CO), play important roles both in physiology and in disease. In recent years, another naturally occurring gas, hydrogen sulfide (H₂S), has been found to be of importance [9]. An abundance of experimental evidence suggests that H₂S plays a prominent role in normal physiology and pathophysiology. Therefore, many therapeutic targets exist for H₂S therapy, including cancer, heart failure, organ
transplant, peripheral artery disease, inflammatory bowel disease, Alzheimer’s disease, acute myocardial infarction (AMI), stroke, atherosclerosis, hypertension, erectile dysfunction, metabolic syndrome, diabetes, and tombosis[10]. Although much less is known about the biological functions of H$_2$S, this gas is suggested to fulfill a wide range of physiological and pathological functions. For instance, H$_2$S opens K$^+$-ATP channels in vascular smooth muscle cells, gastrointestinal smooth muscle cells, cardiomyocytes, neurons, and pancreatic β-cells, therefore regulates vascular tone, intestinal contractility, myocardial contractility, neurotransmission and insulin secretion. In nervous system, H$_2$S promotes hippocampus long-term potentiation (LTP) by enhancing the sensitivity of NMDA receptors to glutamate and plays a role in neurodegenerative diseases [11].

Additional H$_2$S may scavenge reactive nitrogen species (RNS), peroxynitrite (OONOO-), oxygen free radicals and lipid peroxidations, resulting in cardiovascular protection and neuron protection now[12, 13, 14]. H$_2$S is increasingly considered as the third gasotransmitter [15]. Recently, some experiments confirmed that endogenous H$_2$S has been shown to play an important role in the pathogenesis of inflammatory diseases and associated organ injury, such as acute pancreatitis[16], sepsis [17], ischemia/reperfusion injury [18], and lung injury(ventilator-induced lung injury [19], or oleic acid-induced ALI) [20], succeeded in exerting organ protective effects. The experiments aiming at exogenous H$_2$S are fewer [2, 3, 19]. Although the biologic effects of H$_2$S have been described in a variety of in vivo models, the molecular mechanisms by which it elicits these responses implicate a wide array of molecules, yet remain incompletely understood. Some experiments have showed that H$_2$S may participate in the pathogenesis of ALI/ARDS and H$_2$S related therapy may be a potential therapeutic approach in this condition [21]. However, the role of H$_2$S in the pathogenesis of ALI is far from clear. The effect of mitochondria may be one of mechanisms. Major targets clearly include the inhibition of mitochondrial cytochrome c oxidize and activation of endothelial cell K$^+$-ATPase channels, but the
downstream effects of both pathways are highly context-dependent. In addition, injection of NaHS (exogenous H\textsubscript{2}S) to normal rat directly results in lung inflammation and inflammatory damage in a dose-dependent manner [22]. Thus, the aim of the present study was to find out the possible role of H\textsubscript{2}S in the pathogenesis of LPS-induced ALI in rats and its regulatory effects on the lung mitochondria with different doses.

**Methods**

This study was approved by the Institutional Experimental Animal Care and Ethics Committee of Hebei Medical University (Shijiazhuang, China) before the commencement of any intervention. All animal care and experimental protocols complied with the Animal Management Rules of the Ministry of Health of the People’s Republic of China (documentation number 19890503) and the Guide for the Care and Use of Laboratory Animals of Hebei Medical University (Shijiazhuang, China). The study was performed in the Animal Laboratory of our Department of Pharmacology.

**Study Animals**

This study was carried out on forty Male Sprague-Dawley rats, weighing 250–280 g, which were obtained from the Experimental Animal Centre, Hebei Medical University (Shijiazhuang, China) and housed in our Laboratory Animal Husbandry Facility until the experiments. Rats were acclimated for 1 week before experiments, with unrestricted access to deionized water with 2 drinking bottles attached to each cage) and standard rat chow and no other restrictions were performed. Before and throughout the study, the rats were kept at room temperature (22°C), 30%–70% humidity, and 12 hours night/12 hour are day conditions, lights on at 7:00 AM. None deaths occurred before the intervention. Forty rats were included in the study. All procedures were performed in accordance with the Guidelines of Animal Experiments from the Committee of Medical Ethics, National Health Department of China.
**Materials**
The H$_2$S donor NaHS was obtained from Sigma-Aldrich (St Louis, MO, USA). E.coli LPS (serotype 0127:B8) was obtained from Sigma-Aldrich Company Ltd (Poole, Dorset, U.K.). SOD, MDA, GPx and ATPase detection kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). The primary rabbit monoclonal antibodies were obtained from Abcam Company Ltd (Cambridge, U.K.). All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl) from Shijiazhuang No.4 Pharmaceutical Company Ltd (Shijiazhuang, Hebei, China).

