


ORIGINAL ARTICLE

Artesunate Inhibits Renal Ischemia Reperfusion-Stimulated Lung Inflammation in Rats by Activating HO-1 Pathway

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ABSTRACT— Artesunate (AS), a semi-synthetic derivative of Artemisia, has been shown to exert a wide range of pharmacological effects, such as anti-inflammatory and antioxidant functions. However, the protective functions of AS on renal ischemia reperfusion injury (RIR)-stimulated lung inflammation remain unclear. In this research, acute lung injury (ALI) was stimulated by renal ischemia reperfusion injury (RIR). AS (15 mg/kg) was intraperitoneal administrated to rat 1 h before RIR stimulation. Serum and pulmonary NO, MDA, IL-6, MIP-2, and PGE₂ levels, arterial blood gas and biochemistry, lung wet/dry weight ratio and MPO activity, total cell number and protein concentration in BALF, tissue histology, and NF-κB expression were determined. The results indicated that serum and pulmonary NO, MDA, IL-6, MIP-2, and PGE₂ levels, lung wet/dry weight ratio and MPO activity, total cell number, and protein concentration in BALF enhanced after RIR stimulation. These alterations were mitigated by AS. AS attenuated lung wet/dry weight ratio and MPO activity, total cell number, and protein concentration in BALF. AS attenuated RIR-stimulated pulmonary NF-κB phosphorylation. In addition, these previously mentioned actions of AS were antagonized by suppressing HO-1 pathway. However, RIR-stimulated arterial blood gas and biochemistry and lung histopathology were also attenuated by AS. In summary, AS inhibited RIR-stimulated lung inflammation by activating HO-1 pathway.

KEY WORDS: artesunate; renal ischemia reperfusion injury; acute lung injury; HO-1; NF-κB.

INTRODUCTION

Renal ischemia reperfusion injury (RIR) is a common clinical syndrome of kidney inflammation responses with a high rate of complications and death [1]. RIR can result in remote organ injury such as the liver, heart, and lung [2–4]. Lung inflammation are the most common complication

that caused by multiple ischemia reperfusion injury (IR) [5–7]. This experimental model was extensive used to investigate the underling mechanisms of lung inflammation and exploit new lung-protective drugs [1]. Previous reports have demonstrated that RIR-stimulated inflammatory molecules releases in rat model of lung inflammation [8–10]. These inflammatory molecules resulted in lung tissue edema and Pathological damage [11].

Heme oxygenase-1 (HO-1) is a kind of anti-inflammation protein that plays an important role in the development of lung inflammation [12–14]. Nuclear factor-kappa B (NF-κB) signaling plays a critical role in the expression of lung inflammatory mediators mRNA and protein [15, 16]. Researches indicated that NF-κB was activated *via* stimuli, which also enhance the expression

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of HO-1 gene [17]. Upregulates in HO-1 expression in macrophages results in suppression of NF- κ B-induced innate immune responses [18]. Recent studies indicated that enhanced-HO-1 activity ameliorates IR-stimulated lung inflammation [5, 7], and inhibition of NF- κ B activation had protective effects against IR-stimulated lung inflammation [19, 20].

Artesunate (AS), a semi-synthetic derivative of Artemisia, has been shown to possess anti-inflammatory and antioxidant functions [21–25]. Previous researches indicated that AS protects lipopolysaccharide (LPS) or cecal ligation and puncture (CLP)-stimulated lung inflammation through inhibition of NF- κ B activation [21, 25] and attenuates paraquat- and cigarette smoke-induced lung injury by inhibiting the inflammatory response [26, 27]. AS was found to inhibit LPS-induced pro-inflammatory responses in microglia [24]. AS also attenuated hepatic fibrosis stimulated by various inflammation factors *via* inhibiting NF- κ B activation [23]. Moreover, AS has been shown to improve severe acute pancreatitis through inhibition of pro-inflammatory cytokines expression and NF- κ B activation [22]. However, the roles of AS on RIR-stimulated lung inflammation remain unclear. We hypothesized that AS would attenuate the RIR-stimulated lung inflammation *via* activating HO-1 pathway and suppressing NF- κ B pathway.

