Original Article

Fusu mixture alleviates inflammatory reactions in lipopolysaccharide-induced acute respiratory distress syndrome mice via regulation of surfactant-associated protein C, aquaporin 5, and Notch1

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Background: Acute respiratory distress syndrome (ARDS) is a common life-threatening critical illness with high mortality. Fusu mixture (FSM) can improve the mechanical ventilation in ARDS patients. However, the detailed pharmacological mechanisms and active substances of FSM are still unclear. This study aimed to explore the potential pharmacological mechanisms of FSM for treating ARDS and its chemical compositions.

Methods: A lipopolysaccharide (LPS)-induced ARDS mouse model was established, and the mice subsequently received FSM (50 mg/kg) orally for 5 days. Then, the blood samples and lung tissues were collected. Enzyme-linked immunosorbent assay (ELISA) was used to determine the levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in serum, and histopathology examinations were applied to analyze the inflammatory response of lung tissues in ARDS mice. In addition, protein expressions of aquaporin 5 (AQP-5), surfactant-associated protein C (SP-C), and Notch1 were detected by western blot assays and immunohistochemical (IHC) examination. In addition, the chemical compositions of FSM were analyzed by high performance liquid chromatography (HPLC), using standard reference agents.

Results: After LPS induction, the serum levels of IL-6 and TNF-α in ARDS mice were significantly increased (P<0.01, *vs.* Control), and FSM significantly reduced these 2 pro-inflammatory cytokines (IL-6 and TNF-α) compared to the model mice (P<0.01). Histopathology examinations showed FSM significantly attenuated the inflammatory responses in lung tissues. Furthermore, after FSM treatment, the SP-C and AQP-5 were significantly increased, compared to the Model mice (P<0.01), and FSM also up-regulated the Notch1 expressions in lung tissues of ARDS mice (P<0.001, *vs.* Model).

Conclusions: Collectively, it is suggested that FSM alleviates inflammatory reactions and promotes the proliferation of alveolar epithelial cells in LPS-induced ARDS mice via regulation of SP-C, AQP-5, and Notch1 in lung tissues.

Keywords: Acute respiratory distress syndrome (ARDS); Fusu mixture (FSM); alveolar epithelial cells; active substances; inflammatory cytokines

Introduction

Acute respiratory distress syndrome (ARDS), characterized by intractable hypoxia and expiratory dyspnea, is a common life-threatening critical illness in with high mortality (1,2). Importantly, new emerging evidences have revealed that ARDS has a very high morbidity in coronavirus disease of 2019 (COVID-19)-induced respiratory tract infection, which is also a predominant cause for the poor prognosis of patients with severe COVID-19 pneumonia (3-5). The detailed pathogenesis for ARDS is extremely complex and remains uncovered, and the results of previous research showed that ARDS might be closely correlated to lung injury induced by inflammatory cytokines released from several inflammatory cells (6,7). Currently, the available treatment for ARDS predominantly comprises supportive treatment including lung protective ventilation, restricted liquid management, and extracorporeal membrane oxygenation (ECMO). Taraxasterol (8), vitamin D (9), and fraxin (10) may help alleviate ARDS by downregulating inflammatory responses. However, there is a lack of evidence or effective drugs to ameliorate ARDS in the clinical setting. Consequently, there is an urgent need to discover more reliable candidate drugs for the treatment of ARDS (11,12). Natural herbal medicines, particularly traditional Chinese medicines (TCMs), are irrefutably precious resources for the exploration of new drugs for treating various intractable diseases (13,14). Increasing evidences have shown that TCMs could be used to improve COVID-19 pneumonia, and could be also effective to treat ARDS induced by various viruses or bacteria (15-17).