**Equipments**
The equipment include the followings: electric homogenizer (Silentcrusher M, Heidolph company, Germany), cold centrifuge (5417R Eppendorf company, Germany), analytical balance (METTLER-AH240, METTLER TOLEDO company, Switzerland), analytical balance (AE240, Shanghai Precision & Scientific Instrument company, China), a water bath (Beijing Dongcheng District Medical Machinery factory, China), Full wavelength microplate reader (BIOTEK company, USA), 722 spectrophotometer meter (Shanghai Third Analytical Instrument factory, China), Paraffin machine (LEICA company, Germany), Transmission electron microscopy (HITACHI company, Japan), Thermostatic oscillator (SHA-C, Changzhou Guohua Electric Appliance company, China), Multifunction electrophoresis (Multiphor, Pharmacia company, Sweden), Vertical slab electrophoresis device (LOX-1, Beijing sixty-one Instrument factory, China), Electrophoresis tank (DYY-23A, Beijing sixty-one Instrument factory, China), Fishing machine (LEICA company, Germany), DBA Color kit (Beijing Zhongshan Golden Bridge Biotechnology company, China).

**Experimental protocol**
Rats were randomly divided into five groups (n = 8 in each): control
group, LPS injury group, LPS + low-dose NaHS (0.78 mg•kg\(^{-1}\)) group, LPS + middle-dose NaHS (1.56 mg•kg\(^{-1}\)) group and LPS + high-dose NaHS (3.12 mg•kg\(^{-1}\)) group. Rats were anaesthetized with an injection of 100 g/L chloralhydrate (3 ml•kg\(^{-1}\)). Anesthesia depth was evaluated every 2–3 min throughout the study. No supplemental oxygen, fluids, or mechanical ventilation were performed during the anesthesia period. The same dose was administered to the different groups, and was found adequate. After adequate anesthesia, rats were the fixed in dorsal position on a surgical table.

1. Control group: Rats were treated with an equal volume of saline without causing endotoxemia.
2. LPS injury group: Rats were treated with E.coli LPS (5 mg•kg\(^{-1}\) via sublingual vein injections, serotype 0127:B8) was given slowly over 10 min and were an equal volume of saline via intraperitoneal injections 3 h after the induction of LPS.
3. LPS + low-dose NaHS group: Rats were administered NaHS (0.78 mg•kg\(^{-1}\)) via intraperitoneal injections 3 h after the induction of LPS.
4. LPS + middle-dose NaHS group: Rats were administered NaHS (1.56 mg•kg\(^{-1}\)) via intraperitoneal injections 3 h after the induction of LPS.
5. LPS + high-dose NaHS group: Rats were administered NaHS (3.12 mg•kg\(^{-1}\)) via intraperitoneal injections 3 h after the induction of LPS.

All rats were killed 3 h after administration of NaHS or saline and the mitochondria of lung were isolated by differential centrifugation.

**Observation of ultrastructural changes to mitochondria in lung cells**

After rats were killed, lung tissue (1 mm\(^3\)) was resected and fixed in 4% glutaraldehyde in 0.1 mol•L\(^{-1}\) phosphate buffer (pH 7.4) at 4\(^\circ\)C. Tissues were washed three times in dimethyl arsenate buffer and then post-fixed with 1% osmium tetroxide for 1 h, followed by another three washes in dimethyl arsenate buffer and dehydration by passage through graded concentrations of ethylene alcohol. After sequential
treatments with propylene oxide, ultrathin sections were cut using a Leica (Wetzlar, Germany) UCT Ultra Microtome, stained with 1% uranyl acetate and lead citrate and observed under a transmission electron microscope.

**Preparation of isolated mitochondria**
Mitochondria were isolated as previously described. Briefly, after rats had been killed, the lung tissues were removed rapidly into ice-cold isolation medium (0.025 mol•L$^{-1}$ sucrose, 0.075 mol•L$^{-1}$ mannitol, 0.001 mol•L$^{-1}$ EDTA, and pH 7.4). The tissues were finely homogenized using a homogenizer in a glass pestle. The homogenate was centrifuged at 600 g for 7 min at 2°C. The supernatant was collected and then centrifuged again at 1 600 g for 5 min at 2°C. The crude mitochondrial pellet was resuspended in a final volume of 5 mL of 3% Ficoll medium (0.12 mol•L$^{-1}$ mannitol, 0.03 mol•L$^{-1}$ sucrose, 0.025 mol•L$^{-1}$ K$^+$-EDTA, pH 7.4). This suspension was carefully layered onto 10 mL of 6% Ficoll medium (0.24 mol•L$^{-1}$ mannitol, 0.06 mol•L$^{-1}$ sucrose, 0.05 mol•L$^{-1}$ K$^+$-EDTA, pH 7.4) and centrifuged at 12 500 g for 10 min at 2°C. The supernatant was decanted and the slight fluffy layer was removed from the pellet. The mitochondrial pellet was resuspended in isolation medium and centrifuged again at 12 500 g for 10 min at 2°C. The prepared mitochondria were diluted in isolation medium prior to use.