MATERIALS AND METHODS

Ethics Declaration

Experimental procedures were performed in the accordance with the US NIH Guide for the Care and Use of Laboratory Animals and authorized by the Animal Care and Use Ethics Committee of Shanghai First People's Hospital (Approval ID: 20151210-3).

Materials

AS and SnPP (A specific inhibitor for HO-1) were obtained from Sigma (St. Louis, MO, USA). Malonyldialdehyde (MDA) and myeloperoxidase (MPO) assay kits were purchased from R&D Systems (Minneapolis, MN). NO, IL-6, MIP-2, and PGE₂ ELISA kits were provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against NF- κ B, I κ B- α , and β -actin were provided by the Nanjing Jiancheng Biology Engineering Institute (Nanjing, China).

Animals

Adult male Sprague-Dawley rats (200 to 250 g) were provided by the Center of Experimental Animals of Shanghai First People's Hospital (Shanghai, China). All animals were fed with a standard diet and sterile water under specific pathogen-free conditions.

Experimental Scheme

Rat acute lung injury model was stimulated by RIR. To assess the protection functions of AS on RIR-stimulated lung inflammation, 60 rats were randomly divided into five groups: the sham group, the sham plus AS group, RIR group, RIR plus AS group, and RIR plus AS plus SnPP (A specific inhibitor for HO-1, 30 mg/kg), group. AS (15 mg/kg) was given to the rat 1 h before RIR stimulation. The dose of AS chosen was determined by referring to previous studies [21, 28] and our pre-experiments. The serum and lung samples were gathered 24 h after RIR stimulation for subsequent assay.

Measurement of NO, IL-6, MIP-2, and PGE₂

NO, IL-6, MIP-2, and PGE₂ production in serum and lung samples were determined by applying commercial ELISA kits provided by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) in accordance with the manufacturer's manuals.

MDA and MPO Activities Determination

Serum and pulmonary MDA levels were measured by using MDA assay kit in accordance with the manufacturer's manuals (R&D Systems, Minneapolis, MN). MPO activity in lung samples was measured by applying relevant assay kits provided by R&D Systems (Minneapolis, MN) in accordance with the manuals.

Measurement of Lung Inflammation

Broncho alveolar lavage fluid (BALF) and lung tissues were gathered at 24 h after RIR stimulation. The total cell number in the BALF was calculated with a hemacytometer, and the protein level in the BALF was evaluated by the BCA method. The lung samples collected from the left upper lobe were dried out, and then it was weighed to obtain wet weight and then placed in the oven at 80 °C to get dry weight. Wet/dry weight ratio was calculated as a maker of lung edema.

Histological Evaluation

Lung samples were fixed in 10% buffered formalin and then embedded with paraffin. After fixation and stained with hematoxylin-eosin (H&E), characterizing lung injury was observed by microscopic evaluation.

Western Blot

Lung samples were homogenized and centrifuged, and then nuclear and cytoplasmic proteins was collected from the lung samples in accordance with the method instructed by the protein extract kit (Thermo, USA). Protein levels were measured by applying the Bradford protein assay (Bio-Rad, United Kingdom). Protein extracts were heated, denatured, and loaded on 12% sodium dodecyl sulfate polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The membranes were treated overnight at 4 °C with the primary antibodies. After washing three times, the membranes were treated with HRP-conjugated secondary antibodies at 37 °C for 1 h. Ultimately, the membranes were measured by applying the ECL western blot determination system in accordance with the manufacturer's manual.

Statistical Analysis

All experimental data were expressed as mean \pm SD. The biochemical data were determined using one-way analysis of variance (ANOVA) and Student's *t* test applying the GraphPad 5.0 software. A $P < 0.05$ is considered as statistically significant.

