

## Original Article

# Sulforaphane attenuates acute lung injury by inhibiting oxidative stress via Nrf2/HO-1 pathway in a rat sepsis model

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**Abstract:** Sulforaphane (SFN), an antioxidant derived from cruciferous vegetables, exerts antioxidant capacity and protects organ against oxidative damage. However, the effects of SFN on sepsis-induced acute lung injury (ALI) have not been determined. The aim of this study was to investigate the effect of SFN in sepsis-induced ALI and the role of Nrf2/HO-1 in this process. Rats were subjected to either sham-operated or cecal ligation and puncture-induced sepsis without or with SFN. Pulmonary oxidative stress was significantly increased (reduced SOD activity, enhanced 8-OHdG concentration, elevated 15-F2t-isoprostane level, and enhanced 4-HNE expression) in sepsis that were associated with elevated lung injuries (increased lung injury index, elevated lung water content, and reduced endothelial barrier integrity). Supplementation of SFN significantly enhanced Nrf2 and HO-1 protein expression in the lungs in sepsis. Further, SFN dose-dependently reduced pulmonary oxidative stress and attenuated lung injuries in sepsis. However, these beneficial effects of SFN were reduced by HO-1 inhibition. Therefore, we concluded that SFN attenuated ALI in sepsis by reducing oxidative stress through activating Nrf2/HO-1.

**Keywords:** Sulforaphane, acute lung injury, oxidative stress, sepsis

## Introduction

Sepsis, defining as life-threatening multiple organ failure, is the most common cause of mortality in intensive care units [1] and is the second leading cause of death in patients admitted at non-coronary intensive care units [2]. Acute lung injury (ALI) and its most severe form acute respiratory distress syndrome are the frequent complications of sepsis [3] and account for over 38% of death in patients with sepsis [4]. Despite extensive studies on the ALI in sepsis, the molecular mechanism of sepsis-induced ALI still not fully elucidated and effective treatment lacking.

It is generally accepted that oxidative stress-induced damage is the major factor that contribute to multiple organ failure in sepsis [5] including the lung. Indeed, oxidative stress has been found in many forms of lung injuries and that inhibition of oxidative stress has been shown to attenuate lung injuries [6, 7]. Antioxidant capacity is significantly decreased

that is associated with excessive oxidative stress in the lungs in patients with sepsis [8]. Similarly, in cecal ligation and puncture-induced sepsis models in rats, activities of antioxidant enzymes such as SOD, CAT, and GSH-PX are significantly decreased in the lung accompanied by increased 8-isoprostane and superoxide anion formation in the lung [9-11], indicating the critical role of oxidative stress in sepsis-induced ALI. Thus, effective means that reduce oxidative stress in the lung may attenuate ALI in sepsis [12].

Sulforaphane, an antioxidant derived from cruciferous vegetables, has been shown to confer organ protective effects via its antioxidant capacity in many organs such as hearts [13], lungs [14], and livers [15]. Application of sulforaphane reduced doxorubicin-induced oxidative stress and cell death in cardiomyocyte H9C2 cells which possibly via activation of the potent antioxidant molecule, NF-E2-related factor-2 (Nrf2) and its downstream target heme oxygenase-1 (HO-1) [13]. Moreover, pretreat-

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ment with sulforaphane ameliorated  $\beta$ -amyloid peptide-induced oxidative damage and the subsequent cell death in neural SH-SY5Y cells which through activation of Nrf2/HO-1 pathways [16]. Sulforaphane has also been shown to protect the lung against lipopolysaccharide-induced acute lung injury in mice [17]. However, the effect of sulforaphane on ALI in sepsis has not been studied.

In this study, we aimed to study the effects of sulforaphane on sepsis-induced ALI. In addition, given that Nrf2 activation is critical in sulforaphane's antioxidant effects [13], and sulforaphane has been considered as an inducer of Nrf2 [18]. The roles of Nrf2 and its downstream target HO-1 in sulforaphane-mediated pulmonary protective effects on ALI in sepsis has also been investigated in the current study.

### Materials and methods

#### *Animals and reagents*

Sprague-Dawley rats weighing 180-200 g were purchased from the Experimental Animal Center of the Wuhan university (Wuhan, China). All animals were exposed to a 12 h light/12 h dark cycle with room temperature maintained at  $22 \pm 2^\circ\text{C}$  and allowed food and tap water ad libitum in accordance with the Principles of Laboratory Animal Care approved by Wuhan University. All animal experiments were approved by the Animal care and Used Committee of Wuhan University (Wuhan, China) and followed the "Guide for the Care and Use of Laboratory Animals" (NIH Publications no. 8023, revised 1978) guidelines for the treatment of animals.