**Determination of lung mitochondrial MDA content, SOD and GPx and ATPase activity**
The levels of SOD and GPx activity and ATPase activity and MDA content in the lung Mitochondrial were measured using an enzyme-linked immunosorbent assay (ELISA), using commercially available kits (Nanjing Jiancheng Bioengineering, Nanjing, Jiangsu, China) according to the manufacturer’s instructions. Detail of steps (the way to determine the levels of SOD): The kit was maintained at room temperature (20–25°C) prior to testing; and the washing buffer was prepared 15 minutes before using. The 100 µl of SOD standards
and 100 µl of samples were added to the corresponding wells in the plates and shaken gently for 30 seconds before sealing. After 1 hour of incubation at 37°C, liquid was removed; the plates were washed with the washing buffer (350 µl/well) and soaked for a few minutes. Plates were blotted dry by tapping upside down on filter paper. The washing was repeated for five times. The 100 µl of biotin was added to each well of the plates, and left for 1 hour at 37°C. After five additional washing steps, 100 µl of horseradish peroxides (HRP) was added to the wells, and left for 30 minutes at 37°C. The plates were again washed five times and 100 µl of tetramethylbenzidine (TMB) substrate was added to each well and plates were shaken gently for 10 seconds. The mixture was incubated in the dark for (15±10) minutes at 37°C. Optical density (OD) at 450 nm was measured by ELISA reader after adding 100 µl of stop solution to each well and shaking gently for 30 seconds. The standard curve of OD value versus concentration was plotted. The sample data were plotted on the standard curve and the sample concentration of SOD was obtained. Others in samples were obtained in the similar way.

Determination of the swelling of lung mitochondria
Freshly prepared mitochondria without repeated freeze-thaw were at 4 °C prior to the reaction. The mitochondria were removed rapidly into the medium (0.025 mol•L⁻¹ sucrose, 0.0005 mol•L⁻¹ KH₂PO₄, 0.001 mol•L⁻¹ Sodium succinate, and pH 7.2). Mitochondrial protein content was adjusted to 0.5mg•ml⁻¹. Measuring the absorbance values at 540nm on a spectrophotometer at 722. The reaction conditions at 25 °C.

Determination of the lung mitochondria activity
Freshly prepared mitochondria suspension (100µL) without repeated freeze-thaw was removed rapidly into the microtiter plate’s microporous. Add MTT (5g•L⁻¹ 40µL) at 30 °C incubated for 30min. Add isopropanol (100µL) for 20min. Colorimetric analysis was at 570nm. OD570 value size indicates mitochondrial activity.
Determination of lung mitochondrial and cytosol Cyt-c protein expression

Preparation of mitochondria and cytosol
Briefly, after rats were killed, the lung tissues were removed rapidly into ice-cold isolation medium (0.025 mol/L sucrose, 0.075 mol/L mannitol, 0.001 mol/L EDTA, and pH 7.4). The tissues were finely homogenized using a homogenizer in a glass pestle. The homogenate was centrifuged at 600 g for 7 min at 2°C. The supernatant was collected and then centrifuged again at 1 600 g for 5 min at 2°C. The crude mitochondrial pellet was resuspended in a final volume of 5 mL of 3% Ficoll medium (0.12 mol/L mannitol, 0.03 mol/L sucrose, 0.025 mol/L K⁺-EDTA, pH 7.4). This suspension was carefully layered onto 10 mL of 6% Ficoll medium (0.24 mol/L mannitol, 0.06 mol/L sucrose, 0.05 mol/L K⁺-EDTA, pH 7.4) and centrifuged at 12 500 g for 10 min at 2°C. The supernatant was decanted and the slight fluffy layer was removed from the pellet. The mitochondrial pellet was resuspended in isolation medium and centrifuged again at 12 500 g for 10 min at 2°C. The mitochondria and cytosol supernatant were used for analysis of cytochrome c(Cyt-c) by Western blotting assay.