RESULT

AS Suppresses RIR-Stimulated Serum NO, MDA, IL-6, MIP-2, and PGE₂ Production

Serum NO, MDA, IL-6, MIP-2, and PGE₂ production were used to assess the degree of RIR-induced systemic inflammatory responses. In comparison with the sham group, serum NO, MDA, IL-6, MIP-2, and PGE₂ production augmented evidently in RIR group (Fig. 1, $P < 0.05$). Nevertheless, administration of AS suppress RIR-stimulated serum NO, MDA, IL-6, MIP-2, and PGE₂ production (Fig. 1, $P < 0.05$). Meanwhile, the inhibition functions of AS on RIR-stimulated serum NO, MDA, IL-6, MIP-2, and PGE₂ production were reversed by SnPP (A specific inhibitor for HO-1; Fig. 1, $P < 0.05$).

AS Suppresses RIR-Stimulated Pulmonary NO, MDA, IL-6, MIP-2, and PGE₂ Production

Pulmonary NO, MDA, IL-6, MIP-2, and PGE₂ production were used to assess the degree of RIR-induced pulmonary inflammatory responses. In comparison with the sham group, pulmonary NO, MDA, IL-6, MIP-2, and PGE₂ production augmented evidently in RIR group (Fig. 2, $P < 0.05$). Nevertheless, administration of AS suppress RIR-stimulated pulmonary NO, MDA, IL-6, MIP-2, and PGE₂ production (Fig. 2, $P < 0.05$). Meanwhile, the inhibition functions of AS on RIR-stimulated pulmonary NO, MDA, IL-6, MIP-2, and PGE₂ production were reversed by SnPP (A specific inhibitor for HO-1; Fig. 2, $P < 0.05$).

AS Suppresses RIR-Stimulated the Indicators of Pulmonary Inflammation.

The indicators of pulmonary inflammation, such as total cell number and protein in BALF, pulmonary wet/dry weight ratio, and MPO activity, were used to assess the degree of RIR-induced pulmonary inflammation. In comparison with the sham group, the indicators of pulmonary inflammation production augmented evidently in RIR group (Fig. 3, $P < 0.05$). Nevertheless, administration of AS suppress RIR-stimulated the indicators of pulmonary inflammation production (Fig. 3, $P < 0.05$). Meanwhile, the inhibition functions of AS on RIR-stimulated the indicators of pulmonary inflammation production were reversed by SnPP (A specific inhibitor for HO-1; Fig. 3, $P < 0.05$).

Effects of AS on RIR-Stimulated Lung Histopathology

Histological evaluation of lung samples was used to determine the protection functions of AS on RIR-stimulated lung inflammation. As indicated in Fig. 4, lung samples of the RIR group indicated intra-alveolar hemorrhage, alveolar septa edema, and neutrophil exudation. Administration of AS evidently mitigated these pathological alteration stimulated by RIR. However, the inhibition functions of AS on RIR-stimulated lung histopathology were reversed by SnPP (A specific inhibitor for HO-1; Fig. 4, $P < 0.05$).

AS Suppresses RIR-Stimulated Pulmonary NF- κ B Translocation

Pulmonary NF- κ B p65 and p-I κ B- α were used to evaluate the extent of RIR-induced pulmonary NF- κ B translocation. In comparison with the sham group, pulmonary NF- κ B p65 in the nuclear and p-I κ B- α in the