Sulforaphane (LKT Laboratories, Inc., USA) was dissolved in corn oil (Sigma Chemical Company) before use. Zinc protoporphyrin IX (ZnPP) (St. Louis, MO., USA) was dissolved in 0.9% saline before use. Lung tissue protein extraction reagent was obtained from Thermo Scientific Pierce (Rockford, IL, USA). Primary antibodies specific for Nrf2, HO-1, and 4-HNE were purchased from Abcam (Cambridge, MA., US) and Occludin, ZO-1, and Claudin-1 were obtained from Santa Cruze Biotechnology (California USA). Enzyme-linked immunosorbent assay (ELISA) kits of SOD, 8-OHdG, and 15-F2t-isoprostane were purchased from Cayman Chemical Company (Ann Arbor, MI USA).

#### *Cecal ligation and puncture model*

Cecal ligation and puncture (CLP) model was performed to induce sepsis as described previously [19]. Simplify, rats were anesthetized by inhalation 2% isoflurane using an anesthetic mask and a midline abdominal incision was performed. Using an 18-gauge needle, the cecum was then punctured through both sides. The cecum was placed back into the peritoneum after extruding a small amount of feces from the punctured site. 5 mL 0.9% sodium chloride solution was injected subcutaneously in abdominal wall for fluid resuscitation. In the sham group, animals underwent laparotomy and bowel manipulation without ligation and perforation. All rats had free access to water and food after recovery from anesthesia.

#### *Experiment design*

Two sets of experiments were performed. The first set of the experiment was aimed to detect whether SNF could protect lung from CLP-induced lung injury. Rats were randomly assigned to four groups ( $n=8$ , each group). SHAM group received laparotomy and bowel manipulation without ligation and perforation. CLP group received cecal ligation and puncture as described above. CLP+SNF groups received an intraperitoneally given of SNF in different dose (1.25, 2.5, and 5 mg/kg) 1 hour before the operation. SNF (5 mg/kg) was chose for the subsequent detection. The second part of the experiment was designed to exposure the underline mechanism of the protective effects of SNF. CLP+SNF ( $n=8$ ) group received SNF (5 mg/kg) [20] intraperitoneally 1 hour before CLP model created. Rats in CLP+SNF+ZnPP ( $n=8$ ) group were subjected to CLP after SNF (5 mg/kg, intraperitoneally, 1 hour before CLP) and ZnPP (50 mg/kg [21], intraperitoneally, 30 minutes before CLP) treatment. During the surgery, rectal temperatures were measured and heat preservation measures were provided by using an insulation blanket. Then rats were deeply anesthetized by carbon dioxide and sacrificed at 18 hours after CLP. The superior lobe of right lung was collected for histopathology detection. Then the left lung was rapidly excised and preserved in the liquid nitrogen for subsequent tissue protein extraction. The lower lobe of right lung was collected for lung wet/dry weight ratios analysis. Blood samples were collected from abdominal artery.

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## *Pathological assay*

The superior lobe of right lung was obtained and fixed in buffered formalin (10%, Sigma, USA) and then embedded in paraffin. Then the lung tissues were cut into 5- $\mu$ m sections and stained with hematoxylin-eosin (H&E) after de-waxing and hydration. The lung H&E sections were then examined under an optical microscope (Leica, DMLB2, Germany) and the lung injury score was calculated by two pathologists who blind to the experimental classification as previous described [22].

## *Lung water content*

The right middle lobe of the lungs were removed and massed to achieve the lung wet weight data, which then placed in an baking oven at 80°C for 48 hours to determine and got the dry weight data, and the wet/dry (W/D) lung weight ratio were calculated.

## *ELISA assay of 8-OHdG, SOD activity and 15-F2t-isoprostane*

8-OHdG, SOD activity and 15-F2t-isoprostane levels in lung homogenate were determined by ELISA kits (Cayman Chemical Company, Ann Arbor, MI USA) according to the manufacturer's instructions as previous described [23].

