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Shen-su-yin, a Traditional Herbal Medicine, Attenuates Lipopolysaccharide-induced Acute Lung Injury in Rats Through its Anti-inflammatory and Antioxidative Effects

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ABSTRACT The present study was designed to investigate potential effects and mechanisms of Shen-su-yin (SSY) on lipopolysaccharide (LPS) induced acute lung injury (ALI) in rats. Forty-eight rats were randomly divided into 4 groups of control (Ctrl), LPS-induced ALI (ALI), low- (SSY-LD) and high- (SSY-HD) dose SSY-treated ALI group. Twenty-four hours after SSY administration, bronchoalveolar lavage fluid determination, Western blot analysis, and histopathological analysis were performed. The results showed that SSY administration 24 hours post-treatment showed improved lung histopathology, reduced pro-inflammatory and oxidative markers, and increased anti-inflammatory and antioxidative cytokines. SSY also inhibited NF-êB signalling and activated the Keap1-Nrf2-ARE pathway. The high-dose SSY group exhibited significant improvements. The results indicate that the preventive use of SSY can alleviate ALI through the anti-inflammatory and antioxidant effects mediated by inhibition of NF-êB signalling pathway and activation of Keap1-Nrf2-ARE signalling pathway, respectively
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INTRODUCTION

Acute lung injury (ALI) and its severe form, acute respiratory distress syndrome (ARDS), are critical conditions with high mortality rates, primarily caused by inflammation and oxidative stress. Current treatments, including antibiotics, glucocorticoids, and mechanical ventilation, have significant limitations and side effects. Traditional Chinese medicine, such as Shen-su-yin (SSY), has shown promise as a therapeutic option due to its anti-inflammatory and antioxidative properties. This study aims to investigate the therapeutic effects and underlying mechanisms of SSY on LPS-induced ALI in rats.

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The present work is derived from the thesis entitled Shen-su-yin, a Traditional Herbal Medicine, Attenuates Lipopoly saccharide-induced Acute Lung Injury in Rats Through Its Anti-inflammatory and Antioxidative Effects submitted by Peng Xiao at Shengli Clinical Medical College of Fujian Medical University, Fuzhou, Fujian, PR China under the supervision of Wuhong Zheng in the year

Objectives

The primary objectives of this study are to:

1. Determine the effects of SSY on lung histopathology and inflammatory responses in LPS-induced ALI rat models.
2. Evaluate the role of SSY in modulating oxidative stress in ALI.
3. Explore the molecular mechanisms underlying the potential therapeutic effects of SSY in ALI.

Recent Literature Review

Recent studies have highlighted the pivotal role of inflammation and oxidative stress in the pathogenesis of ALI/ARDS (Fan et al. 2018; Will-

iams et al. 2021; Bezerra et al. 2023). Traditional treatments, while effective to some extent, are associated with significant adverse effects (Fan et al. 2018; Williams et al. 2021). In contrast, Chinese traditional medicine preparations like YuPingFeng-San and Xuanfei Baidu formula have shown efficacy in animal models of ALI (Li et al. 2023; Wang et al. 2023; Zhu et al. 2023). SSY, with its recorded use in respiratory inflammatory diseases (Jeon et al. 2015; Kim et al. 2020; Kim et al. 2021), presents a novel therapeutic approach that has not been explored for ALI/ARDS. This gap in research motivated the current study to examine the potential benefits of SSY in treating ALI and to elucidate its underlying mechanisms.

Acute lung injury (ALI) is a life-threatening disease caused by a variety of intrapulmonary and extrapulmonary factors, leading to progressive hypoxemia, lung oedema, and acute respiratory distress syndrome (ARDS), accounting for approximately 10 percent of intensive care unit admissions worldwide (Fan et al. 2018). Evidence indicates that inflammation and oxidative stress are the critical mechanisms behind it, resulting in diffuse alveolar exudation, hyaline membrane formation, and alveolar and capillary epithelial damage and gradually initiating a pathophysiological process of ALI, including a ventilation/perfusion defect, worsened compliance, and reduction of lung volume (Williams et al. 2021; Bezerra et al. 2023).

Current therapies for ALI mainly focus on anti-infection using anti-pathogenic microorganism drugs, anti-inflammation with glucocorticoids, and respiratory support with oxygen therapy and/or mechanical ventilation, and have disadvantages including development of opportunistic infection, immunodeficiency, gastrointestinal haemorrhage, and ventilator associated lung injury, which are vital reasons for high mortality of ALI/ARDS (Fan et al. 2018; Williams et al. 2021). Chinese traditional medicine preparations such as YuPingFengSan, Xuanfei Baidu formula, and Jinhua Qinggan granules have emerged as promising therapies for ALI in animal models due to their high efficiency, low-cost, easy accessibility, and low adverse effect rates (Li et al. 2023; Wang et al. 2023; Zhu et al. 2023). Shen-su-yin (SSY), which is originally recorded in the Prescriptions of the Bureau of Taiping People's Welfare Pharmacy during the Song Dynasty in China, is a traditional Chinese herb formula consisting of 13 kinds of Chinese herbs. It is mainly used in the treatment of respiratory inflammatory diseases, including common cold, pneumonia, laryngitis, and pharyngitis, and has a wide range of pharmacological activities, including anti-allergic, anti-inflammatory, anti-asthmatic, and immunomodulatory effects (Jeon et al. 2015; Kim et al. 2020; Kim et al. 2021).

Considering these properties, the researchers hypothesised that SSY might provide beneficial effects for ALI. However, as far as is known, there are no current studies reporting the therapeutic effects of SSY on ALI, nor yet its possible molecular mechanisms. Therefore, this study was conducted to investigate its potential effects and mechanisms on rats with lipopolysaccharide (LPS)-induced ALI.

