Protective effects of exogenous hydrogen sulfide on seawater aspiration induced-acute lung injury in rats

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Abstract: Aim: Seawater drowning induced-acute lung injury (ALI) is a serious clinical condition characterized by refractory hypoxemia, enhanced alveolar-capillary permeability, hemorrhage, and uncontrolled overwhelming inflammatory responses. Hydrogen sulfide (H$_2$S) is an endogenous gaseous mediator that plays an important role in the physiological and pathophysiological conditions, especially in some inflammatory diseases. However, the beneficial effects of sodium hydrosulfide (NaHS), a H$_2$S donor, on seawater aspiration-induced ALI remains to be elucidated.

Material and methods: Two hundred and forty rats were equally assigned to the naive group, normal saline group, seawater group, or seawater + NaHS group. NaHS or vehicle was intraperitoneally administered immediately after seawater aspiration. Arterial blood gas analysis was performed with a gas analyzer at baseline, 30 min, 1 h, 4 h, and 24 h after the seawater aspiration, respectively. Histological scores, computed tomography scan, TUNEL-positive cells assay, Toll-like receptor 4, nuclear factor kappa B p65, caspase-3, tumor necrosis factor-alpha, interleukin (IL)-1β, IL-6, IL-10, wet-to-dry weight ratio, myeloperoxidase activity, malondialdehyde, and superoxide dismutase levels in the lung were assessed at 30 min after the seawater aspiration. Results: NaHS treatment alleviated ALI as demonstrated by the increased arterial partial oxygen tension and deceased lung histological scores, which was accompanied by decreased TUNEL-positive cells, Toll-like receptor 4, nuclear factor kappa B p65, caspase-3, tumor necrosis factor-alpha, IL-1β, myeloperoxidase activity, and malondialdehyde in the lung. Conclusions: Our study suggested that the protective effects of NaHS on seawater aspiration-induced ALI might be associated with its anti-inflammatory, anti-oxidative, and anti-apoptotic properties.

Keywords: Seawater, acute lung injury, hydrogen sulfide, cytokines, apoptosis

Introduction

Seawater drowning induced-acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) is a serious clinical condition characterized by refractory hypoxemia, enhanced alveolar-capillary permeability, hemorrhage, and uncontrolled overwhelming inflammatory responses [1-3]. Lung edema is a major feature in seawater aspiration induced-ALI because high osmotic seawater can pull water through vascular endothelial cells and alveolar epithelial cells [1-3]. Although mechanical ventilation is currently the basic therapeutic option for seawater aspiration-induced ALI [5], there is a need to search alternative agents for the treatment of seawater induced-ALI.

Hydrogen sulfide (H$_2$S), regarding as the third ‘gasotransmitter’ alongside nitric oxide and carbon monoxide, has received much attention because of its multiple physiological and pathophysiological roles in various body systems [6-8]. H$_2$S is naturally synthesized in the lung from the L-cysteine mainly by cystathionine-γ-lyase at low micromolar concentrations [9], although it is considered to be toxic at high concentrations due to its ability to interfere with the mitochondrial electron transport chain [10]. As an endogenous molecule, H$_2$S has been identified to participate in the regulation of various physiological processes as well as in inflammatory responses [6-9]. Administration of gaseous H$_2$S or H$_2$S donors has anti-inflammatory effects in an animal model of sepsis [9], neurodegenerative diseases [6], and ischemia-reperfusion injury [11]. In addition, exogenous H$_2$S administration has been implicated to play critical roles in the pathogenesis of various lung diseases, including chronic obstructive pulmonary disease [12] and inflammatory lung injury [13]. However, its role in seawater aspiration induced-ALI is currently unknown.
In the present study, we hypothesized that sodium hydrosulfide (NaHS), a H\textsubscript{2}S donor, would protect against seawater aspiration induced-ALI. Furthermore, the possible mechanism underlying the protective effects of NaHS was also explored.

**Materials and methods**

**Animals and ethics**

All animal experiments were approved by the Ethics Committee of Jinling Hospital, Nanjing University. Animals were housed in standard conditions and maintained in a 12 h light/12 h dark cycle with food and water ad libitum. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health. Two hundred and forty male Sprague-Dawley rats (8-10 weeks) weighing 250-350 g were purchased from the Animal Center of Nanjing University, Nanjing, China.

**Drug and reagents**

The H\textsubscript{2}S donor NaHS was purchased from Sigma-Aldrich (St Louis, MO, USA). Seawater (osmolality 1300 mmol/L, pH 8.2, specific weight 1.05, NaCl 26.518 g/L, MgSO\textsubscript{4} 3.305 g/L, MgCl\textsubscript{2} 2.447 g/L, CaCl\textsubscript{2} 1.141 g/L, KCl 0.725 g/L, NaHCO\textsubscript{3} 0.202 g/L) was provided by Chinese Ocean Bureau.