## *Immunofluorescence staining of 4-HNE*

Frozen sections of lung tissue were fixed for 1 hour at room temperature with phosphate-buffered saline (PBS) containing 5% bovine serum albumin and 0.3% Triton X-100. Next, Lung sections were incubated at 4°C overnight with anti-4-HNE (1:150, Abcam®, UK) antibody. After washing with PBS for three times, the sections were incubated for 1 hour at 25°C with a fluorescently labeled secondary antibody (1:100). The cover slips were then observed using a fluorescent microscope (Leica, DMLB2, Germany).

## *Western blot assay*

Nuclear and cytoplasmic proteins were extracted from frozen lung tissues on ice with the Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit according to the manufacturer's instructions as previous described [24]. Protein concentrations were assayed by using the BCA method (KeyGenBioTech, China).

Equal amounts of protein lysates (50  $\mu$ g/Lane) were fractionated on sodium dodecyl sulfate polyacrylamide gels-10% and transferred to 0.45  $\mu$ m pore size hydrophilic polyvinylidene fluoride (PVDF) membranes. The PVDF membranes were blocked for 1 hour at room temperature by incubation in phosphate-buffered saline containing 0.1% Tween-20 (PBST) and 5% non-fat milk. The membranes were then incubated with rabbit anti-HO-1 antibodies (1:1000, Santa Cruz Biotechnology, USA), rabbit NQO-1 (1:500, Santa Cruz Biotechnology, USA), mouse anti-Brg1 (1:1000, Abcam®, UK), mouse anti-Nrf2 (1:1000, Abcam®, UK), rabbit anti-Occludin (1:1000, Santa Cruz Biotechnology, USA), mouse-anti Claudin-1 (1:1000, Santa Cruz Biotechnology, USA), and mouse-anti ZO-1 (1:1000, Santa Cruz Biotechnology, USA) overnight. The membranes were subsequently washed in PBST once for three times for 10 minutes. After washing, the membranes were incubated for 1 hour at room temperature with anti-rabbit immunoglobulin IgG (1:2000) or anti-mouse IgG (1:2000) and then they washed in PBST once for three times for 10 minutes. Protein bands were visualized by enhanced chemiluminescence detection ECL kit (KGP-1125, Nanjing KeyGen Biotech. Co., Ltd.). The membranes were then stripped and re-incubated with an anti-H2A antibody (for nuclear proteins, diluted 1:1000; Santa Cruz Biotechnology, USA) or an anti-GAPDH antibody (for whole cell splitting proteins, diluted 1:2000; Santa Cruz Biotechnology, USA) to ensure equal loading.

## *Statistical analysis*

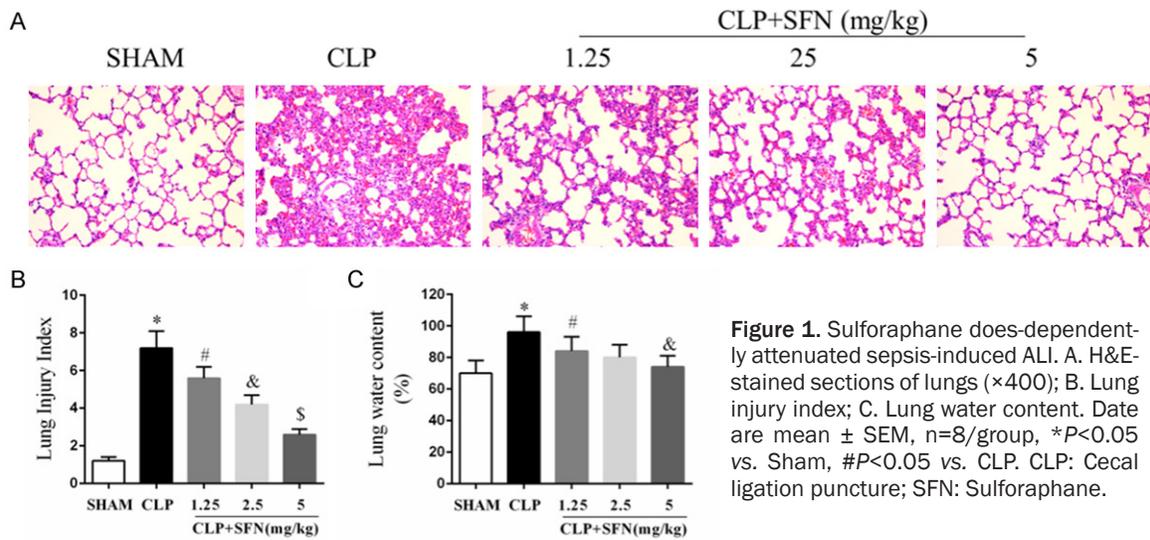
GraphPad Prism 7.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS13.0 software (SPSS, Inc., Chicago, IL, USA) were used for statistical analysis. All data were expressed as mean  $\pm$  SEM. The statistical analyses were performed using *Student's t-test*, *one-way ANOVA with Tukey post hoc multiple comparisons test* for unpaired values. The significant differences were considered at  $P < 0.05$ .