Western blotting assay
Protein extractions in each group were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were treated with blocking solution (5% skim milk in TBST) and incubated overnight at 4°C with the primary rabbit multilocal antibodies respectively (Cyt-c, 1:1000; abcam, U.K.). Immunoblots were developed with horseradish peroxidase-conjugated secondary antibodies, and visualized using an enhanced chemiluminescence reagent (Millipore, Billerica, MA, USA) and quantified by densitometry using a ChemiDoc XRS (Bio-Rad, Berkeley, California, USA). The band density was normalized to β-actin. The percentage increase or decrease of the proteins was
estimated by comparison to vehicle control (100%).

**Statistical analysis**

Based on the assumed differences and variability in the data marking a biologic effect of treatment in each group, numbers of animals per group were estimated before the study. All data are presented as the mean ± SD. Differences between groups were assessed by one-way ANOVA followed by Tukey’s post hoc multiple comparison test using SPSS version 13.0 (SPSS, Armonk, NY, USA). Two-sided P value of less than 0.05 was considered to be statistically significant.

**Results**

**Effects of H\textsubscript{2}S on the ultrastructure of mitochondria in lung cells**

There were significant differences in the ultrastructure of mitochondria in lung cells in the control group and LPS. In contrast, the mitochondria in lung cells from the LPS injury group were swollen with disrupted or disintegrated cristae and the osmiophilic lamellar bodies were fusion or disappeared. This mitochondrial damage was lighty mitigated in the LPS + low-dose NaHS group. These effects were reversed by middle-dose NaHS, but were aggravated by high-dose NaHS. (Fig. 1).

**Effects of H\textsubscript{2}S on MDA content and mitochondrial ATPase, SOD and GSH-P\textsubscript{X} activity**

Compared with control group, the content of MDA was significantly increased ($P < 0.01$), and the activities of ATPase, SOD and GSH-P\textsubscript{X} were significantly decreased ($P < 0.01$) in lung mitochondria in LPS injury group. Compared with LPS injury group, the content of MDA was significantly decreased, and the activities of ATPase, SOD and GSH-P\textsubscript{X} were significantly increased in LPS+low, middle and high dose NaHS groups ($P < 0.05$ or $P < 0.01$). (Tab.1, 2) (Fig.2, 3, 4, 5)
Effects of H$_2$S on the swelling and activity of the lung mitochondria

The swelling extent of the mitochondria was significantly increased and the activity of the mitochondria was significantly decreased in LPS injury group compared with the control group ($P < 0.01$). In LPS + low-dose NaHS group, LPS + middle-dose NaHS group and LPS + high-dose NaHS group, the swelling of the mitochondria was markedly decreased and the activity of the mitochondria was markedly increased compared with the LPS injury group ($P < 0.05$ or $P < 0.01$) (Tab. 3) (Fig. 6, 7).

Effects of H$_2$S on lung mitochondrial and cytosol Cyt-c protein expression

The band intensity showed the expression of the Cyt-c protein. The Cyt-c protein expression of the lung mitochondria was significantly decreased in the LPS injury group compared with the control group ($P < 0.01$). In the LPS + low-dose NaHS group, LPS + middle-dose NaHS group and the LPS + high-dose NaHS group, Cyt-c protein expression of the lung mitochondria was markedly increased compared with the LPS injury group ($P < 0.01$). The Cyt-c protein expression of cytosol was significantly increased in the LPS injury group ($P < 0.01$) (Tab. 4) (Fig. 8).

Discussion

ALI / ARDS is a common clinical illness. It has led to a significant increase of the social and economic burden, which is comparative with breast cancer, AIDS, asthma, or myocardial infarction. The Current ALI mortality rate is still as high as 35%-40%, which reaches to 50% in ARDS, accordingly [23, 24]. The pathogenesis of ALI is complex and there are still a lot of controversy before we get the definitive conclusion [25]. Increased oxidative stress has been implicated in the
pathogenesis [26, 27]. The lung-protective, low-tidal-volume ventilation strategy increases survival rate through limiting the alveolar damage and consequent biotrauma [28]. But, no ideal targeted drug therapies have been shown to be curative, and treatment is mainly supportive. Therefore, alternative strategies are urgently needed to improve the care.