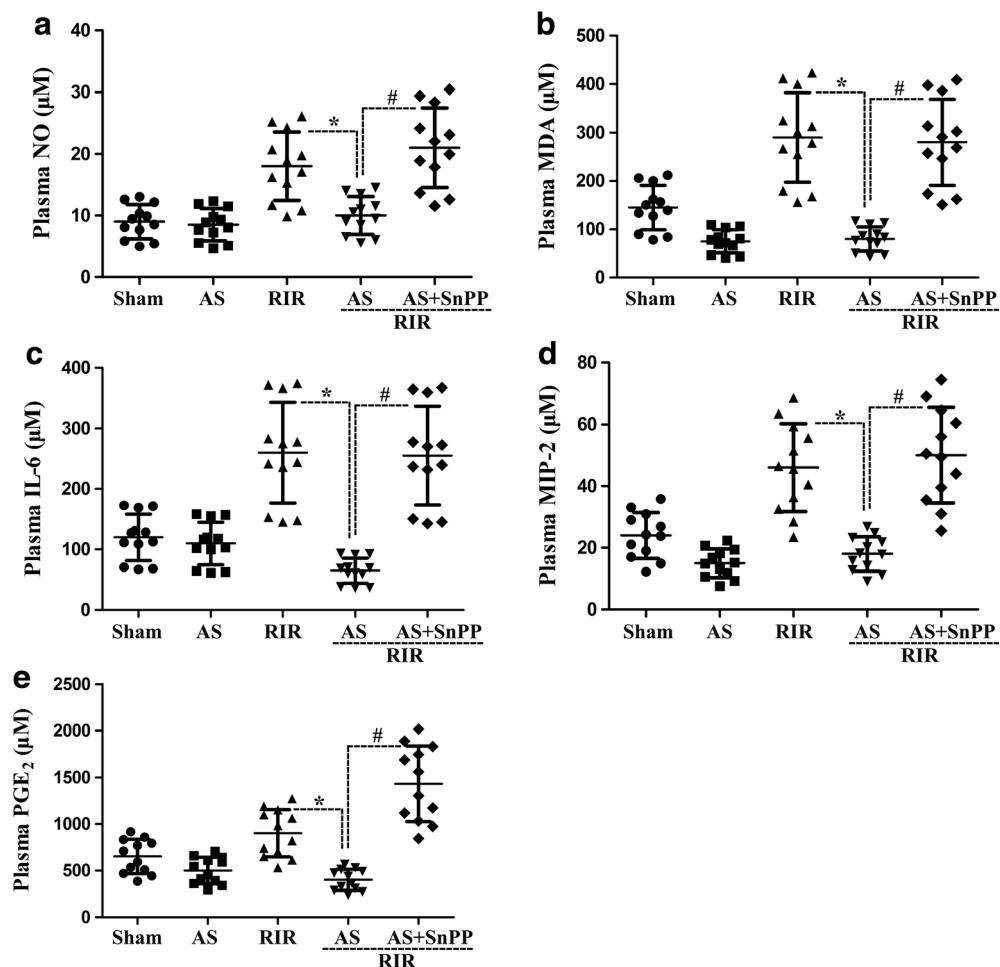


Fig. 1. Effects of artesunate (AS) on serum inflammatory molecule levels after renal ischemia reperfusion injury (RIR) in rats. AS markedly reduces the serum levels of NO (a), MDA (b), IL-6 (c), MIP-2 (d), and PGE₂ (e) in the rat model of RIR. Data were shown as mean \pm SD. * $P < 0.05$ vs. sham group, # $P < 0.05$ vs. RIR group, [§] $P < 0.05$ vs. RIR + AS group.

cytosolic augmented evidently in RIR group (Fig. 5, $P < 0.05$). Nevertheless, administration of AS suppress RIR-stimulated pulmonary NF- κ B translocation (Fig. 5, $P < 0.05$). Meanwhile, the inhibition functions of AS on RIR-stimulated pulmonary NF- κ B translocation were reversed by SnPP (A specific inhibitor for HO-1; Fig. 5, $P < 0.05$).

DISCUSSION

AS, a derivative of artemisinin, has been shown to exert antioxidant and anti-inflammatory functions [28]. Nevertheless, the previous studies did not examine whether AS inhibits the lung inflammation induced by RIR, and

effects the HO-1 level and NF- κ B activity in RIR-induced ALI. RIR-stimulated ALI model in rat was commonly used to investigate original lung protection strategies [29]. In this research, we explored the protective functions of AS on RIR-stimulated ALI in rat. The results have demonstrated that AS attenuated serum and pulmonary inflammatory molecules production, the markers of the degree of lung inflammation. Moreover, histopathological injury evaluation directly demonstrated that AS mitigated RIR-stimulated ALI. AS represents an important therapeutic reagents for mitigating ALI.