Fusu mixture (FSM) is an effective TCM formula for treating ARDS or septic shock in the clinic that is derived from the ancient prescriptions of *Qian-yang-dan* recorded in a TCM monograph of *Yilizhenchuan* written in the Qing dynasty (18,19). FSM comprises 7 herbal medicines, including *Aconiti Lateralis Radix Praeparata* (Fuzi; 30 g), *Amomi Fructus* (Sha Ren; 15 g), *Testudinis Carapax et Plastrum* (Gui Jia; 30 g), *Ephedrae Herba* (Ma Huang; 10 g), *Zingiberis Rhizoma* (Gan Jiang; 10 g), and *Glycyrrhizae Radix et Rhizoma* (Gan Cao; 12 g). Our previous clinical research results suggested that FSM can improve the mechanical ventilation in ARDS patients via increase of the arterial partial pressure of oxygen (PaO_2) and oxygenation index [PaO_2/fraction of inspired oxygen (FiO_2)] (18,19). In the previous clinical practice, we found that the TCM FSM could supplement the basic treatment of Western medicine, clinically reduce the extravascular lung water index of patients with sepsis-induced ARDS, and improve the prognosis of patients (19). However, the detailed pharmacological mechanisms and active substances of FSM are still unclear. So, in our present study, we established an ARDS animal model to explore the potential pharmacological mechanisms of FSM for treating ARDS, and we also further explored the chemical compositions of FSM analyzed by high performance liquid chromatography (HPLC). We present this article in accordance with the ARRIVE reporting checklist (available at https://jtd. amegroups.com/article/view/10.21037/jtd-23-367/rc).

Highlight box

Key findings

- It is suggested that Fusu mixture (FSM) alleviates inflammatory reactions and promotes the proliferation of alveolar epithelial cells in LPS-induced ARDS mice via regulation of SP-C, AQP-5, and Notch1 in lung tissues.

What is known and what is new?

- FSM can improve the mechanical ventilation in ARDS patients.
- Our findings contribute the detailed pharmacological mechanisms and active substances of FSM in LPS-induced ARDS mice.

What is the implication, and what should change now?

- It provides evidence that FSM is an effective treatment for treating ARDS and other respiratory diseases, providing guidance for clinical medication. Future works might be devoted to the investigation of the activities of these monomers in FSM on ARDS and the related mechanisms.

Methods

Chemicals and reagents

Lipopolysaccharide (LPS), hematoxylin and eosin (H&E) staining, and pentobarbital sodium were purchased from Sigma-Aldrich (Shanghai, China); enzyme-linked immunosorbent assay (ELISA) kits for interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Boster Biotech (Wuhan, China); primary antibodies of surfactant-associated protein C (SP-C), aquaporin 5 (AQP-5), and Notch1 were purchased from Thermo Fisher Inc. (Shanghai, China).

Preparation of water extracts of FSM

All the 7 herbal medicines, obtained from the Chinese

Pharmacy of our hospital, were powdered and soaked in purified water for 30 minutes. Then, all the herbal medicines were decocted for 1.5 hours. The extracts were subsequently filtrated and concentrated at 50 ℃ using a rotary evaporator in vacuum to afford the water extracts of FSM.

Animal model and grouping

A total of 30 mice (20±2 g), purchased from the Dossy Experimental Animals Co. Ltd. (Chengdu, China), were used to establish the ARDS model. All mice were male in order to avoid interference with the female estrus cycle and obtain more stable experimental results. All animals were kept in a specific pathogen free (SPF) environment with temperature and humidity of 22–24 ℃ and 50–60%, respectively. The animals had free access to food and water. Sterilized and residue-free wood shavings were used for animal bedding. Animal experiments were performed under a project license (No. 2021DL-003) granted by the Experimental Animal Ethics Committee of the Hospital of Chengdu University of Traditional Chinese Medicine, in compliance with institutional guidelines for the care and use of animals. A protocol was prepared before the study without registration. After 1 week of adaptive feeding, the research began.

In the specific experimental mouse modeling and grouping process, 20 mice were anesthetized by pentobarbital sodium at the dose of 50 mg/kg (i.p.), and subsequently received the LPS via the respiratory tract by nasal drip. Another 10 mice received the same operation with normal saline instead of LPS, and were used as the Normal control mice. Then, the ARDS mice were divided into 2 groups (n=10), including a Model (LPS) and a Treatment group (LPS+FSM). The selection of the above experimental animals is random.

The Control and Model (LPS) group were administered orally with normal saline (10 mL/kg), and the Treatment group (LPS+FSM) were received FSM at the dose of 50 mg/kg orally. All the mice received a 5 days' treatment. At the end of the treatment, blood samples were collected from the abdominal aorta under anaesthetization by pentobarbital sodium at the dose of 50 mg/kg (i.p.). After blood sampling, mice were sacrificed via decapitation and the lung tissues were collected for further determination.