**Animal model and experimental protocols**

Seawater group: The seawater aspiration animal model was established according to the method described in one previous study with minor modifications [3]. Briefly, the rats were...
anesthetized with sodium pentobarbital (50 mg/kg) intraperitoneally. A catheter was inserted into the right femoral artery to obtain blood samples. The rats were maintained in the supine position during experiments with the head elevated 30 degree. After exposure of the trachea and a 20 min stable baseline period, a 1 ml syringe was gently inserted into the trachea approximately 1.5 cm above the carina. After then, seawater (4 ml/kg) was instilled at a steady speed within 4 min into the both lungs.

Naive group: The rats received neither seawater, nor normal saline, nor NaHS in this group. The other treatment was the same as the seawater group. Normal saline group: The rats received the same volume of normal saline and the other treatment was the same as the seawater group. Seawater + NaHS group: NaHS was intraperitoneally administered immediately after the seawater aspiration and the other treatment was the same as the seawater group. The flow chart of the experimental protocol is shown in Figure 1A.

Drug administration

For the dose-response study, NaHS at the dose of 2.5, 5, or 10 mg/kg was administered intraperitoneally immediately after the seawater aspiration to determine the optimal dose of NaHS to attenuate seawater induced ALI. Our data suggested that 5 mg/kg was the optimal dose with the highest desired pharmacologic effect, as reflected by the increased arterial

Figure 2. NaHS treatment attenuated PaO$_2$ in rats induced by seawater aspiration. Seawater aspiration caused a significant decrease in PaO$_2$, whereas NaHS treatment significantly increased PaO$_2$. However, no difference in PaCO$_2$ was observed among the groups (n = 8-10). PaO$_2$, arterial partial oxygen tension; PaCO$_2$, arterial carbon dioxide tension; NaHS, sodium hydrosulfide. *P < 0.05 vs. the naive group; #'P < 0.05 vs. the seawater group.
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Arterial blood gas analysis

Arterial blood samples were obtained from the right femoral artery at the indicated time-points of the study for the analysis of PaO$_2$ and arteri- al carbon dioxide tension (PaCO$_2$) by a gas analyzer (GEM Premier 3000, Instrumentation laboratory, USA).

Histological analysis

For the lung histological studies, the same right lower lung lobe from each rat was excised and immediately immersed into 10% formalin. The samples were sectioned and stained with hematoxylin and eosin for light microscopy. One slice from each rat was taken and 10 fields observed under the microscope (× 400) and evaluated by a semi-quantitative histological index for quantitative assessment. The severity of microscopic injury was graded from 0 (normal) to 4 (severe) based on the following categories: interstitial edema, hemorrhage, necrosis, neutrophil infiltration and atelectasis. The sum of all scores was combined to calculate a composite score.

Computed tomography (CT) scan

CT scan was performed at 30 min after the seawater aspiration. The rats were anesthetized, and placed on a plate (20 cm × 15 cm) in the supine position. The plate was then placed ver-
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Figure 4. CT scan of lung in rats induced by seawater aspiration. Sweater aspiration induced alveolar septal thickening and edema, whereas NaHS treatment alleviated these abnormal changes in the lung (n = 6).

Western blotting analysis

The lung was subjected to western blotting analysis as described in our previous studies [14]. Briefly, tissues were homogenized on ice using immunoprecipitation buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, and 0.5% Nonidet P-40) plus protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A). The lysates were collected, centrifuged at 10,000 g at 4°C for 10 min. The supernatant was removed, and protein concentration was determined using the Pierce bicinchoninic acid Protein Assay kit (Pierce Technology Co., Iselin, NJ) with a bovine serum albumin standard. Equal amounts of protein were electrophoretically separated on 4-12% NuPAGE Novex Bis-Tris gradient gels (Invitrogen, NY, USA) and transferred to the nitrocellulose membranes. After blocking with 5% non-fat milk for 1 h at room temperature, membranes were incubated with respective antibodies overnight at 4°C followed by horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Pittsburgh, PA) for 2 h at room temperature. TLR-4 (1:1000; Cell Signaling, USA), nuclear factor (NF)-κB p65 (1:1000; Santa Cruz, USA), Cleaved caspase-3 (1:1000; Cell Signaling, USA), and β-actin (1:1000; Cell Signaling, USA) were used to detect TLR-4, NF-κB p65, caspase-3, and β-actin expressions, respectively. The protein bands were detected by enhanced chemiluminescence and the quantitation of bands was undertaken using the Image J software (NIH Image, USA).

TUNEL staining

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to measure the extent of DNA fragmentation as a measurement of apoptosis in paraffin-embedded sections. The assay was performed according to the manufacturer’s instructions (Boehringer, Mannheim, Germany). Fluorescein-conjugated dUTP incorporated in nucleotide polymers were detected and quantified using fluorescence microscopy (Zeiss LSM 410, Wetzlar, Germany).