## **Results**

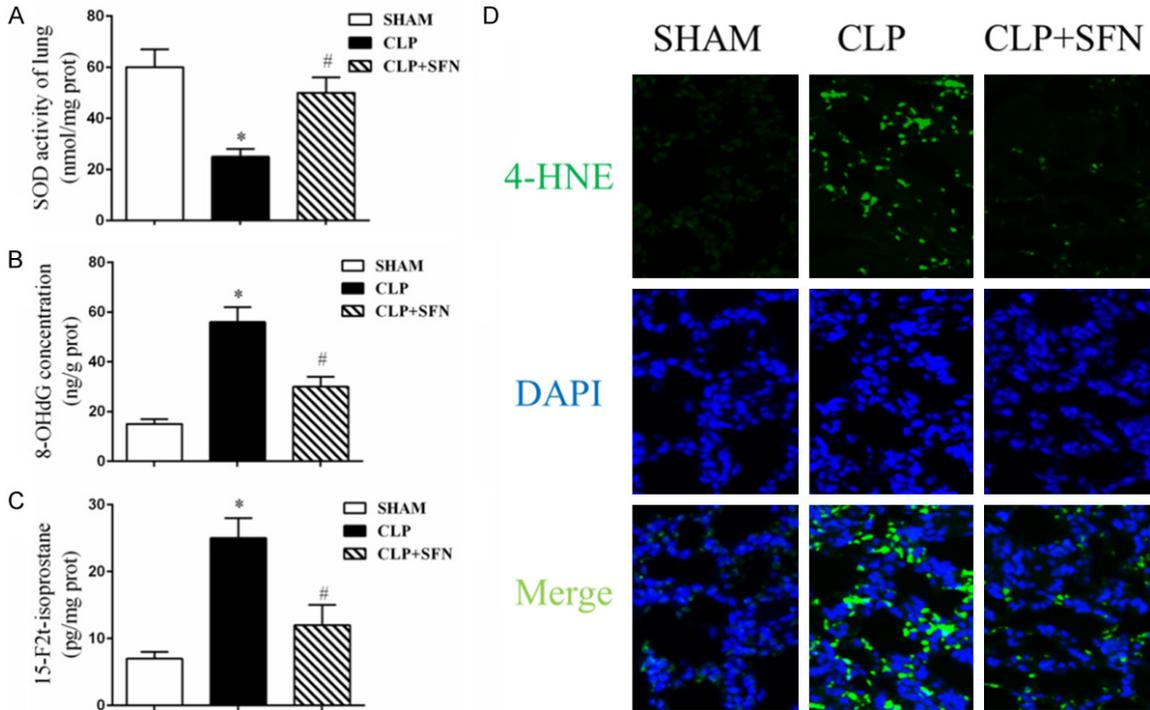
### *Sulforaphane does-dependently attenuated sepsis-induced ALI*

As shown in **Figure 1**, sepsis induced significant lung injury manifested as sever pathological damage (**Figure 1A**), increased lung injury

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**Figure 1.** Sulforaphane dose-dependently attenuated sepsis-induced ALI. A. H&E-stained sections of lungs ( $\times 400$ ); B. Lung injury index; C. Lung water content. Data are mean  $\pm$  SEM,  $n=8$ /group,  $*P<0.05$  vs. Sham,  $\#P<0.05$  vs. CLP. CLP: Cecal ligation puncture; SFN: Sulforaphane.