ELISA assays

Serum samples were prepared by centrifugation at 10,000 rpm

for 10 minutes, then levels of TNF-α and IL-6 in serum were determined by commercial ELISA kits following the standard protocols of instruction.

Histopathology examination

Histopathology examinations were applied to analyze the inflammatory response of lung tissues in ARDS mice following the reported method with minor modifications (20). Lung tissues were collected and fixed in 4% paraformaldehyde for 24 hours, and then embedded in paraffin. After a series of standard protocols for tissue section preparation, the lung tissue section was cut into 5 µm and stained with H&E. The histopathological changes of lung tissues were analyzed and the representative figures were captured using an optical microscope (Olympus, Tokyo, Japan).

Immunohistochemical (IHC) examination

Conduct IHC experiments on mice from the three groups mentioned above. After the lung tissue section (5 µm) was prepared as described in previous method, protein expressions of AQP-5, SP-C, and Notch1 were detected by IHC examination. Then, the IHC determination was carried out with primary antibodies of AQP-5, SP-C, and Notch1. Primary antibodies included: AQP-5 (1:200, PA5-36529, Thermo Fisher), SP-C (1:200, PA5-76631, Thermo Fisher), and Notch1 (1:200, PA5-32522, Thermo Fisher), β-actin (1:5,000, AC026, ABclonal, USA). Briefly, the deparaffinized tissue sections were treated with citric acid and antigenic unmasked at 98 ℃ for 10 minutes and subsequently incubated with primary antibodies overnight at 4 ℃, followed by incubation with the secondary antibody at room temperature for 1 hour. Finally, the tissue sections were further stained with 3,3'-diaminobenzidine (DAB) solution. The target protein positive expressions of lung tissues were analyzed and the representative figures were captured using an optical microscope (Olympus, Japan).

Western blot assay

Total proteins of the lung tissues were extracted using radioimmunoprecipitation assay (RIPA) solution and the protein concentration was quantified by bicinchoninic acid (BCA) reagents. After separation of the protein bands by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the target protein bands were blotted on a polyvinylidene fluoride (PVDF) membrane, and
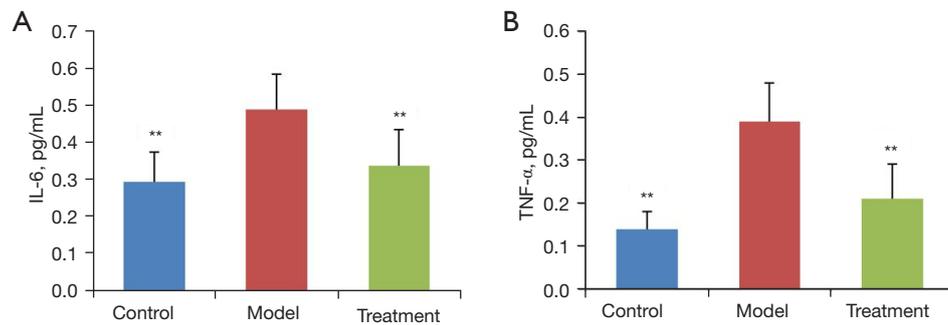
Figure 1 FSM decreased pro-inflammatory cytokines in serum of ARDS mice. (A) Results of the ELISA assays of IL-6 in Control, Model, and Treatment groups, respectively; (B) results of the ELISA assays of TNF-α in Control, Model, and Treatment groups, respectively. Data were expressed as mean ± SD (n=10), **, P<0.01 indicated *vs.* Model. IL-6, interleukin-6; TNF-α, tumor necrosis factor-α; FSM, Fusu mixture; ARDS, acute respiratory distress syndrome; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

subsequently incubated with primary antibodies of AQP-5, SP-C, and Notch1, respectively. Then, the PVDF membrane was incubated with HRP-conjugated secondary antibodies, and followed visualized by chemiluminescence with the enhanced chemiluminescence (ECL) chemicals.