Previous reports indicated that lipid peroxidation might participate in RIR-stimulated ALI [30]. MDA, an end product of oxidative injury, enhanced evidently in rat subjected to RIR [31]. In this report, we demonstrated that

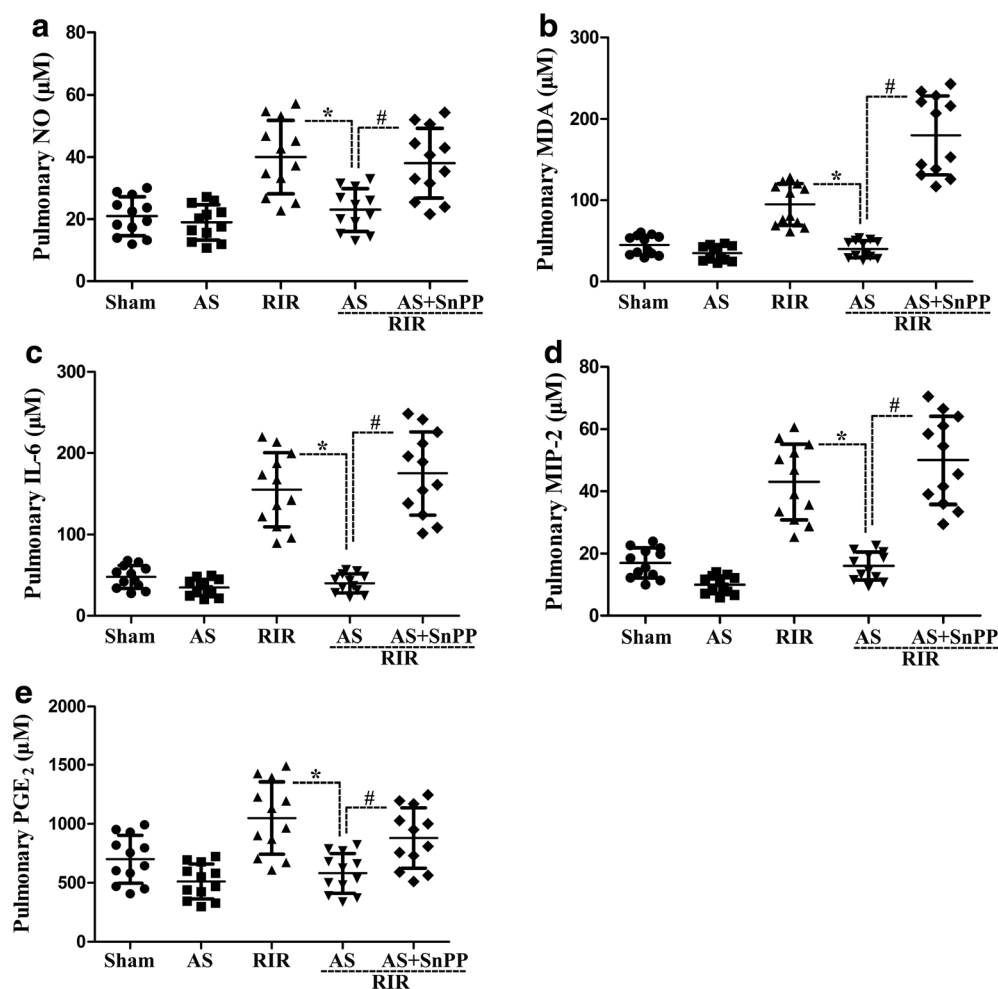


Fig. 2. Effects of artesunate (AS) on pulmonary inflammatory molecule levels after renal ischemia reperfusion injury (RIR) in rats. AS markedly reduces the lung levels of NO (a), MDA (b), IL-6 (c), MIP-2 (d), and PGE₂ (e) in the rat model of RIR. Data were shown as mean \pm SD. * $P < 0.05$ vs. Sham group, # $P < 0.05$ vs. RIR group, ^S $P < 0.05$ vs. RIR + AS group.