MATERIAL AND METHODS

Preparation of SSY

All the herbs purchased from Beijing Tong Ren Tang Co. Ltd. (Beijing, China) were prepared according to the original SSY prescription (Table 1) in the Prescriptions of the Bureau of Taiping People's Welfare Pharmacy. The crude herbs were chopped, mixed, and soaked with 1000 ml distilled water (DW) at room temperature for 30 minutes. Then, they were boiled at 100°C for another 30 minutes in an extractor to obtain SSY decoction. The researchers collected, filtered, and evaporated the decoction to 50 ml (5.0 g of crude herbs were extracted to 1 ml decoction) for further investigation.

Animals

7-week-old male Sprague–Dawley (SD) rats (200–230 g) were purchased from the Shanghai SLACCAS Laboratory Animal Co. Ltd. (Shanghai, China). They had free access to chow and water and were housed in a standard animal facility (temperature between 22°C and 24°C; humidity at 50 percent-60 percent; day and night cycle of 12:12). After 7-day acclimatisation, the researchers randomly divided 48 rats into 4 groups of 12 rats each as control (Ctrl group), LPS-induced ALI (ALI group), and low-dose (SSY-LD group) and high-dose (SSY-HD group) SSY-treated ALI group. The researchers injected rats in ALI and SSY-treated groups with a single dose of 7.5 mg/kg LPS (Sigma-Aldrich Co. St. Louis, MO, USA) through the

Table 1: The prescription of Shen-su-yin

Chinese herbal pieces	*Chinese Pinyin*	*Weight (g)*	*Proportion(%)*
Ginseng Radix et Rhizoma	Renshen	23.4375	9.3750
Perillae Folium	Zisuye	23.4375	9.3750
Puerariae Lobatae Radix	Gegen	23.4375	9.3750
Peucedani Radix	Qianhu	23.4375	9.3750
Poria	Fuling	23.4375	9.3750
Pinelliae Rhizoma	Banxia	23.4375	9.3750
Zingiberis Rhizoma Recens	Shengjiang	15.6250	6.2500
Jujubae Fructus	Dazao	15.6250	6.2500
Aurantii Fructus	Zhiqiao	15.6250	6.2500
Platycodonis Radix	Jiegeng	15.6250	6.2500
Citri Reticulatae Pericarpium	Chenpi	15.6250	6.2500
Glycyrrhizae Radix et Rhizoma Praeparata Cum Melle	Zhigancao	15.6250	6.2500
Aucklandiae Radix	Muxiang	15.6250	6.2500
Total		250.0000	100.00

tail vein to induce ALI as described by Hagiwara et al. (2009) and injected rats in the Ctrl group with an equivalent volume of normal saline (NS). Immediately after LPS administration, the researchers administered SSY-LD group rats an animal equivalent dose (AED) of SSY (5.2 ml/kg) + 10.4 ml/kg DW and SSY-HD group rats 3 times AED of SSY (15. 6 ml/kg) by gavage, and rats in Ctrl and ALI groups were given 15.6 ml/kg DW. AED was calculated as follows:

AED (g/kg) = Human dose of crude herbs (g/kg) ×6.2 (Nair et al. 2016).

Human dose of crude herbs used in this study was converted into 4.2 g/kg (Human dose of crude herbs = Total weight of crude herbs/Body weight=250 g/60 kg=4.2 g/kg) according to the Prescriptions of the Bureau of Taiping People's Welfare Pharmacy.

After 24 hours, the researchers anaesthetised all rats with 1 percent pentobarbital sodium (40 mg/kg) and measured their mean arterial pressure (MAP) and heart rate (HR) from the carotid artery using a PowerLab data acquisition system (AD Instruments, Sydney, Australia). After collecting hemodynamic data, the researchers collected the arterial blood for blood gas analysis and lactic acid determination. The researchers calculated oxygenation index (OI) using the following equation:

OI=Arterial partial pressure of oxygen (PaO_2)/ Fraction of inspired oxygen (FiO_2).

Finally, the researchers sacrificed all animals by exsanguination.

The animal protocol was in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health and was approved by the Laboratory Animal Welfare and Ethics Committee of the Fujian Provincial Hospital (Fuzhou, China).

Bronchoalveolar Lavage Fluid (BALF) Tests

The researchers performed bronchoalveolar lavage (BAL) in the left lungs of rats, repeated them twice using 2 ml NS each time, and mixed the retrieved lavage samples and centrifuged (200×*g* for 10 minutes at 4°C) them before further investigations. The researchers detected protein concentration and cytokines levels (TNF-α, IL-1β, IL-6, CXCL2, and IL-10) in the supernatant using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA, USA) and enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, MA, USA), according to the manufacturers' introductions. The researchers re-suspended the pellets in 1.0 ml phosphate buffered solution (PBS) and used a cell counter to determine the total number of nucleated cells in BALF. The re-suspended BALF samples were then centrifuged (200×*g* for 10 minutes at 4°C) onto slides. The researchers stained the slides with a Wright-Giemsa stain kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) to further count the number of neutrophils using a haemocytometer.

Histopathology and Wet-to-dry (W/D) Weight Ratio Analysis

The researchers excised and immediately weighed the middle lobe of the right lung to mea-

sure its wet weight and placed the lung sample in an oven at 65°C for 60 hours for dry weight detection. For histopathological analysis, the researchers harvested lung tissues and fixed them with 10 percent neutral formalin (pH 7.40), embedded them into paraffin and sectioned them (4-μm slices). After deparaffinization, the researchers stained the sections with haematoxylin and eosin (Nanjing Jiancheng Bioengineering Institute) for microscopic observation (Nikon, Tokyo, Japan). The researchers semi-quantitatively scored the injury degree of each tissue slice using previously published criteria, with some modifications, like:

- 0 for no injury
- 1 for injury less than or equal to 10 percent of the field
- 2 for injury more than 10 percent but less than or equal to 20 percent of the field
- 3 for injury more than 20 percent but less than or equal to 30 percent of the field
- 4 for injury more than 30 percent but less than or equal to 40 percent of the field
- 5 for injury more than 40 percent but less than or equal to 50 percent of the field
- 6 for injury more than 50 percent but less than or equal to 60 percent of the field
- 7 for injury more than 60 percent but less than or equal to 70 percent of the field
- 8 for injury more than 70 percent but less than or equal to 80 percent of the field
- 9 for injury more than 80 percent but less than or equal to 90 percent of the field
- 10 for diffuse injury (Li et al. 2022).