HPLC analysis

The main compositions in FSM were determined by HPLC extracts (21,22). Separation was carried out using the Agilent 1200 High-Performance Liquid Chromatograph (Agilent, Santa Clara, CA, USA) with a Shimadsu InertSustain C_{18} column (250 mm × 4.6 mm, 5 µm; Shimadsu, Kyoto, Japan) at 30 ℃ using a gradient elution at a flow rate of 1.0 mL/min. The acetonitrile (B) and 0.1% formic acid-water (A) was used as the mobile phase and the gradient program was as following: 0–15 min, 5–20% A; 15–40 min, 20–25% A; 40–50 min, 25–35% A; 50–80 min, 35–65% A; 80–90 min, 65–65% A. The volume of injected sample was 10 µL and the detection wavelength was set at 230 nm.

Statistical analysis

All values were described as the mean ± standard deviation. The software SPSS 22.0 (IBM Corp., Armonk, NY, USA) was used for statistical testing, and significance was determined by one-way analysis of variance (ANOVA) with the least significant difference (LSD) test. Statistical significance was defined as P<0.05.

Results

FSM decreased pro-inflammatory cytokines in serum of ARDS mice

As shown in *Figure 1*, after LPS induction, the serum levels of IL-6 and TNF-α in ARDS mice were significantly increased (P<0.01, *vs.* Control). Interestingly, FSM treatment significantly reduced these 2 pro-inflammatory cytokines compared to the model mice (P<0.01).

FSM improved pro-inflammatory responses in lung tissues of ARDS mice

As shown in *Figure 2*, after LPS induction, the alveolar septum was significantly broadened and obvious inflammatory reactions and pink homogenous edema fluids were observed in the alveolar cavity. In addition, the results also showed focal pulmonary dilatation, alveoli collapse, and multifocal hemorrhage. Interestingly, FSM significantly attenuated the inflammatory responses in lung tissues; no obvious alveoli collapse and multifocal hemorrhage were observed in the treatment group mice.

FSM increased AQP-5, SP-C, and Notch1 in lung tissues of ARDS mice

Subsequently, we explored the potential mechanisms of FSM for treating ARDS via western blot assays and IHC examinations. We determined the protein expressions of
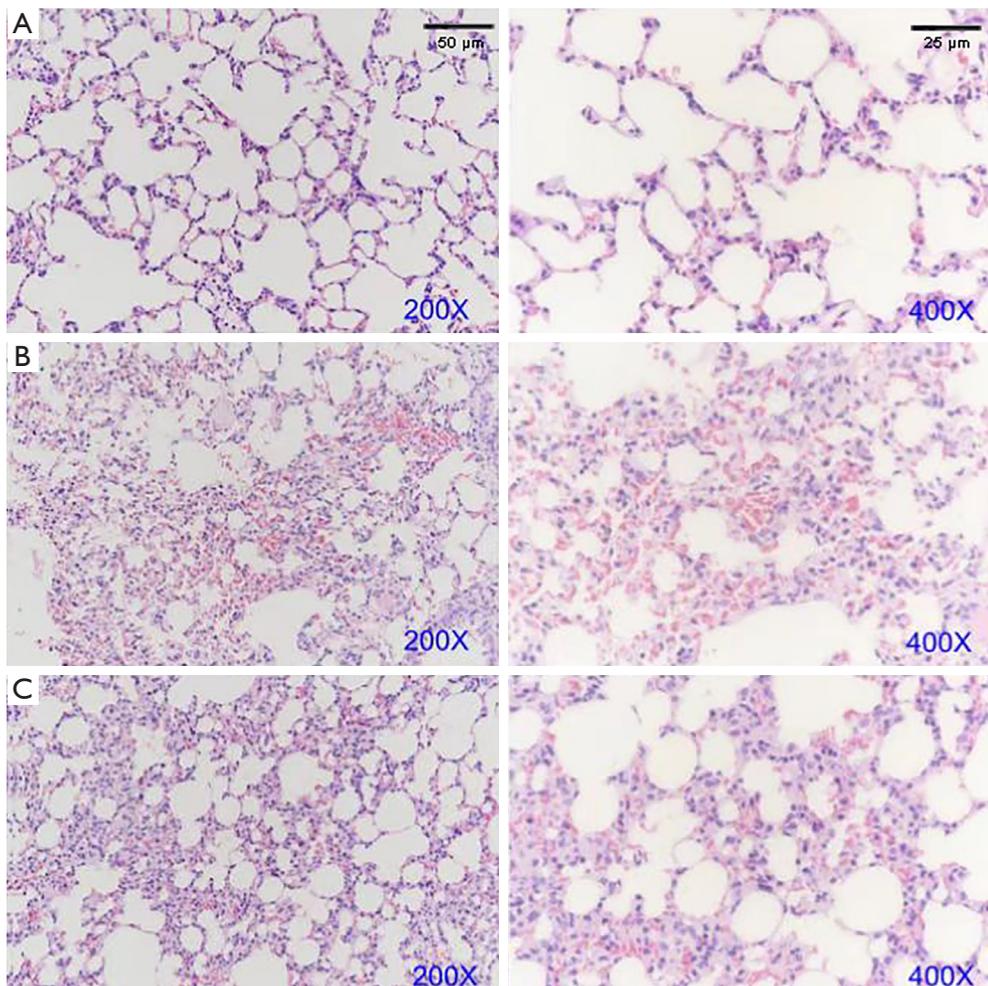
Figure 2 FSM improved pro-inflammatory responses in lung tissues of ARDS mice. (A-C) H&E-staining results of the histopathology examinations in Control, Model, and Treatment groups, respectively. FSM, Fusu mixture; ARDS, acute respiratory distress syndrome; H&E, hematoxylin and eosin.