Measurement of pro-inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA)

The levels of TNF-α, IL-β, IL-6, and IL-10 were measured with TNF-α, IL-β, IL-6, and IL-10 ELISA kits according to the manufacturer’s instructions. Briefly, portions of lung tissues were homogenized in cool phosphate-buffered saline (lung tissue to normal saline 1:5). The concentration of total protein in tissue lysate was detected and then all samples were made to
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the same concentration by adding phosphate-buffered saline. After then, the concentration of pro-inflammation cytokines was detected in each sample.

Malondialdehyde (MDA), and superoxide dismutase (SOD), and myeloperoxidase (MPO) activity assay

Myeloperoxidase (MPO) activity, a marker for polymorphonuclear neutrophil infiltration into the lung, was determined using a MPO (Nanjing Jiancheng Bioengineering, Nanjing, Jiangsu, China) assay kit according to the manufacturer’s instructions. MDA levels were determined by the thiobarbituric acid method and SOD activities were evaluated by the xanthine oxidase method as previously described [15]. The absorbance was measured at 532 and 550 nm for MDA and SOD, respectively. MDA concentrations are expressed as nanomoles per milligram of protein and SOD activities are expressed as units per milligram of protein, respectively.

Lung wet-to-dry weight (W/D) ratio

To evaluate the severity of pulmonary edema, the lung W/D ratio was measured. The left lower lobe of the lung was harvested, weighed, and dried for 48 hours in a heated stove (50°C). W/D ratio was calculated by dividing wet by the dry lung weight.

Statistical analysis

The Statistical Program for Social Sciences software (Version 16.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Data are expressed as the mean ± standard error of the mean (S.E.M.). Normal distribution for the continuous variable was tested using Kolmogorov-Smirnov test. Statistical significance was determined by one-way, two-way, or repeated-measures of analysis of variance (ANOVA) followed by a Bonferroni test as appropriate. A P value < 0.05 was regarded as statistically significant difference.

Results

NaHS treatment increased PaO2 in rats induced by seawater aspiration

As shown in Figure 2, seawater aspiration caused a marked decrease in the PaO2, whereas NaHS treatment significantly increased PaO2 compared with that of seawater group (two-way repeated-measures of ANOVA, P < 0.05), especially at 30 min. Therefore, 30 min after seawater aspiration were used for the following experiments. However, no difference was observed in PaCO2, K+, and Na+ concentrations among the four groups over time (two-way repeated-measures of ANOVA, P > 0.05).

NaHS treatment attenuated ALI in rats induced by seawater aspiration

To investigate the protective effects of NaHS on seawater aspiration-induced ALI, we assessed the pulmonary histological scores changes. After seawater aspiration, there were serious lung injuries such as hemorrhage, the markedly thickened alveolar wall, and the infiltration of
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inflammatory cells in alveolar spaces (one-way ANOVA, \( P < 0.05 \), Figure 3B), whereas treatment with NaHS significantly reduced the abnormality of lung structure.

To further assess the effects of NaHS on seawater aspiration-induced ALI, we performed the lung CT scan of each group. Lung CT scans of the lungs in the seawater aspiration group showed marked alveolar septal thickening and edema, while NaHS treatment alleviated this lung edema (Figure 4).

NaHS treatment decreased pulmonary NF-κB p65 and caspase-3 levels in rats induced by seawater aspiration

The expression of NF-κB p65 and caspase-3 was significantly increased in the seawater group compared with the naive group, while NaHS treatment significantly inhibited these parameters (one-way ANOVA, \( P < 0.05 \), Figure 5). However, there was no difference in TLR4 among the four groups (one-way ANOVA, \( P > 0.05 \)).
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To determine whether NaHS treatment could affect seawater aspiration induced pulmonary apoptosis, we performed TUNEL staining on lung sections. As shown in Figure 6, few cells staining positive for TUNEL were detected in the naïve group. Seawater aspiration significantly increased pulmonary TUNEL-positive cells, while NaHS treatment resulted in a marked reduction in the number of these cells.

NaHS treatment decreased pulmonary pro-inflammatory mediators levels in rats induced by seawater aspiration

As shown Figure 6, TNF-α and IL-1β levels increased markedly after seawater aspiration. Compared with the seawater group, NaHS treatment significantly inhibited the expressions of these pro-inflammation cytokines (one-way ANOVA, \( P < 0.05 \), Figure 7).