Three pathologists blinded to the experimental procedures analysed each slice, and the researchers calculated the average score as the final lung injury score.

Oxidative Stress and Superoxide Dismutase (SOD) Activity Measurements

The researchers homogenised lung tissues in PBS, centrifuged (5000×*g* for 10 minutes at 4°C) the samples, collected the supernatant for detecting peroxidation products using hydroxyl free radical (.OH; Nanjing Jiancheng Bioengineering Institute), hydrogen peroxide (H_2O_2; Abcam), and malondialdehyde (MDA; Abcam) assay kits, and evaluated antioxidant activity using a commercial SOD activity assay kit (Abcam), according to the manufacturers' instructions.

Western Blot Analysis

The researchers homogenised the stored frozen lung tissues in an ice-cold lysis buffer and centrifuged (12000×*g* for 10 minutes at 4°C) the samples. After 10 minutes of boiling, the researchers determined protein concentrations using a BCA protein assay kit (Thermo Fisher Scientific), separated the protein samples (20 μg) in 8 to 10 percent SDS-PAGE and transferred them onto PVDF membranes. The researchers blocked membranes with 5 percent fat-free milk at room temperature for 1 hour, and then incubated overnight at 4°C with their respective primary antibodies [mouse anti-β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-NF-κB p65/Phospho-NF-κB p65/IκBα/Phospho-IκBα (1:1000/1:1000/1:1000/1:1000; Cell Signalling Technology, Beverly, MA, USA); rabbit anti-IKKβ/Phospho-IKKβ (1:1500/1:500; Abcam) or rabbit anti-Keap1/Nrf2/NQO1/GCLC/HO-1 (1:500/1:1500/1:10000/1:400/1:2000; Abcam)]. After three washes, the blots were incubated with HRP-labelled goat anti-mouse/rabbit IgG secondary antibody (1:5000/1:2000; Abcam) for 1 hour at room temperature. Then, the researchers washed the membranes three times again and exposed them to a chemiluminescence luminol solution. Finally, the researchers obtained the blots using a ChemiDoc XRS imaging system (Bio-Rad, California, American) and measured the band densities using an ImageJ software (National Institutes of Health, USA). β-actin was used as an internal reference.

Statistical Analysis

The researchers first expressed quantitative data as the mean ± standard deviation (SD). Data normality and homogeneity of variances were verified by Shapiro-Wilk test and Levene test, respectively. If data have a normal distribution and homogeneity of variance, one-way analysis of variance (ANOVA) was performed, followed by a least significant difference (LSD) test for multiple comparisons, otherwise, the data were expressed as the median (lower- and upper-quartile) [median (LQ, UQ)] and Kruskal-Wallis test was applied, followed by a *post-hoc* analysis of all pairwise comparisons with Bonferroni correction. The researchers analysed all statistical

data using the SPSS 19.0 software (IBM, Armonk, NY, USA). P-values < 0.05 were considered statistically significant.

RESULTS

Effects of SSY on Vital Signs and on Blood Lactate Level

All animals survived 24 hours after LPS-induction. LPS-injected rats presented a series of physical signs of illness, for example, polypnea, bradykinesia, and lethargy. SSY-LD and SSY-HD group rats showed similar signs but with less severity (especially SSY-HD group rats). Twenty-four hours after LPS induction, the OI and MAP levels in ALI rats significantly decreased, and the HR and blood lactate levels (a marker for histanoxia and circulatory disturbances) increased in comparison with those of control rats. Compared with ALI group rats, SSY-LD group rats had increased OI and MAP levels and presented reduced HR and blood lactate levels. Moreover, the OI and MAP levels further increased and the HR and lactate levels were lower in the SSY-HD group than in the SSY-LD group (Fig. 1A-D). Overall, these results showed that SSY could relieve the respiratory and circulatory dysfunctions in LPS-induced ALI rats.

Figure 1A-D presents a clear trend of improvement in respiratory and circulatory parameters with increasing SSY doses. The data are presented in a graphical format, which makes it easy to visualise the differences between groups. The x-axis represents the groups, while the y-axis indicates the physiological parameters (OI, MAP, HR, blood lactate levels). The bars represent the mean values, with error bars showing the standard deviation. The clear separation of the SSY-treated groups from the ALI group emphasises the therapeutic potential of SSY. SSY can significantly improve vital signs and reduce blood lactate levels in LPS-induced ALI rats, suggesting that SSY has the potential to mitigate the severity of ALI and its associated complications.

SSY Improved Lung Histopathological Changes in ALI Rats

After 24 hours, the lung tissues from rats with untreated ALI showed severe epithelial cell necrosis, alveolar exudation, haemorrhage, and inflammatory cell infiltration, with capillary congestion, interstitial oedema, thickened alveoli septa, and small airway occlusion. Conversely, although SSY-treated group rats also displayed slight-to-moderate lung injury, the severity (especially in SSY-HD group) was less than that in untreated ALI group rats (Fig. 1E). Semi-quantitative analysis further showed that the level of lung injury score in the SSY-HD group was significantly reduced in comparison with the ALI group. Compared with the ALI group, although the level of lung injury score in the SSY-LD group had no statistical significance, it had a trend towards lower levels (Fig. 1F). The researchers further quantified the effect of SSY on LPS-induced lung injury by calculating values for the lung W/D ratio (an index for tissue oedema) and measuring the total protein concentration in BALF (an epithelial permeability marker). LPS caused significantly higher levels of W/D ratio and protein exudation in ALI rats than in control rats. Both of them decreased, especially at a high dose, after SSY treatment, indicating that SSY alleviated the lung tissue pathology in ALI rats (Fig. 1G-H).

Figures 1E-H provide visual and quantitative evidence of the protective effects of SSY on lung histopathology in ALI rats. Figure 1E shows histopathological images of lung tissue sections stained with hematoxylin and eosin (H and E), with clear differences in injury severity between the groups. The arrows indicate specific pathological features. Figure 1F presents the semi-quantitative lung injury scores, which are a numerical representation of the severity of lung injury observed in Figure 1E. The error bars represent the standard deviation, and the asterisks indicate statistically significant differences between the groups.