AQP-5, SP-C, and Notch1 (*Figure 3*) in the lung tissues of ARDS mice. Our results showed that after FSM treatment, the SP-C and AQP-5 were significantly increased, compared to the Model mice (P<0.01). Besides, FSM treatment also up-regulated the expression of Notch1 in the lung tissues of ARDS mice (P<0.001, *vs.* Model). In addition, similar results with the western blot assays were also observed from the IHC examinations on AQP-5 (*Figure 4*), SP-C (*Figure 5*), and Notch1 (*Figure 6*) in lung tissues of ARDS mice.

Chemical compositions of FSM

As shown in *Figure 7*, over 20 chromatography peaks were obtained from the HPLC chromatogram, and 8 constituents

of FSM were identified by using standard reference agents, including (I) liquiritin, (II) isoliquiritin, (III) benzoylmesaconine, (IV) mesaconitine, (V) liquiritigenin, (VI) hypaconitine, (VII) glycyrrhizic acid, and (VIII) 6-gingerol, respectively.

Discussion

FSM is a clinical empirical TCM formula for treating ARDS and other respiratory diseases. In the present study, we reported experimental animal evidences for FSM against ARDS for the first time, in addition, we also explored the related potential mechanisms.
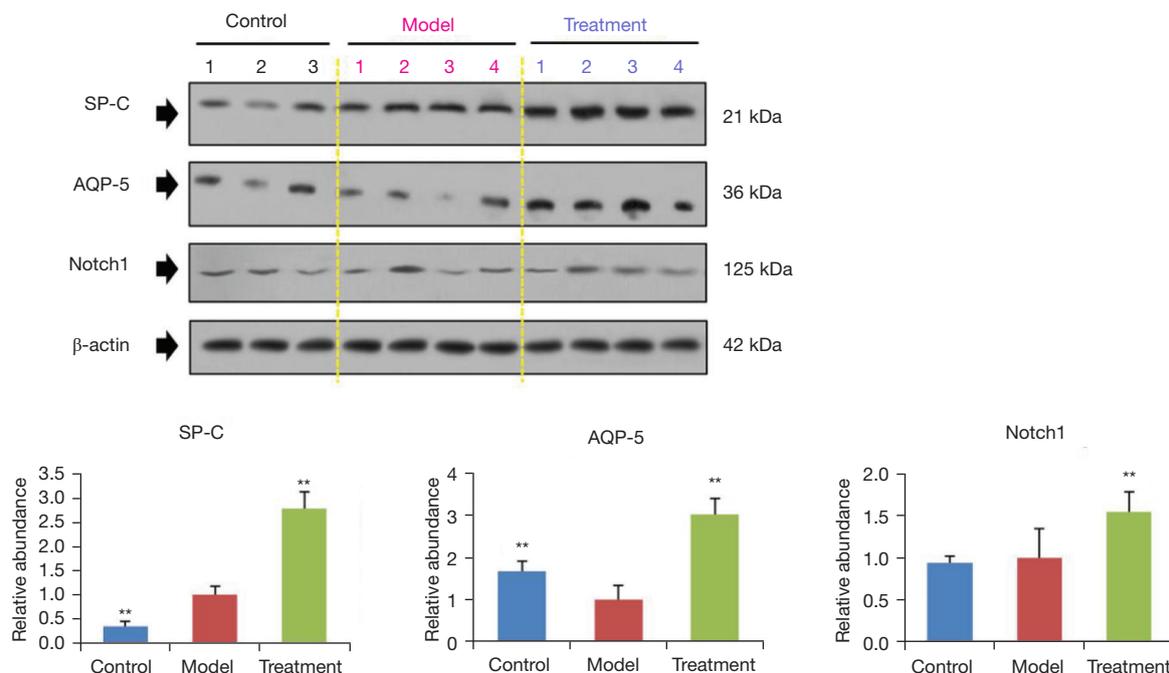
The mouse model of LPS-induced ARDS is a consistent