In recent years, some researchers found that mitochondrial dysfunction plays an important role in the course of ALI [29]. Mitochondria of cells has a very important role to maintain the normal function. Mitochondria utilize approximately 98% of total body oxygen consumption [30, 31]. This would maintain tissue oxygen levels by decreasing demand, and protect against cell death [32]. Studies have shown that mitochondrial dysfunction is the key factor to cell damage [33]. In sepsis, mitochondrial dysfunction of vital organs leading to cell organisms lack of energy, and may develop into multiple organ failure [34]. Lung is a special organ. Gas exchange in it, meanwhile it accepts all in cardiac output of blood. There is a wealth of capillary endothelial cells and alveolar epithelial cells in it. Respiratory or circulatory system from invading the body of harmful substances, the earliest damage both target cells [35]. Lung is a organ which is easier injured. Mitochondria is a complex and sensitive organelle. ALI can lead to the abnormal mitochondrial structure and function, and tend to cause abnormal mitochondria organelles and other changes in the entire cell, thereby increasing the degree of ALI. Meanwhile, mitochondrial dysfunction is also prone to ALI/ARDS [36].

Including the case of LPS-induced lung injury, the lung tissue can produce large radical NO, O$^{-2}$, ONOO$^{-}$, and the mitochondrial film which is rich in unsaturated fatty acids is a major target of free radical attack. These lead mitochondrial membrane swelling expansion, increased lipid oxidation, membrane fluidity decreased, activity changes of mitochondrial ATP enzyme, mitochondrial activity decreased and decreased ATP production and so on. The mitochondria has intrinsic defense mechanisms to protect against damage induced by ROS through its large array of antioxidants (e.g., superoxide
dismutase, glutathione, thioredoxin) [37]. However, these can be overwhelmed in pathological processes generating large amounts of ROS. Under physiological conditions, the mitochondrial SOD, GSH-Px content rich, timely removal of the metabolism of oxygen free radicals. When ALI, produce large amounts of oxygen free radicals, resulting in mitochondrial SOD, GSH-Px activity decreased while consumption increased, MDA content increased, resulting in decreased activity of ATP, ATP decreased production. Ultimately these lead to the destruction of mitochondrial structure [38].

H₂S, a potent toxin, is a gaseous mediator that has created great excitement as a therapy for preserving organ function-and life-during suspended animation in in vivo models [39]. H₂S is one of important regulators of mitochondrial signaling in health [40]. Major targets clearly include the inhibition of mitochondrial cytochrome c oxidase and activation of endothelial cell K⁺-ATPase channels, but the downstream effects of both pathways are highly context-dependent. For example, moderate inhibition of cytochrome c oxidase at low H₂S concentrations induces the “suspended animation” (i.e., hypometabolism, hypothermia) that has been associated with protection. Potential utility of H₂S in sepsis has been demonstrated in several animal studies with improvements in organ function and survival [41, 42, 43]. God does not create “waste”. As one of gas signaling molecules, H₂S benefits may be derived from its anti-inflammatory actions although, arguably. It may act predominantly through promoting a protective metabolic shutdown triggered by decreased energy availability akin to the intrinsic adaptive process argued previously [32].

The experimental results show that acute lung injury in rats after LPS, rat lung tissue showed mitochondrial swelling, decreased activity, membrane fluidity reduced total lung mitochondrial ATP enzyme, SOD, GSH-Px activity was significantly decreased mitochondrial MDA content was significantly increased; The Cyt-c protein expression of the lung mitochondria was significantly decreased and the Cyt-c protein expression of cytosol was
significantly increased (Cyt-c was released from mitochondria); Mitochondrial ultrastructure damage. NaHS treatment group compared with the ALI group, the total mitochondrial ATP enzyme, SOD, GSH-Px activity were significantly increased, MDA content decreased; The Cyt-c protein expression of the lung mitochondria was significantly increased and the Cyt-c protein expression of cytosol was significantly decreased (suppress the release of Cyt-c from mitochondria); The mitochondrial ultrastructure damage lighter. Meanwhile, these therapeutic role is in dose-dependent manner (positively correlated with the dose). Our results add an important information to the role of H$_2$S in ALI induced by LPS. The mechanism maybe that H$_2$S suppress the mitochondrial cytochrome c oxidase, decrease the level of mitochondrial lipid peroxidation and protect cell structure. But some conflicting data exist in models of cecal ligation and puncture demonstrating aggravation [44, 45]. As well as substantial reduction of the resulting lung injury in response to application of H$_2$S donors [46, 47]. It is likely that the purity of H$_2$S donors, the route of administration, timing, and dosage may be accountable for the inconsistent data. Our experiment confirmed that H$_2$S can reduce the mitochondrial oxidative damage by free radicals, thereby reducing ALI lung tissue damage and play a therapeutic role in dose-dependent manner.