AS inhibited RIR-stimulated MDA production. Meanwhile, the levels of total cell number and protein in BALF, pulmonary wet/dry weight ratio, and MPO activity suppressed by RIR were increased by administration of AS. NF- κ B is a transcription factor that adjusts inflammatory molecule gene production, which has been shown to exert anti-inflammatory effects [32]. Reports indicated that inhibition of NF- κ B had protection functions against RIR-stimulated lung inflammation [8]. Consequently, we deduced that AS represented anti-inflammatory functions by inhibiting NF- κ B signaling pathway. In order to validate this hypothesis, the roles of AS on NF- κ B activation were determined. Our results indicated that AS attenuated the activation and translocation of NF- κ B. These data

indicated that AS attenuated RIR-stimulated lung inflammation by inhibiting NF- κ B signaling pathway.

Growing evidences showed that inflammatory molecules, such as NO, IL-6, MIP-2, and PGE₂, had vital functions in the initiating and maintenance of lung inflammation [33]. In this research, elevated NO, IL-6, MIP-2, and PGE₂ production were determined in RIR-induced ALI model. In addition, AS attenuated RIR-stimulated NO, IL-6, MIP-2, and PGE₂ levels. NF- κ B signaling pathway is in charge of processing and regulation of the HO-1 activity [34]. To clarify whether the lung protective action of AS is *via* the activation of HO-1 activity. The actions of AS on RIR-stimulated HO-1 activity were determined in this paper. Our