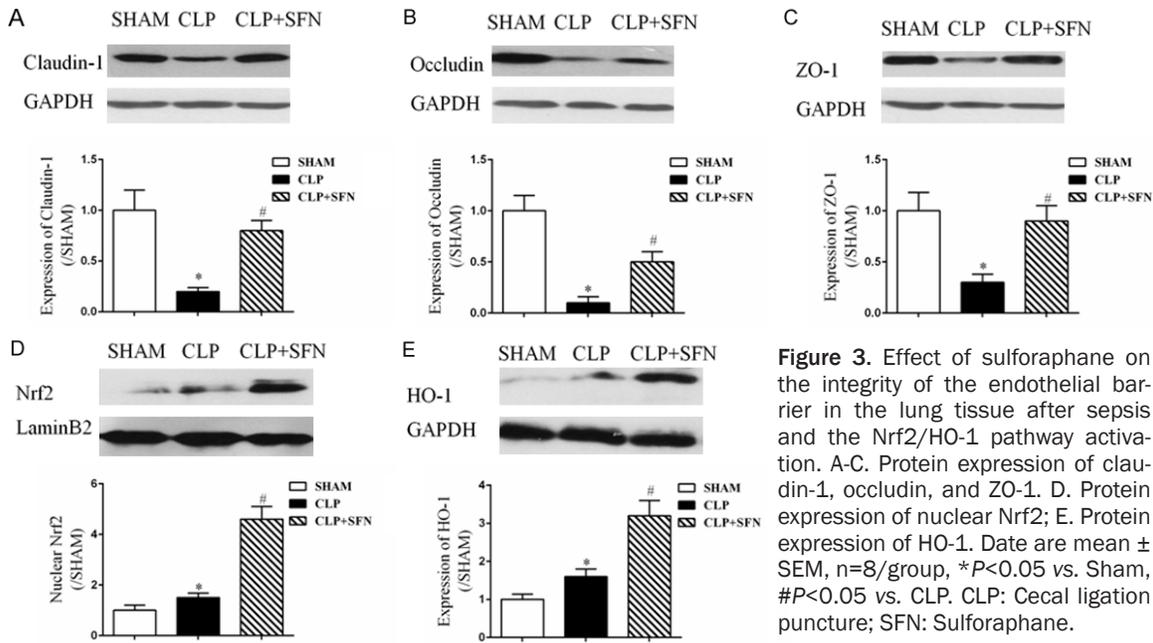
**Figure 2.** Sulforaphane reduced pulmonary oxidative stress induced by sepsis. A. Superoxide dismutase (SOD) activity in the lungs assessed by using an enzyme-linked immunoassay kit; B. 8-OHdG concentration in the lungs determined by using an enzyme-linked immunoassay kit; C. 15-F2t-isoprostane in the lung tissues was measured by using an enzyme-linked immunoassay kit; D. Visualization of expression of 4-hydroxynonenal (4-HNE). Data are mean  $\pm$  SEM,  $n=8$ /group,  $*P<0.05$  vs. Sham,  $\#P<0.05$  vs. CLP. CLP: Cecal ligation puncture; SFN: Sulforaphane.

index, and elevated lung water content (**Figure 1B, 1C**). Application of sulforaphane (SFN) in doses ranging from 1.25 to 5 mg/kg dose dependently attenuated sepsis-induced lung injury evidenced by reduced pathological damage in the lungs, decreased lung injury index, and reduced lung water content (**Figure 1A-C**).

### *Sulforaphane reduced sepsis-induced oxidative stress in the lung*

Oxidative stress is the main cause of sepsis-induced lung injury, we then determined the effects of SFN on sepsis-induced oxidative stress in the lungs. As shown in **Figure 2**, sepsis

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**Figure 3.** Effect of sulforaphane on the integrity of the endothelial barrier in the lung tissue after sepsis and the Nrf2/HO-1 pathway activation. A-C. Protein expression of claudin-1, occludin, and ZO-1. D. Protein expression of nuclear Nrf2; E. Protein expression of HO-1. Data are mean  $\pm$  SEM,  $n=8$ /group, \* $P<0.05$  vs. Sham, # $P<0.05$  vs. CLP. CLP: Cecal ligation puncture; SFN: Sulforaphane.

induced significant oxidative stress in the lung manifested as down-regulated pulmonary SOD activity (**Figure 2A**), enhanced 8-OHdG concentration (marker of oxidative damage to DNA) and 15-F2t-isoprostane level (marker of ROS-induced oxidative stress) in the lungs (**Figure 2B, 2C**), which were associated with enhanced pulmonary 4-HNE expression (marker of lipid peroxidation) (**Figure 2D**). This sepsis-induced oxidative stress was attenuated by SFN pretreatment (**Figure 2A-D**).