Figures 1G and 1H display the lung W/D ratio and BALF protein concentration, respectively, as quantitative measures of lung edema and epithelial permeability. The y-axis represents the values of these indices, while the x-axis denotes the different treatment groups. The bars indicate the mean values, and the error bars represent the standard deviation. The statistical significance is indicated by asterisks, with different symbols denoting the level of significance. The histopathological analysis and the quantitative measures of lung injury presented in Figures 1E-H provide compelling evidence that SSY treatment can significantly improve the histopathological changes associated with LPS-induced ALI. This improvement is dose-de-

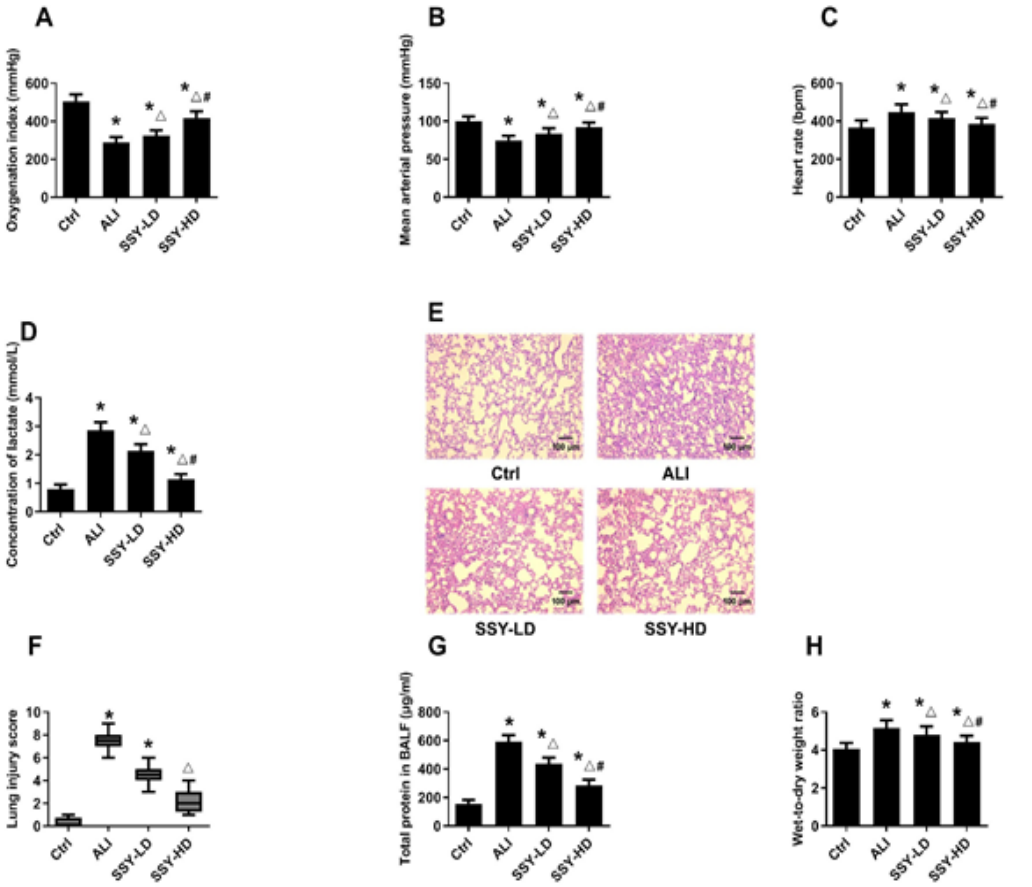

Fig. 1. SSY improved respiratory and circulatory disorders in ALI rats

(A-D) SSY improved the oxygenation index and mean arterial pressure in ALI rats, and reduced the heart rate and lactate levels. (E, F) 24 hours after LPS administration, the HE (×100) staining showed that lung sections of SSY-treated rats were less injured than those of untreated ALI rats. The level of lung injury score in SSY-HD group was lower than that in ALI group. Scale bar, 100 μm. Boxes and horizontal lines represent the 25–75 percent interquartile range and the median, respectively; the up and down whiskers represent the highest and lowest lung injury scores, respectively. (G, H) The BALF protein content and the wet-to-dry weight ratio were significantly reduced in the ALI rats given SSY than in those receiving only distilled water. Data are presented as mean ± SD or median (LQ, UQ) (n=12 per group). $^{*}P$ <0.05 vs. Ctrl group, ΔP <0.05 vs. ALI group, $^{\#}P$ <0.05 vs. SSY-LD group. BALF, bronchoalveolar lavage fluid; SSY, Shen-su-yin; ALI, acute lung injury; LD, low-dose; HD, high-dose; SD, standard deviation; LQ, lower-quartile; UQ, upper-quartile

pendent, with the SSY-HD group showing the most pronounced beneficial effects. These results support the hypothesis that SSY has a protective effect on the lungs, potentially due to its anti-inflammatory and anti-edema properties.

SSY Attenuated Lung Inflammation Associated with LPS

LPS caused lung infiltration of total leukocytes and neutrophils and increased expression of pro-

inflammatory mediators including CXCL2 (MIP-2 in mouse), TNF-α, IL-1β, and IL-6 in the BALF after 24 hours. SSY partially prevented rats in the ALI group from inflammatory reactions, and the effects were observed more obvious in the SSY-HD group than in the SSY-LD group (Fig. 2A-F). LPS also led to an increase in IL-10 levels, which is a kind of anti-inflammatory cytokine. Surprisingly, SSY intervention further increased the IL-10 levels. Moreover, the IL-10 level in the SSY-HD group was higher than that in the SSY-LD group (Fig. 2G).