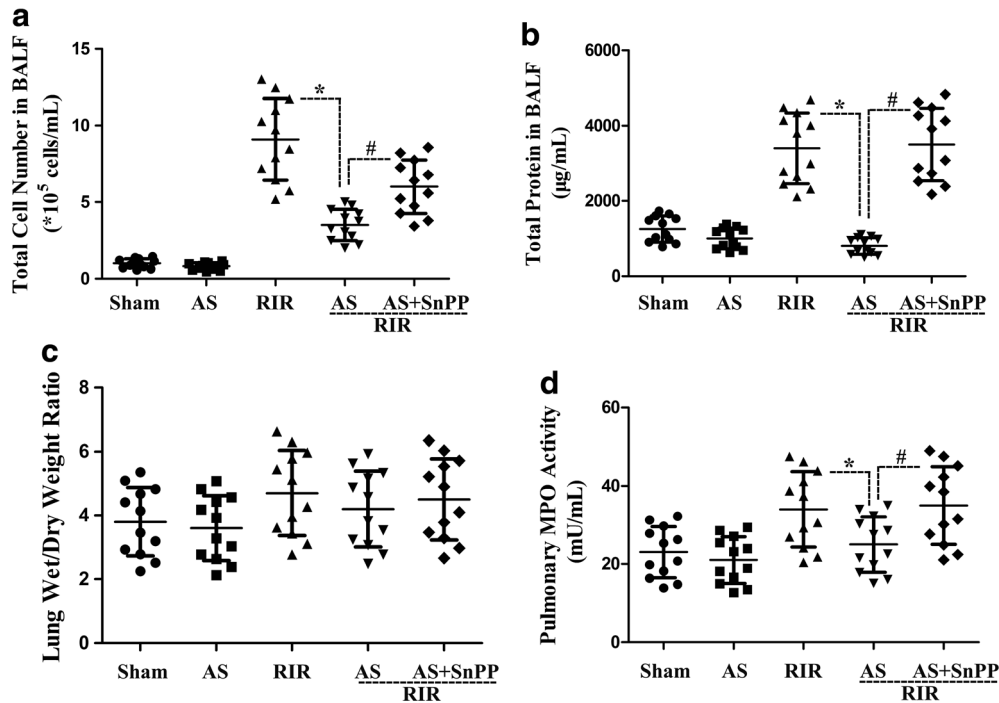


Fig. 3. Effects of artesunate (AS) on pulmonary inflammatory response levels after renal ischemia reperfusion injury (RIR) in rats. AS markedly reduces the BALF levels of total cell number (a) and total protein concentration (b) and the lung levels of wet/dry weight ratio (c) and MPO activity (d) in the rat model of RIR. Data were shown as mean \pm SD. * $P < 0.05$ vs. Sham group, # $P < 0.05$ vs. RIR group, $^{\$}P < 0.05$ vs. RIR + AS group.

results indicated that HO-1 activity enhanced evidently following RIR administration. AS enhanced RIR-stimulated HO-1 activity. Lately, it has been shown

that HO-1 adjusted NF- κ B phosphorylation in RIR-stimulated lung inflammation. Reports indicated that HO-1 activity was indispensable to the NF- κ B

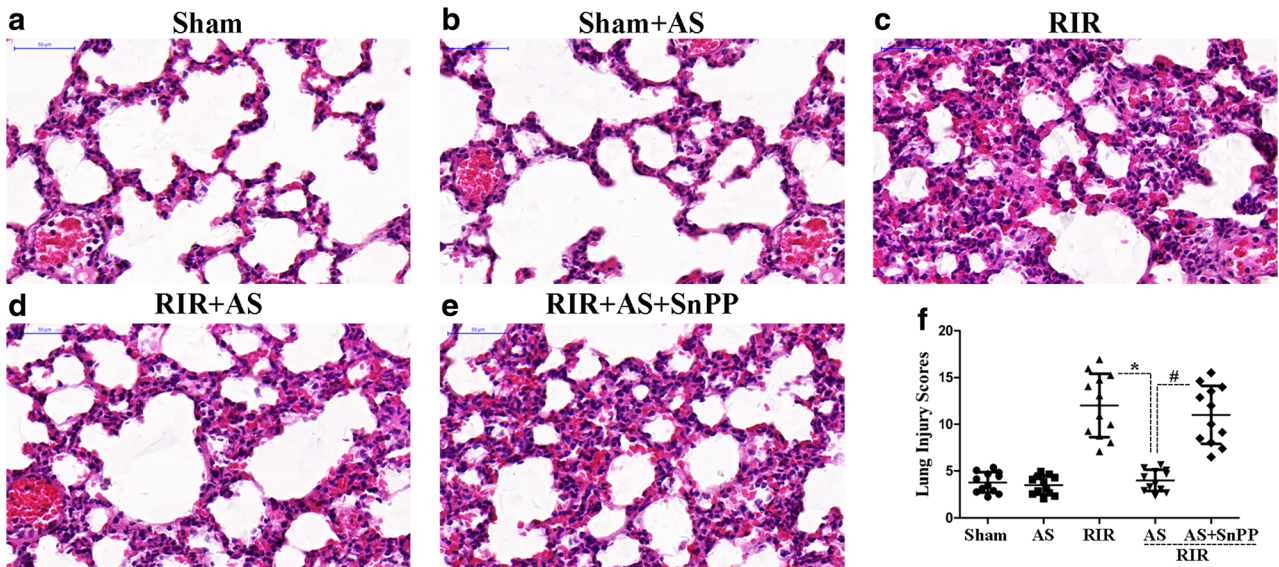


Fig. 4. Artesunate (AS) ameliorate RIR-induced histologic changes in the lung. a Sham group. b AS group. c RIR group. d RIR + AS group. e RIR + AS + SnPP group (HE $\times 200$). f The lung injury scores. Data were shown as mean \pm SD. * $P < 0.05$ vs. Sham group, # $P < 0.05$ vs. RIR group, $^{\$}P < 0.05$ vs. RIR + AS group.