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Figure 3 Western blot assays suggested that FSM increased SP-C, AQP-5, and Notch1 in lung tissues of ARDS mice. **, P<0.01 indicated *vs.* Model. SP-C, surfactant-associated protein C; FSM, Fusu mixture; AQP-5, aquaporin 5; ARDS, acute respiratory distress syndrome.

and reproducible ARDS animal model which can mimic the pathophysiology of ARDS in humans (23,24). In the real-world study, the proinflammatory cytokines TNF-α, IL-6, and IL-8 are among the most promising as biomarkers for predicting morbidity and mortality (25,26). In our present study, we established the LPS-induced ARDS mouse model to evaluate the curative effects of FSM against ARDS, and the results showed that FSM could significantly attenuate the ARDS symptoms of mice. The pathological characteristics of ARDS include increased pulmonary capillary permeability, uncontrolled local inflammatory responses, and inflammatory cytokines release, finally leading to systemic inflammatory response (23,27). Currently, it is generally recognized that uncontrolled inflammatory reactions are closely related to the development of ARDS, and the over-produced inflammatory cytokines of IL-1, IL-6, and TNF-α further exacerbated the injury of alveolus (26,27). Recently, a study showed that the inhibition of miR-129-5p may induce autophagy and inhibit the inflammatory response by promoting the expression of the PPAR-γ, thereby relieving ARDS (28). Our present results showed that FSM treatment could remarkably attenuate the inflammatory responses and injury of lung tissues induced by LPS challenge, including

reduction of inflammatory exudation, decrease of alveoli collapse and multifocal hemorrhage, and so on. In addition, FSM treatment could also reduce the serum levels of IL-6 and TNF-α in ARDS mice.