Figures 2A-G present the inflammatory response in the lungs of ALI rats and the effect of SSY treatment. Figures 2A-F show the levels of the pro-inflammatory mediators CXCL2, TNF-á, IL-1â, and IL-6, with the x-axis representing the treatment groups and the y-axis representing the levels of these mediators. The bars represent the mean values, and the error bars represent the standard deviation. The asterisks denote statistically significant differences between the groups. Figure 2G shows the levels of IL-10, an anti-inflammatory cytokine, with similar

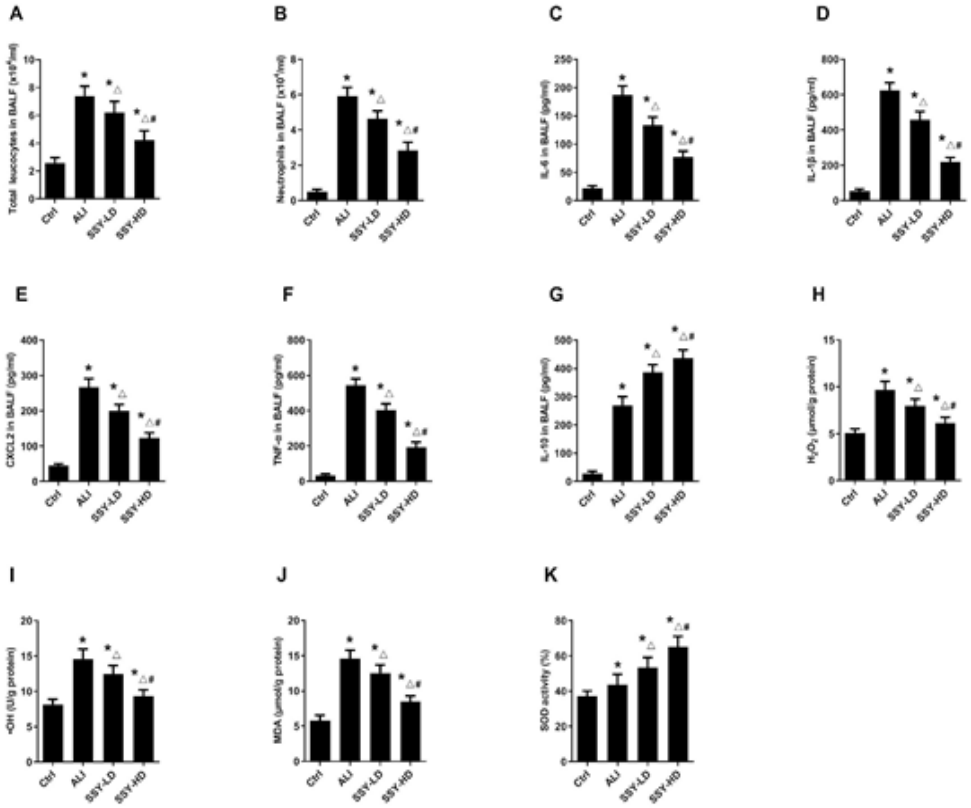

Fig. 2. SSY alleviated inflammation and oxidative stress in LPS-induced ALI rats

(A-G) SSY reduced levels of pro-inflammatory mediators and promoted expression of anti-inflammatory cytokine in ALI rats. (H-J) SSY suppressed levels of oxidative stress in SSY-treated ALI rats. (K) LPS increased SOD activity, and the activity was further elevated in SSY-treated groups. Data are presented as mean ± SD (n=12 per group). $^{*}P$ <0.05 vs. Ctrl group, ΔP <0.05 vs. ALI group, $^{\#}P$ <0.05 vs. SSY-LD group. BALF, bronchoalveolar lavage fluid; IL, interleukin; CXCL2, chemokine (C-X-C motif) ligand 2; TNF, tumor necrosis factor; H_2O_2, hydrogen peroxide; ·OH, hydroxyl free radical; MDA, malondialdehyde; SOD, superoxide dismutase; SSY, Shen-su-yin; ALI, acute lung injury; LD, low-dose; HD, high-dose; SD, standard deviation

formatting as Figures 2A-F. The increased levels of IL-10 in the SSY-treated groups suggest that SSY may stimulate the production of this cytokine, which could be part of its mechanism of action in reducing inflammation.The data presented in Figures 2A-G indicate that SSY has a significant anti-inflammatory effect in LPS-induced ALI, reducing the levels of pro-inflammatory mediators and enhancing the levels of anti-inflammatory cytokines. This dual action on the inflammatory response suggests that SSY could be a promising therapeutic agent for the treatment of ALI.

SSY Alleviated Oxidative Stress Levels in Rats with ALI

The levels of H_2O_2, ·OH, and MDA (oxidative stress indicators) were higher in lung tissues of ALI rats than those of control rats, and these alterations obviously alleviated in SSY-treated ALI rats in comparison with ALI rats (Fig. 2H-J). Compared with control group rats, the SOD activity (a biomarker for anti-oxidation) of ALI group rats was significantly increased, and, interestingly, SSY seemed to further enhance its activity (Fig. 2K). In addition, levels of oxidative stress were lower and antioxidant activity was higher in SSY-HD group rats than in SSY-LD group rats (Fig. 2H-K).

Figures 2H-J illustrate the levels of oxidative stress markers H_2O_2, ·OH, and MDA in lung tissues, with the x-axis representing the treatment groups and the y-axis representing the levels of these markers. The bars represent the mean values, and the error bars represent the standard deviation. The asterisks indicate statistically significant differences between the groups. Figure 2K presents the SOD activity as a marker of antioxidant defence. The formatting is consistent with Figures 2H-J, with clear visualisation of the enhanced antioxidant activity in the SSY-treated groups compared to the ALI group. The data presented in Figures 2H-K demonstrate that SSY has a potent antioxidant effect in LPS-induced ALI, reducing oxidative stress markers and enhancing antioxidant enzyme activity. This dual action on oxidative stress suggests that SSY could play a significant role in protecting lung tissues from oxidative damage, contributing to its therapeutic potential in ALI.