**Limitations**

The most common models of experimental ALI caused by sepsis include injection (local or systemic) of products of bacteria such as endotoxin (LPS), a commonly used organism being Escherichia coli, or lipoteichoic acid. Other models include extrusion of faecal contents from surgically manipulated areas of gut [usually cecum, cecal ligation and puncture (CLP)]; the colon ascendens stent peritonitis (CASP) which causes an intraperitoneal leak of faeces, leading to polymicrobial sepsis, similar to what is seen in the CLP model. Each of these models has its advantages and disadvantages. Dramatic differences in the timing of responses (such as when
cytokine/chemokine levels peak in plasma). In rodents injected with LPS, plasma mediators peak in the first several hours versus in CLP with mediator peaks much more slowly developing, over the first 48 h. To what extent can these two models be compared is questionable as is whether either model mimics events developing in humans with ALI caused by sepsis. Thus, this study has limited clinical relevance. In future studies, we will use other models.

This study does not elucidate the effect by other models. Lung tissue H$_2$S concentrations were not measured in this study. Comparing the lung tissue concentrations of sulfide species in future studies may help explain the differential biologic effects of intravascular NaHS and their dose-dependency.

**Conclusions**

In our model, LPS resulted in lung mitochondrial structure injury and loss of function in ALI rats. Hydrogen sulfide reduced LPS-induced lung mitochondrial oxidative damage and protect mitochondrial structure and function. This therapeutic role is in dose-dependent manner. Its regulatory effect on the lung mitochondrial is positively correlated with the dose. Detailed mechanism needs further study.

**Key messages**

- The results of this in vivo experimental study demonstrate that hydrogen sulfide (considered as the third gaseous transmitter) donor, NaHS, prevented mitochondrial oxidative damage and protect mitochondrial structure and function in a model of ALI induced by LPS in rats.
- NaHS reduced ALI-induced oxidative stress.
- NaHS reduced ALI-induced mitochondrial dysfunction.
- NaHS up-regulated the lung mitochondria Cyt-c protein expression and down-regulated cytosol the Cyt-c protein expression (suppress the release of Cyt-c from mitochondria) in this model.
- NaHS reduced ALI-induced mitochondrial structure damage.
- Regulatory effect of NaHS on the lung mitochondria is positively
correlated with the dose.

**Abbreviations**

Acute lung injury: ALI; acute respiratory distress syndrome: ARDS; Lipopolysaccharide: LPS; systemic inflammatory response syndrome: SIRS; polymophonuclear neutrophil: PMN; nitric oxide: NO; carbon monoxide: CO; hydrogen sulfide: H₂S; NaHS: sodium hydrosulfide; acute myocardial infarction: AMI; long-term potentiation: LTP; ROS: radical oxygen species; peroxynitrite: OONO⁻; Malondialdehyde: MDA; superoxide dismutase: SOD; adenosine triphosphatase: ATPase; glutathione peroxidase: GPx; cytochrome c: Cyt-c; SD: standard deviation.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

QD designed the study, participated in the laboratory investigations and the design of the protocol, and wrote the manuscript. CW carried out the Western blotting, participated in the laboratory investigations and performed the statistical analysis and helped to draft the manuscript. NZ and GL participated in the laboratory investigations and wrote the manuscript. MZ, QZ and LL participated in analysis and interpretation. JZ directed the study. All authors read and approved the final version of the manuscript.

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**References**


Table 1 The activities of ATPase and SOD from rats lung mitochondria following treatment with NaHS for 3h after acute lung injury 3h in rats (x ±s, n=8)

<table>
<thead>
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<th>ATPase (U.mg⁻¹pro)</th>
<th>SOD (U.mg⁻¹pro)</th>
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<tr>
<td>control group</td>
<td>9.92±0.65</td>
<td>27.44±1.97</td>
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<tr>
<td>LPS injury group</td>
<td>4.83±0.25**</td>
<td>18.78±1.13**</td>
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<td>20.13±0.85#</td>
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<tr>
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</tbody>
</table>

**P<0.01. Compared with control group ; #P<0.05. ##P<0.01. Compared with LPS injury group; L: LPS+low-dose NaHS group ; M: LPS+middle-dose NaHS group ; H: LPS+high-dose NaHS group

Table 1 Compared with control group, the activities of ATPase and SOD were significantly decreased (all P < 0.01) in lung mitochondrial in LPS injury group. Compared with LPS injury group, the activities of ATPase and SOD were significantly increased in LPS+low, middle and high-dose NaHS groups (P < 0.05 or P < 0.01).
Table 2: The activities of GSH-Px and the contents of MDA from rats lung mitochondria following treatment with NaHS for 3h after acute lung injury 3h in rats $(\bar{x} \pm s, n=8)$.