Discussion

The present study demonstrated that (1) seawater aspiration induced ALI, as evidenced by the deterioration of gas exchange, pulmonary edema, vascular leakage, neutrophil infiltration, and alveolar congestion/collapse; (2) treatment with NaHS significantly attenuated the seawater aspiration-induced ALI, with concomitant reduced pulmonary pro-inflammatory

NaHS treatment decreased pulmonary MDA, SOD, myeloperoxidase (MPO) activity and W/D ratio in rats induced by seawater aspiration

Seawater aspiration increased pulmonary MDA, SOD, MPO activity, and W/D ratio when compared with the naïve group. However, NaHS treatment attenuated MDA, MPO activity, and W/D ratio compared with the seawater group (one-way ANOVA, all \( P < 0.05 \), Figure 8). No difference was detected in SOD levels between the seawater group and the seawater + NaHS groups (one-way ANOVA, \( P > 0.05 \)).
cytokines, reactive oxygen species, and apoptosis.

Drowning is one of the major causes of injury-related death, especially in children and young adults worldwide [1-3]. Seawater drowning represents one of the most important causes of ALI [2]. In the pathogenesis of ALI, disruption of the alveolar epithelial-endothelial capillary barrier, which consists of alveolar epithelial cells, interstitium cells and vascular endothelial cells, is considered as a central process [16]. In seawater aspiration induced-ALI, high osmotic seawater can pull water through vascular endothelial cells and alveolar epithelial cells and leads to disruption of blood-gas barrier, which in turn triggers the transmigration of inflammatory cells into the alveolar spaces and release several molecules, such as pro-inflammatory cytokines, reactive oxygen species [1-3]. Thus, strategy targeting at these pathological aspects may be promising for the treatment of seawater aspiration-induced ALI.

H$_2$S is an endogenous gaseous mediator that has recently been the focus of intense investigation, which has been suggested to play an important role in the physiological and pathophysiological conditions [5-8]. H$_2$S is reported to possess potent anti-oxidant, anti-inflammatory, as well as other functions [5-9]. However, conflicting data exist in models of cecal ligation and puncture demonstrating aggravation [17] and improvement of the resulting lung injury in response to H$_2$S treatment [18-20]. This discrepancy might be attributed to the route of administration, dosage, timing, and the purity of H$_2$S donors. The inflammatory response in ALI/ARDS is characterized by the transmigration of immune-competent cells (mostly neutrophils) into the lung interstitium and the alveolar space, and the release of numerous pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 [21-23]. Among several transcriptional factors activated by inflammatory responses after seawater aspiration, TLR4 and its downstream signaling NF-κB play key roles in the increased
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expressions of cytokines, chemokines, adhesion molecules, acute phase proteins, and inducible effectors enzymes [14, 23]. In the present study, we showed that NaHS treatment reversed the enhanced nuclear NF-κB p65 expressions and pulmonary concentration of inflammatory cytokines after seawater aspiration, suggesting NF-κB activation plays a key role in the enhanced inflammatory response. Our data were consistent with previous studies that H₂S decreases the activation of NF-κB and other pro-inflammatory cytokines [24, 25] and that NaHS attenuates LPS-induced inflammation by inhibition of p38 MAPK and p65 NF-κB in rodent microglia [6].

Activated neutrophil infiltration into the lungs is known to play a significant role in the progression of ALI [26]. Our results confirmed that MPO activity, a marker for polymorphonuclear neutrophil infiltration into the lung, was significantly decreased after NaHS treatment in seawater aspiration-induced ALI. Furthermore, we demonstrated that NaHS treatment significantly alleviated membrane lipid peroxidation levels in the lung after seawater aspiration, which was supported by the observation that administration of H₂S caused a significant decrease in MDA content in an animal model of ischemia/reperfusion [11]. The attenuation of neutrophil transmigration and MDA by administration of H₂S has also been shown in other models of ALI, such as ventilator-induced lung injury [27] and myocardial ischemia/reperfusion injury [28]. These findings strongly support our results that NaHS substantially inhibits pulmonary oxidative stress and thereby limits seawater aspiration-induced ALI.

Accumulating evidence has suggested that cell apoptosis plays a key role in the pathogenesis of ALI, as demonstrated by the alveolar epithelial cells apoptosis early in the course of ARDS [29]. Apoptosis of the alveolar epithelial cells could increase permeability of alveolar capillaries and damage the alveolar capillary barrier effect [29, 30]. In the present study, activation of apoptotic pathways in the lung was evaluated by measuring the caspase-3 enzymatic activity. NaHS treatment significantly decreased caspase-3 activity after seawater aspiration. In parallel, treatment with NaHS effectively reduced seawater aspiration-induced increase in pulmonary TUNEL-positive cells. Our data combined with previous studies suggested that reduced apoptosis may account for the protective effects of H₂S against seawater-induced ALI.

In conclusion, our study provides the evidence that administration of NaHS protects against seawater aspiration induced-ALI. The protective mechanism may be partly due to the inhibition of inflammation, oxidative stress, and apoptosis following NaHS treatment.

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Disclosure of conflict of interest

None.

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