Anti-inflammatory Effects of SSY Could be Attributed to NF-κB Signalling Pathway Suppression

The NF-κB signalling pathway is pivotal during inflammation (Fiordelisi et al. 2019). After 24-hour LPS induction, the phosphorylation levels of IKKβ, IκBα, and p65 (a subunit of NF-κB) in ALI group rats were remarkably elevated. Besides, the total IκBα level had notably reduced in ALI rats in comparison with control rats, suggesting enhanced degradation. However, intervention with both SSY doses could markedly suppress these alterations, and a more pronounced effect was observed in the SSY-HD group than in the SSY-LD group (Fig. 3A-E).

Figures 3A-E depict the phosphorylation levels of NF-κB pathway components in the ALI rats and their response to SSY treatment. The x-axis represents the treatment groups, and the y-axis represents the levels of phosphorylation. The bars represent the mean values, and the error bars represent standard deviation. The asterisks denote statistically significant differences between the groups. The clear reduction in phosphorylation levels of IKKβ, IκBα, and p65 in the SSY-treated groups, particularly in the SSY-HD group, provides strong evidence that SSY can suppress the NF-κB signalling pathway, thereby mitigating the inflammatory response in ALI. This mechanism is likely to be one of the underlying reasons for the anti-inflammatory effects of SSY. The findings suggest that the anti-inflammatory properties of SSY are mediated, at least in part, by the suppression of the NF-κB signalling pathway. This discovery could have important implications for the development of new therapeutic strategies for the treatment of ALI and other inflammatory diseases.

Nrf2 Signalling Pathway Activation by SSY Mediated Its Antioxidant Effects

The Keap1-Nrf2-ARE signalling pathway is a vital molecular mechanism associated with antioxidant stress (Bellezza et al. 2018). LPS downregulated Keap1 expression and upregulated expression of Nrf2, NQO1, GCLC, and HO-1 in lung tissues of LPS-treated rats. Remarkably, SSY treatment further weakened Keap1 expression and promoted expression of Nrf2, NQO1, GCLC, and HO-1. Moreover, expression of Keap1 was significant-

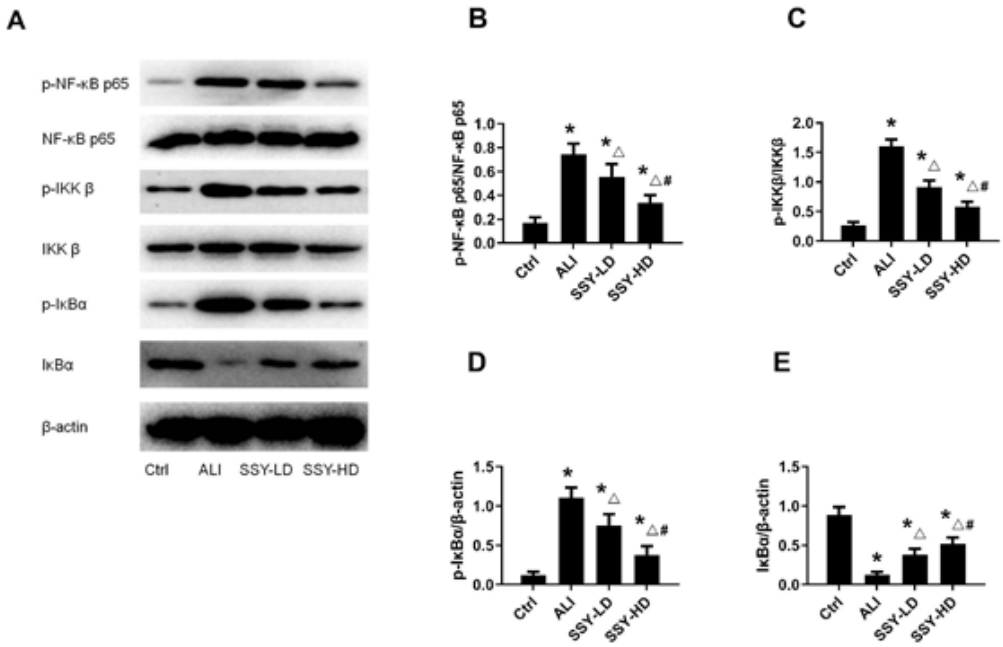

Fig. 3. SSY suppressed NF-êB signalling pathway in rats with ALI

(A) Representative western blots of p-NF-κB p65, NF-κB p65, p-IKKβ, IKKβ, p-IκBα and IμBα expression in lung tissues. (B-E) SSY decreased the phosphorylation of NF-κB p65 (B), IKKâ (C) and IκBα (D) as well as the degradation of IκBα (E) in ALI rats. We used β-actin as an internal control. Data are presented as mean ± SD (n=8 per group). $^{*}P$ <0.05 vs. Ctrl group, $^{\triangle}\Delta P$ <0.05 vs. ALI group, $^{\#}P$ <0.05 vs. SSY-LD group. IκBα, NF-κB inhibitor α; IKKβ, IβB kinase β; SSY, Shen-su-yin; ALI, acute lung injury; LD, low-dose; HD, high-dose; SD, standard deviation

ly lower and levels of Nrf2, NQO1, GCLC, and HO-1 were higher in the SSY-HD group than in the SSY-LD group (Fig. 4A-F).

Figures 4A-F visually depict the expression levels of key components of the Keap1-Nrf2-ARE pathway in lung tissues of the ALI rats and their response to SSY treatment. The x-axis represents the treatment groups, and the y-axis represents the levels of protein expression. The bars represent the mean values, and the error bars represent the standard deviation. The asterisks indicate statistically significant differences between the groups. The downward trend in Keap1 expression and the upward trend in the expression of Nrf2, NQO1, GCLC, and HO-1, particularly in the SSY-HD group, illustrate the potentiation of the Nrf2 pathway by SSY. This pathway activation is a crucial aspect of SSY's antioxidant mechanism, as it leads to the induction of protective enzymes that counteract oxidative stress and its damaging effects on cellular components. The results indicate that SSY activates the Keap1-Nrf2-ARE pathway, which is a key mediator of cellular antioxidant defence. This activation is another mechanism by which SSY may contribute to the alleviation of oxidative stress and inflammation in LPS-induced ALI, providing a potential therapeutic target for the development of new strategies for treating this condition.