SP-C, one of the pulmonary surfactants of the lung tissues, is important for maintaining the normal functions of lung tissues, such as regulation of alveolar surface tension and local defense system of lung (29). Importantly, recent studies have suggested that pulmonary surfactants could attenuate the ARDS both in children and adults (30,31). Aquaporins (AQP) are water channels with the function of regulating the balance of intracellular and intercellular water fluid (32). Current findings have shown that AQPs are closely correlated to the development of ARDS, and upregulation of AQPs are beneficial for improving ARDS (33,34). Notch/DLK1 is a key signaling pathway for the development of type II alveolar epithelial cells, which could be beneficial for the repair of lung function after ARDS (35-38). In our present study, we found that FSM treatment significantly up-regulated the protein expressions of SP-C, AQP-5, and Notch1 in lung tissues of ARDS mice. In addition, we also analyzed the main composition of FSM, and 8 potential active constituents were identified including (I) liquiritin, (II) isoliquiritin, (III) benzoylmesaconine, (IV)
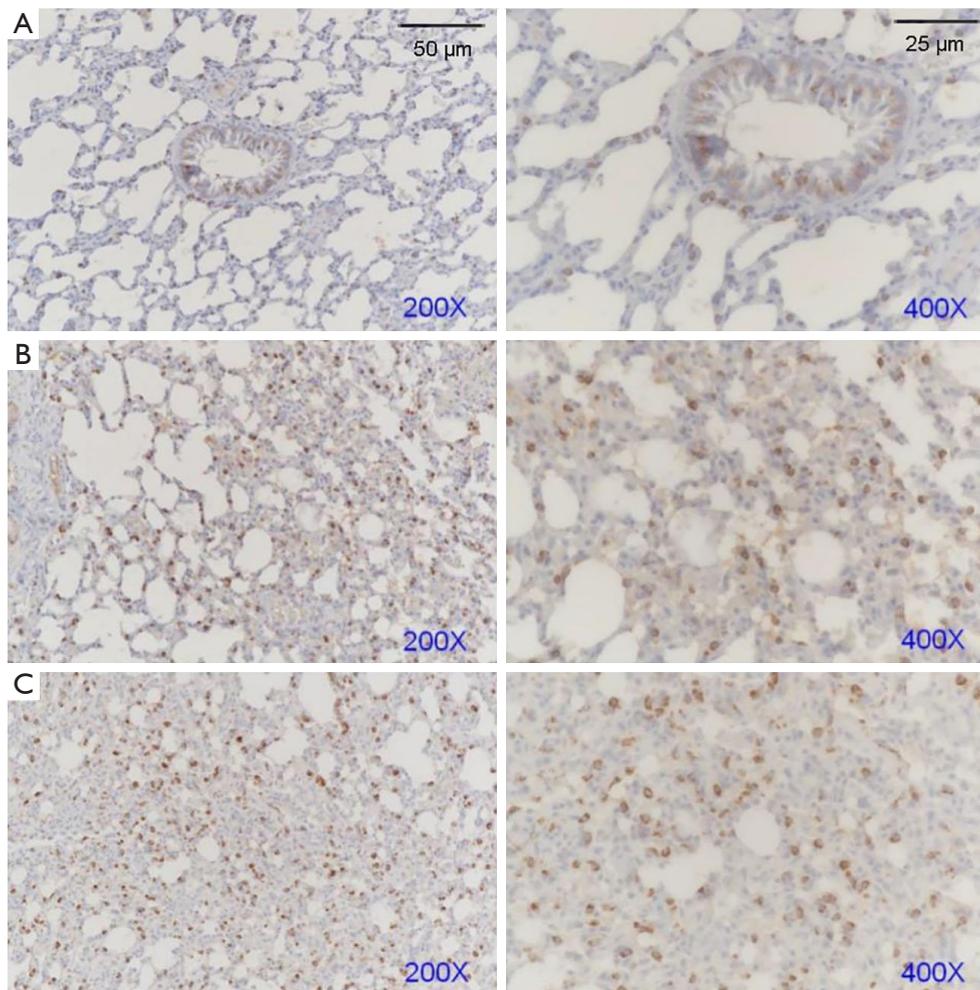
Figure 4 Immunohistochemical assays revealed that FSM increased AQP-5 in lung tissues of ARDS mice. (A-C) Immunohistochemical assays results of AQP-5 in Control, Model, and Treatment groups, respectively. FSM, Fusu mixture; AQP-5, aquaporin 5; ARDS, acute respiratory distress syndrome.

mesaconitine, (V) liquiritigenin, (VI) hypaconitine, (VII) glycyrrhizic acid, and (VIII) 6-gingerol. Consequently, future works might be devoted to investigating the activities of these monomers on ARDS and the related mechanisms.

In our study, we found that SP-C proteins were significantly elevated in the model group, either as a result of compensatory expression of the *SP-C* gene in the model construct or as a consequence of pulmonary epithelial cell damage in the model construct, which activates the repair mechanism of the body. Notably, the tissue expression of SP-C was further increased after pharmacological intervention, indicating that the intervention induced pulmonary epithelial cell proliferation accompanied by an up-regulation of SP-C protein expression. To determine the specific mechanisms of SP-C protein expression in the intervention arm, it is necessary to confirm this by histological analysis and specific staining in subsequent studies.