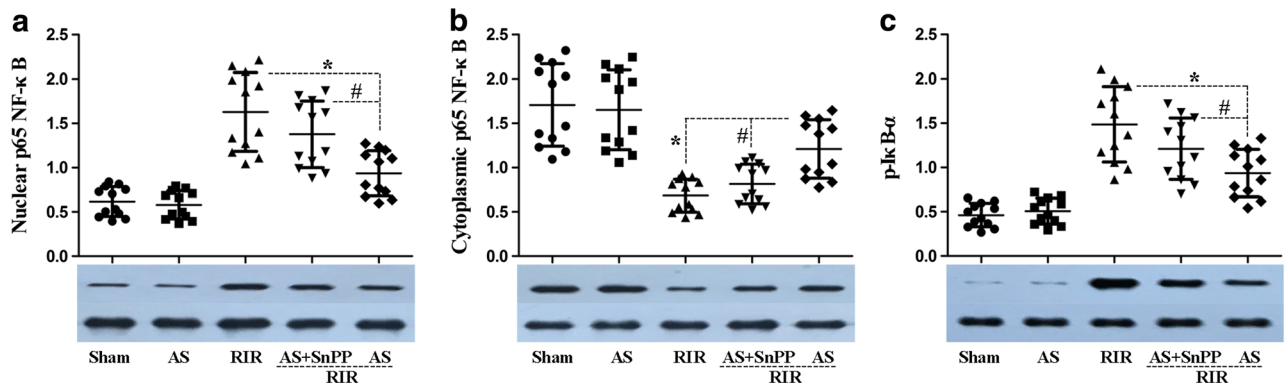


Fig. 5. Effects of artesunate (AS) on pulmonary NF- κ B activation after renal ischemia reperfusion injury (RIR) in rats. NF- κ B (p65) in the nucleus (a), NF- κ B (p65) in the cytosol (b), and p-I κ B- α (c) in the rat model of RIR was detected by western blot. Data were shown as mean \pm SD. * P < 0.05 vs. Sham group, # P < 0.05 vs. RIR group, § P < 0.05 vs. RIR + AS group.

phosphorylation [35]. To further elucidate the potential mechanism, the functions of AS on RIR-stimulated HO-1 activity were determined. The results demonstrated that AS mitigated HO-1 activity was stimulated by RIR. And the upregulation of AS on the HO-1 stimulated by RIR may result from the decreased expression of NF- κ B phosphorylation.

An important limitation of the current study is the pretreatment with AS. It would be more clinically relevant if administered after RIR. In addition, the *in vivo* nature of the findings was an important limitation of the current study. We will add cell mechanism studies in future experiments. In this paper, we are not trying to multiple doses of AS *in vivo*. Therefore, the results showed the inhibition effects of AS on RIR-induced ALI were not dose-dependent. Investigation on the multiple doses of AS *in vivo* is warranted in the future studies. The tolerance dose of AS may be different between humans and animals; thereby, the clinical use of AS for its anti-inflammatory property still needs further careful evaluation.

In summary, our studies indicated that AS serves as a novel approach to protect RIR-stimulated lung inflammation. AS confers protection against RIR-stimulated lung inflammation by up-regulating the HO-1 signal pathway and suppressing NF- κ B phosphorylation. AS may be an important therapeutic strategy for mitigating RIR-induced ALI.

AUTHORS' CONTRIBUTIONS

Zhaohui Liu: conception, design, analysis and interpretation of data; writing the manuscript. Junjie Zhang: conception, design, analysis and interpretation of data;

writing the manuscript. Shitong Li: analysis and interpretation of data; writing the manuscript. Jihong Jiang: conception, design, analysis and interpretation of data; writing the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

All procedures were performed in accordance with the Declaration of Helsinki of the World Medical Association. The study was approved by the ethics committee of Shanghai First People's Hospital, Shanghai, China.

Conflicts of Interest. The authors declare that they have no conflict of interest.

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