### Effect of sulforaphane on the integrity of the endothelial barrier in the lung tissue after sepsis

Maintenance the integrity of endothelial barrier plays the central role in protecting the lung against acute lung injury, we then examined the effect of SFN pretreatment on endothelial barrier in sepsis-induced lung injury. As shown in **Figure 3**, sepsis induced sever endothelial barrier dysfunction in the lungs manifested as down-regulated the expression of three tight junction proteins, claudin-1, occludin, and ZO-1 (**Figure 3A-C**). All these changes were reversed by SFN pretreatment (**Figure 3A-C**).

### Pulmonary protective effects of sulforaphane were associated with activation of Nrf2/HO-1 pathway

The Nrf2/HO-1 signaling pathway plays important role in regulating the balance of antioxidative capacity and oxidative stress, induction of which protects the lung against acute injury.

We then explored the effect of SFN on Nrf2 and HO-1. As shown in **Figure 3**, sepsis induces significant increases of Nrf2 and HO-1 protein expression in the lungs (**Figure 3D, 3E**), while the protein expression of these two proteins were further increased by SFN, indicating that SFN conferred pulmonary protective effects via Nrf2/HO-1 signaling.

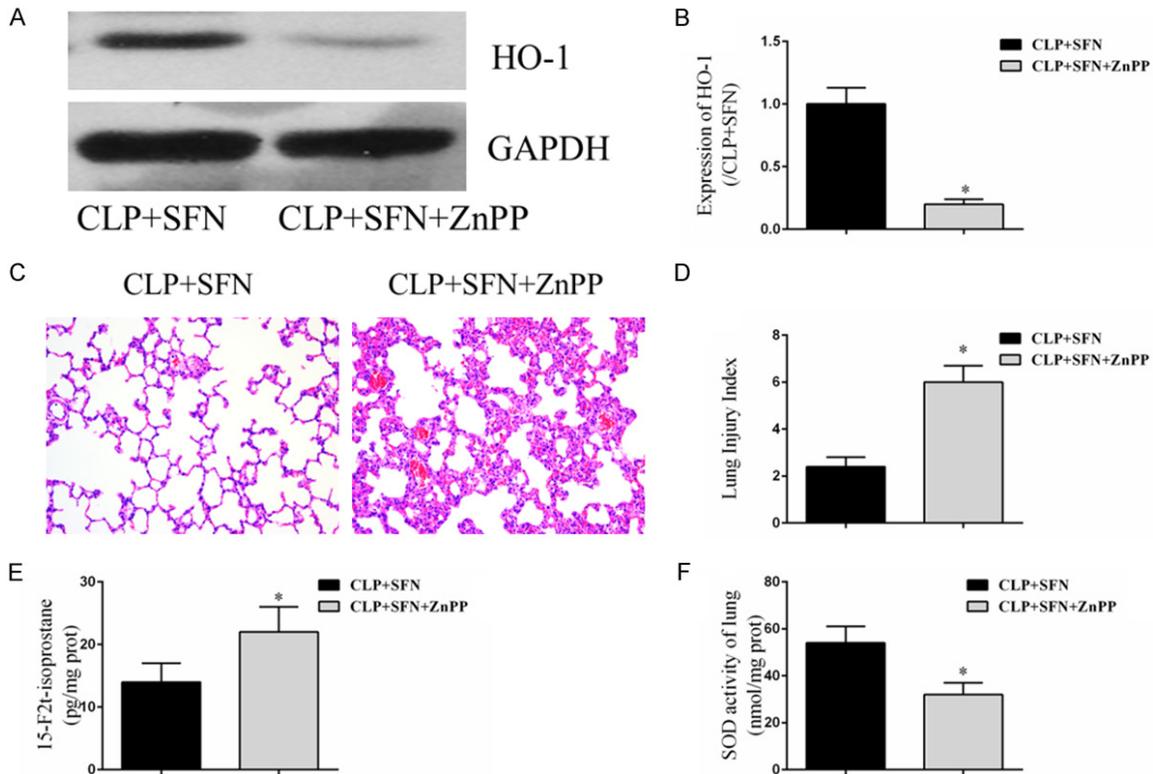
### HO-1 inhibition abolished pulmonary protective effects of sulforaphane

We have showed that SFN conferred its pulmonary protective effects in the lungs from sepsis that were associated with increased of Nrf2/HO-1, we then use HO-1 inhibitor ZnPP to further confirm the role of HO-1 in SFN-induced pulmonary protective effects in sepsis. As shown in **Figure 4**, application of ZnPP significantly decreased SFN-induced HO-1 protein expression in the lung (**Figure 4A, 4B**). Furthermore, HO-1 inhibition abolished SFN-induced pulmonary protective effects evidenced by increased pathological damage in the lungs, elevated lung injury index, enhanced 15-F2t-isoprostane, and decreased SOD activity in the lungs (**Figure 4C-F**).