DISCUSSION

The findings support the hypothesis that SSY has a therapeutic effect on LPS-induced ALI by alleviating lung pathology, modulating inflammation, and reducing oxidative stress. These effects were dose-dependent, with the high dose of SSY

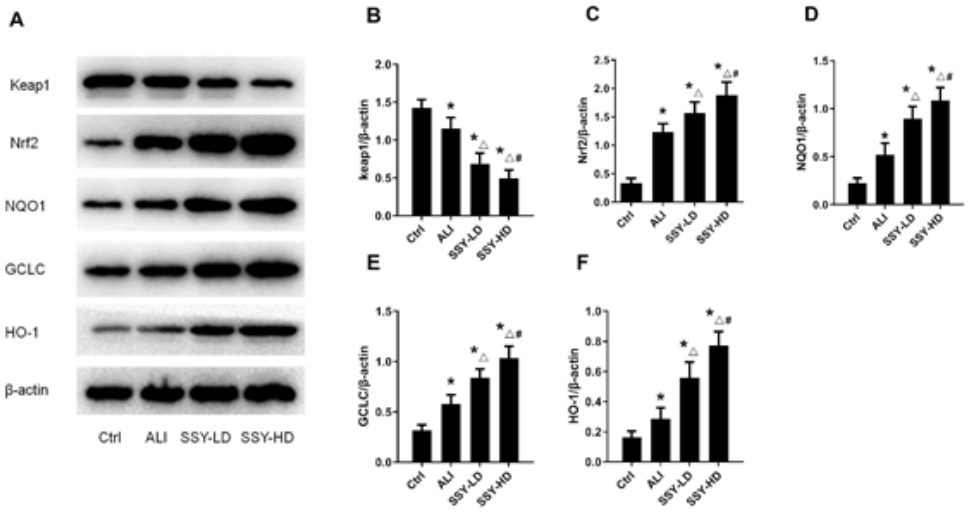

Fig. 4. SSY activated Keap1-Nrf2-ARE signalling pathway in rats with ALI

(A) Representative western blot analyses of Keap1, Nrf2, NQO1, GCLC, and HO-1 in each group. (B-F) LPS administration downregulated expression of Keap1 (B) and upregulated expression of Nrf2 (C), NQO1 (D), GCLC (E), and HO-1 (F). The expression of Keap1 (B) was further decreased, while those of Nrf2 (C), NQO1 (D), GCLC (E), and HO-1 (F) were further increased after 24 hours of the SSY treatment. We used β-actin as an internal control. Data are presented as mean ± SD (n=8 per group). $^{*}P$ <0.05 vs. Ctrl group, ΔP <0.05 vs. ALI group, $^{\#}P$ <0.05 vs. SSY-LD group. Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NQO1, NAD(P)H:quinine oxidoreductase 1; GCLC, glutamate-cysteine ligase catalytic subunit; HO-1, heme oxygenase 1; SSY, Shen-su-yin; ALI, acute lung injury; LD, low-dose; HD, high-dose; SD, standard deviation

(SSY-HD) showing more pronounced benefits compared to the low dose (SSY-LD). The mechanisms underlying these effects appear to involve the suppression of the NF-êB signalling pathway and the activation of the Keap1-Nrf2-ARE pathway, both of which are crucial in inflammatory and oxidative stress responses.

The experiments showed that LPS treatment resulted in severe microstructure damage, oedema, and elevated lung tissue permeability, leading to respiratory and circulatory disorders as previously reported (Hagiwara et al. 2009; Li et al. 2022; Li et al. 2023). The researchers demonstrated for the first time that prophylactic treatment using SSY prevented respiratory and circulatory functions from damage by LPS through alleviating pathological changes of the lungs in ALI rats. The underlying mechanisms possibly involved anti-inflammation via inhibition of NF-êB signalling pathway and antioxidant stress via activation of Keap1-Nrf2-ARE signalling pathway, which seemed more effective at a high dose.

ALI triggers excessive inflammation and uncontrolled oxidative stress, producing several pro-inflammatory cytokines and free radicals, but also promotes anti-inflammatory and antioxidative responses, possibly acting as compensatory mechanisms to defend against these harmful reactions (Dirami et al. 1999; Li et al. 2012; Bellezza et al. 2018; Fiordelisi et al. 2019). Consistent with other studies, the researchers found that LPS exposure resulted in high-level inflammatory infiltration and oxidative stress and led to elevated levels of anti-inflammatory and anti-oxidative factors (Dirami et al. 1999; Li et al. 2012). However, the compensating mechanisms are often insufficient and ALI exacerbations are common. If inadequately treated, it causes gradual dysfunction of extrapulmonary organs (such as heart, liver, and brain) and subsequently causes multiple organ failure and death (Bomsztyk et al. 2015; Mai et al. 2017).