<table>
<thead>
<tr>
<th>Group</th>
<th>GSH-Px (U.mg(^{-1})pro)</th>
<th>MDA (nmol.mg(^{-1})pro)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control group</td>
<td>128.15±3.47</td>
<td>11.16±1.20</td>
</tr>
<tr>
<td>LPS injury group</td>
<td>63.91±1.99**</td>
<td>26.30±1.45**</td>
</tr>
<tr>
<td>L</td>
<td>82.06±1.65##</td>
<td>21.89±1.23##</td>
</tr>
<tr>
<td>M</td>
<td>101.45±2.14##</td>
<td>17.63±1.56##</td>
</tr>
<tr>
<td>H</td>
<td>117.80±2.12##</td>
<td>12.19±1.30##</td>
</tr>
</tbody>
</table>

**P<0.01, Compared with control group ; #P<0.05, ##P<0.01, Compared with LPS injury group; L: LPS+low-dose NaHS group ; M: LPS+middle-dose NaHS group ; H: LPS+high-dose NaHS group

Table2 Compared with control group, the content of MDA was significantly increased ( all $P<0.01$), and the activities of GSH-Px was significantly decreased ( $P<0.05$ or $P<0.01$) in lung mitochondrial in LPS injury group. Compared with LPS injury group, the content of MDA was significantly decreased, and the activities of GSH-Px was significantly increased in LPS+low, middle and high-dose NaHS groups ( $P<0.01$).
Table 3 Effects of NaHS on the lung mitochondrial swelling and activity in acute lung injury rats (\(\bar{x} \pm s, n=8\)).

<table>
<thead>
<tr>
<th>Group</th>
<th>Swelling of mitochondria (OD\textsubscript{540})</th>
<th>Activity of mitochondria (OD\textsubscript{570})</th>
</tr>
</thead>
<tbody>
<tr>
<td>control group</td>
<td>0.273±0.023</td>
<td>0.319±0.045</td>
</tr>
<tr>
<td>LPS injury group</td>
<td>0.182±0.012**</td>
<td>0.164±0.025**</td>
</tr>
<tr>
<td>L</td>
<td>0.195±0.008#</td>
<td>0.194±0.018#</td>
</tr>
<tr>
<td>M</td>
<td>0.219±0.017##</td>
<td>0.230±0.032##</td>
</tr>
<tr>
<td>H</td>
<td>0.249±0.018##</td>
<td>0.297±0.038##</td>
</tr>
</tbody>
</table>

**P<0.01. Compared with control group; *P<0.05. **P<0.01. Compared with LPS injury group; L: LPS+low-dose NaHS group; M: LPS+middle-dose NaHS group; H: LPS+high-dose NaHS group

Table 3 The swelling of the mitochondria was significantly increased and the activity of the mitochondria was significantly decreased in the LPS injury group compared with the control group (all \(P < 0.01\)). In LPS+low, middle and high-dose NaHS groups, the swelling of the mitochondria was markedly decreased and the activity of the mitochondria was markedly increased compared with the LPS injury group (\(P < 0.05\) or \(P < 0.01\)).
Table 4 Effects of NaHS on lung mitochondrial and cytosol Cyt-c protein expression in acute lung injury rats (\(\bar{x} \pm s, n=8\)).

<table>
<thead>
<tr>
<th></th>
<th>Cyt-c (mitochondria)</th>
<th>Cyt-c (cytosol)</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(Grayscale scan value)</td>
<td>(Grayscale scan value)</td>
<td>(Grayscale scan value)</td>
</tr>
<tr>
<td>control group</td>
<td>178.51</td>
<td>96.992</td>
<td>171.47</td>
</tr>
<tr>
<td>LPS injury group</td>
<td>41.177**</td>
<td>293.45**</td>
<td>183.66</td>
</tr>
<tr>
<td>L</td>
<td>62.846##</td>
<td>226.91##</td>
<td>182.52</td>
</tr>
<tr>
<td>M</td>
<td>88.344##</td>
<td>203.38##</td>
<td>196.02</td>
</tr>
<tr>
<td>H</td>
<td>115.27##</td>
<td>111.84##</td>
<td>169.28</td>
</tr>
</tbody>
</table>

**P<0.01. Compared with control group; ##P<0.01. Compared with LPS injury group; L: LPS+low-dose NaHS group; M: LPS+middle-dose NaHS group; H: LPS+high-dose NaHS group.