## Discussion

In the current study, we demonstrated that supplementation of sulforaphane, activated Nrf2 and HO-1, which subsequently reduced pulmonary oxidative damage and attenuated endothelial barrier dysfunction, and eventually at

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**Figure 4.** HO-1 inhibition abolished pulmonary protective effects of sulforaphane. A, B. Representative images and quantitation of HO-1 protein expression; C. H&E-stained sections of lungs ( $\times 400$ ); D. Lung injury index; E. 15-F2t-isoprostane in the lung tissues was measured by using an enzyme-linked immunoassay kit; F. Superoxide dismutase (SOD) activity in the lungs assessed by using an enzyme-linked immunoassay kit. Data are mean  $\pm$  SEM,  $n=8$ /group, \* $P<0.05$  vs. Sham, # $P<0.05$  vs. CLP. CLP: Cecal ligation puncture; SFN: Sulforaphane.

tenuated acute lung injury (ALI) in cecal ligation and puncture-induced sepsis in rats. We provided evidences that in rats with sepsis, oxidative stress-induced damage was existed at different levels in the lung tissues including oxidative damage to DNA, lipid and protein, which can be attenuated by sulforaphane treatment. We further showed that sulforaphane conferred this pulmonary effects were through Nrf2/HO-1 signaling pathway. This was confirmed by using HO-1 inhibitor which showing that inhibition of HO-1 cancelled the beneficial effects of sulforaphane on ALI in sepsis.

Oxidative stress has been recognized as the main factor that contribute to the development of ALI in sepsis [5]. Increase of oxidative stress was associated with lung injuries, while inhibition of oxidative stress attenuates lung injuries in sepsis [6, 7], thus, reduction of oxidative stress by enhancing antioxidant capacity has been considered as an effective treatment in combating lung injuries in sepsis including ALI. Recent studies demonstrated that activation of

Nrf2, a protein with potent antioxidative property, can attenuate ALI [25, 26]. Activation of Nrf2 by direct Nrf2 adenovirus injection or indirect interventions like ischemic postconditioning have been shown to reduce lung injuries in several diseases models such as intestinal ischemia-reperfusion-induced lung injury [27] and paraquat-induced lung injury [28] in mice. All these indicate the critical role of Nrf2 activation in the attenuation of ALI. However, interventions like ischemic postconditioning have to manipulate the lung which itself may induce injury to the lung, while direct injection of adenovirus may cause ethical related issue when applying into the clinics at its current stage. As such, researchers have focus on exploring active phytochemical compound that may activate Nrf2 and thereby attenuate ALI in sepsis. Indeed, most recent studies have shown that zerumbone, the major active phytochemical compound of Zingiber, and gastrodin, a phenolic glucoside derived from *Gastrodia elata* Blume, were effective in activating Nrf2 and attenuated lung injury in lipopolysaccharide (LPS)-

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induced ALI in mice [25, 26]. In the current study, we provided additional evidence that, SFN, an antioxidant derived from cruciferous vegetables, protect the lung against sepsis-induced ALI. Given that SFN has been considered as the inducer/activator of Nrf2 [29, 30], results from our current study support the notion that, application of SFN, would be potent strategy for the treatment of ALI by direct activation of Nrf2. We further showed that this SFN-induced Nrf2 and the subsequent reduction of ALI in sepsis were mainly through inducing HO-1.

Of note, although different doses of SFN was examined in our current study, however, in our study, only one time point of SFN supplementation (before sepsis) was examined, it is possible that supplementation of SFN before may also effective in reducing ALI in sepsis, more investigations are needed to be addressed this concern.

In conclusion, this study demonstrated that SFN, by activating Nrf2, activated HO-1, resulting in reduction of oxidative stress and subsequently attenuated ALI in sepsis.

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### Disclosure of conflict of interest

None.

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