Most Chinese herbs present in SSY, including Aurantii Fructus (Li et al. 2018), *Ginseng Radix et Rhizoma* (Abdelfattah-Hassan et al. 2019), *Perillae Folium* (Takada-Takatori et al. 2019), J*ujubae Fructus* (Kim et al. 2020), *Platycodonis Radix* (Kim et al. 2020), *Glycyrrhizae Radix et Rhizoma* (Ko et al. 2021), *Poria* (Gui et al. 2021), *Zingiberis Rhizoma Recens* (Morvaridzadeh et al. 2021), *Citri Reticulatae Pericarpium* (Zou et al. 2022), *Aucklandiae Radix* (Tousson et al. 2023), and *Puerariae Lobatae Radix* (Yen et al. 2023) have shown anti-inflammatory and/or antioxidative effects. Additionally, Peucedani Radix, another component of SSY, exhibits a wide spectrum of pharmacological activities including anti-infective, antitussive, anti-asthmatic, and expectorant effects, which help relieve symptoms and improve ALI prognosis (Song et al. 2015). In addition, some components of SSY can alleviate multiple organ dysfunction triggered by ALI. For example, *Pinelliae Rhizoma*, possessing sedative, hypnotic, and anticonvulsant effects, can relieve neuropsychiatric symptoms (Wu et al. 2011), and *Ginseng Radix et Rhizoma*, as well as *Glycyrrhizae Radix et Rhizoma,* enjoys popularity for their hepatoprotective effects (Abdelfattah-Hassan et al. 2019; Wahab et al. 2021). Based on these pharmacological characteristics, it is reasonable to speculate that the mechanisms resulting in ALI attenuation by SSY involve anti-inflammation and antioxidation, as the experimental results suggest. To compare different doses of SSY treatment, the researchers used low and high SSY doses for intervention after LPS injection. Both doses could restore lung tissue impairment, but the high dose had a more pronounced curative effect, suggesting a potential dose-response relationship between SSY and its therapeutic effects. The characteristic early lesions in ALI include mostly tissue injury (rather than necrocytosis) and mild-to-moderate (reversible) organ function damage (Lin et al. 2016; Patel et al. 2013). If left to progress, ALI results in tissue necrocytosis, causing serious and irreversible organ dysfunctions that are difficult to reverse medically (Fan et al. 2018; Lin et al. 2016; Patel et al. 2013), and thus, the researchers preventively treated rats in the early stage of the ALI instead of those with middle or advanced stages of the disease.

The inactive form of NF-κB, a nuclear transcription factor, is located in the cytoplasm and bound by a variety of inhibitory proteins, the NF-κB inhibitors (IκBs) family. Upon exposure to various stimuli, the IκB kinase complex (IKK) is activated into phospho-IKK, which further phosphorylates IêBs and leads to their ubiquitination and degradation. Without inhibition of IêBs, NF-κB is phosphorylated and translocates to the nucleus, promoting transcription of inflammation-related genes (Fiordelisi et al. 2019). The results are consistent with those of studies showing that LPS stimulates phosphorylation of IKKβ, IκBα, and NF-κB p65 and promotes expression of pro-inflammatory factors, indicating NF-κB signalling pathway activation (Hagiwara et al. 2009; Li et al. 2022; Li et al. 2023). The results suggest that SSY suppresses this signalling pathway markedly, especially at a high dose. Among the medicinal herbal concoctions based on SSY, some have been reported to promote IL-10 expression (Li et al. 2018; Sun et al. 2018). For example, the mechanisms that *Aurantii Fructus* exhibited anti-inflammatory effects in LPS-treated mice were partially through stimulating IL-10 expression (Li et al. 2018). Overall, the LPS-induced high level of IL-10 (Li et al. 2012; Li et al. 2018), which is further increased by SSY treatment in the study, suggests an amplification of the anti-inflammatory reaction.

Numerous antioxidative metabolites such as NQO1, GCLC, and HO-1 are produced as a result of the body's antioxidant system being activated by ALI to protect against oxidative stress damage. Nrf2, which is kept in the cytoplasm by Keap1 under unstressed conditions, is a transcription factor that regulates expression of antioxidant proteins such as NQO1, GCLC, and HO-1 by travelling to nucleus where it binds to antioxidant response elements (AREs) and initiates transcription of antioxidant genes (Bellezza et al. 2018). The results agree with those of published studies showing that LPS leads to increased production of oxidative metabolites, activating the Keap1-Nrf2-ARE pathway and increasing antioxidant activity (Dirami et al. 1999; Li et al. 2012; Bellezza et al. 2018). An innovative finding is that prophylactic treatment by SSY further increases antioxidant activity in LPS-treated rats and reduces oxidative stress levels, and the evidence suggests this occurs by SSY's activation of the Nrf2 signalling pathway in a possible dose-dependent manner.

The results are in accordance with previous studies that inhibition of both inflammation and oxidative stress can attenuate LPS-induced ALI

effectively, and the molecular mechanisms involve NF-κB and Nrf2 signalling pathway, respectively (Shi et al. 2022; Zhang et al. 2023). Besides, their synergistic effects in attenuation of ALI have also been reported (Köhler et al. 2016; Ma et al. 2020).

The major limitation of this study is that the rodent model can not fully mimic human disease. Although the results of the current study are encouraging, the clinical significance remains to be further investigated. SSY has been used in China for nearly a thousand years. Nevertheless, many questions about its effects are still unclear, especially regarding pharmacological properties. Further research is needed to elucidate pharmacological activities and side effects of the components in the herbal formula.

CONCLUSION

This study has demonstrated that SSY, when used preventively at both low and high doses, can stabilise vital signs and significantly attenuate lung histopathology in a rat model of LPS-induced ALI. The high dose of SSY was found to be more effective in these protective effects. The therapeutic benefits of SSY are attributed to its anti-inflammatory and antioxidant properties, which are mediated by the inhibition of the NF-êB signalling pathway and the activation of the Nrf2 signalling pathway, respectively. These findings suggest that SSY may offer a promising therapeutic strategy for the treatment of ALI.

RECOMMENDATIONS

Based on the study's findings, further research is recommended to explore the potential of SSY as a therapeutic agent for ALI. This includes investigating the optimal dosing regimen, examining the long-term effects of SSY treatment, and conducting clinical trials to assess its safety and efficacy in human patients. Additionally, it would be beneficial to study the specific components of SSY that contribute to its therapeutic effects and to explore the synergistic interactions between these components. Future research should also aim to identify any potential side effects of SSY and to develop formulations that maximise its therapeutic benefits while minimising adverse reactions.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

COMPETING INTERESTS

No conflict of interest exists in this manuscript.

CONSENT FOR PUBLICATION

Manuscript is approved by all authors for publication.

AVAILABILITY OF DATA AND MATERIALS

The data and materials of this experiment are available.

AUTHOR CONTRIBUTIONS

Peng Xiao was responsible for conception and design. Haijun Zhou and Wuhong Zheng were responsible for manuscript writing. Zongcun He was responsible for collection and assembly of data. Fei Lin and Wuhong Zheng were responsible for data analysis and interpretation. All authors were responsible for manuscript writing. All authors were responsible for the final approval of the manuscript.

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