Table 4 The band intensity of lung mitochondria Cyt-c protein was significantly decreased in LPS injury group compared with the control group (\(P<0.01\)). In LPS+low, middle and high-dose NaHS groups, the band intensity of lung mitochondria Cyt-c protein was markedly increased compared with the LPS injury group (\(P<0.05\) or \(P<0.01\)).

The band intensity of cytosol Cyt-c protein was significantly increased in the LPS injury group compared with the control group (\(P<0.01\)). In LPS+low, middle and high-dose NaHS groups, the band intensity of cytosol Cyt-c protein was markedly decreased compared with LPS injury group (\(P<0.05\) or \(P<0.01\)).
Fig. 1 Lung Ultrastructure. Effect of H\textsubscript{2}S on mitochondrial ultrastructure in lung cells, as determined by transmission electron microscopy analysis, in LPS-Induced ALI rat model. There were significant differences in the ultrastructure of mitochondria in lung cells in the control, LPS injury, LPS + low-dose NaHS (L), middle-dose NaHS (M) and LPS + high-dose NaHS (H) groups. In contrast, the mitochondria in lung cells from the LPS group were swollen with disrupted or disintegrated cristae and the osmiophilic lamellar bodies were fusion or disappeared. These effects were reversed by low-dose NaHS, but were aggravated by middle and high-dose NaHS. Arrows indicate mitochondria in nerve cells. Bar, 500 nm.
**Fig. 2 Effect of H<sub>2</sub>S on the content of MDA in lung mitochondria in rats.** Compared with control group, the content of MDA was significantly increased ($P < 0.01$) in lung mitochondria in LPS injury group. Compared with LPS injury group, the content of MDA was significantly decreased in LPS+low, middle and high dose NaHS groups ($P < 0.01$).

**P<0.01 vs control; ##P<0.01 vs LPS**
**Fig. 3** Effect of H₂S on the activity of ATPase in lung mitochondria in rats. Compared with control group, the activities of ATPase were significantly decreased (P < 0.01) in lung mitochondria in LPS injury group. Compared with LPS injury group, the activities of ATPase were significantly increased in LPS+low, middle and high dose NaHS groups (P < 0.01).

**P<0.01 vs control; #P<0.01 vs LPS**
Fig. 4 Effect of H$_2$S on the activity of SOD in lung mitochondria in rats. Compared with control group, the activities of SOD were significantly decreased ($P < 0.01$) in lung mitochondria in LPS injury group. Compared with LPS injury group, the activities of SOD were significantly increased in LPS+low, middle and high dose NaHS groups ($P < 0.05$ or $P < 0.01$).

**$P<0.01$ vs control; #P<0.05, ##P<0.01$ vs LPS
**Fig. 5 Effect of H$_2$S on the activity of GSH-P$_x$ in lung mitochondria in rats.** Compared with control group, the activities of GSH-P$_x$ were significantly decreased ($P < 0.01$) in lung mitochondria in LPS injury group. Compared with LPS injury group, the activities of GSH-P$_x$ were significantly increased in LPS+low, middle and high dose NaHS groups ($P < 0.01$).

**P<0.01 vs control; ##P<0.01 vs LPS**
Fig. 6 Effect of H$_2$S on the swelling of lung mitochondria in rats. The swelling extent of the mitochondria was significantly increased in the LPS injury group compared with the control group ($P < 0.01$). In LPS + low-dose NaHS group, LPS + middle-dose NaHS group and LPS + high-dose NaHS group, the swelling of the mitochondria was markedly decreased with the LPS injury group ($P < 0.05$ or $P < 0.01$)

**$P<0.01$ vs control; # $P<0.05$, ## $P<0.01$ vs LPS
**Fig. 7 Effect of H₂S on the activity of lung mitochondria in rats.** The activity of the mitochondria was significantly decreased in the LPS injury group compared with the control group ($P < 0.01$). In LPS + low-dose NaHS group, LPS + middle-dose NaHS group and LPS + high-dose NaHS group, the activity of the mitochondria was markedly increased compared with the LPS injury group ($P < 0.05$ or $P < 0.01$).

**$**P<0.01$ vs control; **$P<0.05$, **$P<0.01$ vs LPS**
Fig. 8 The expression of lung mitochondrial Cyt-c and cytosol Cyt-c were detected by Western blotting. 

- a: control group
- b: LPS injury group
- c: LPS + low-dose NaHS group
- d: LPS + middle-dose NaHS group
- e: LPS + high-dose NaHS group.