In the model group, AQP-5 was significantly reduced, indicating that the inhibition of the protein was significant in the modeling process, whereas in the intervention group, we found that AQP-5 was significantly elevated, even higher than in the control group. This suggests that the intervention not only affects the protein by improving the tissue status but is likely to have a regulatory effect on the *AQP-5* gene. In subsequent studies, we need to validate
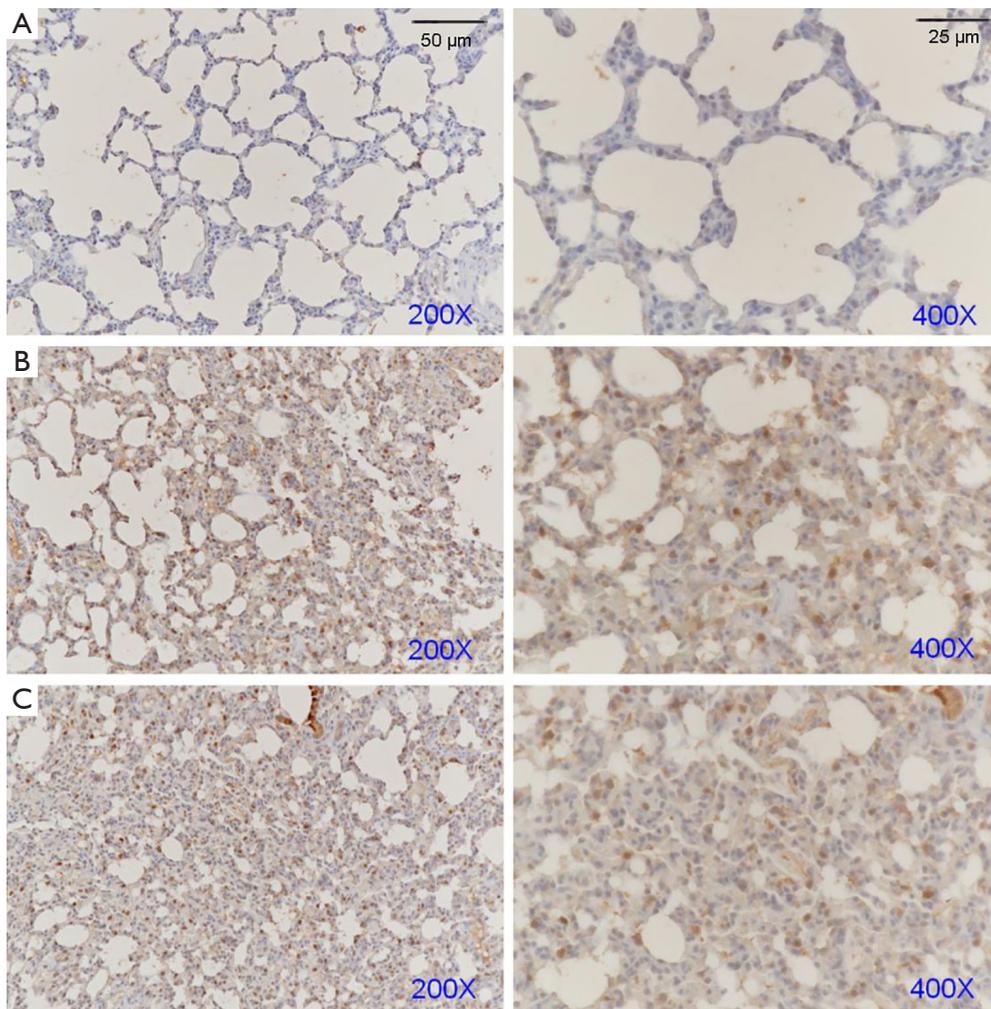
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Figure 5 Immunohistochemical assays revealed that FSM increased SP-C in lung tissues of ARDS mice. (A-C) Immunohistochemical assays results of SP-C in Control, Model, and Treatment groups, respectively. FSM, Fusu mixture; SP-C, surfactant-associated protein C; ARDS, acute respiratory distress syndrome.

the mechanisms by which the *AQP-5* gene is regulated in primary cells.

Notch signaling pathway has a critical role in regulating cell fate determination, proliferation, and differentiation during development and tissue regeneration (38). In the adult, Notch is involved in repair and regeneration of several airway cells types. Finn reported that Notch1 expression was affected in type II pulmonary epithelial cells (39). Similarly, in the present study, FSM up-regulated the Notch expressions in lung tissues of ARDS mice, attenuating the inflammatory responses in lung tissues.

There are several limitations in this study. Firstly, there are too few inflammatory cytokine detections. Secondly, the research of signaling pathway is limited, due to the limited funds and conditions. In the future, we will continue to explore the relationship between FSM and the proliferation of alveolar epithelial cells.

Conclusions

In our study, FSM alleviates inflammatory reactions and promotes the proliferation of alveolar epithelial cells in
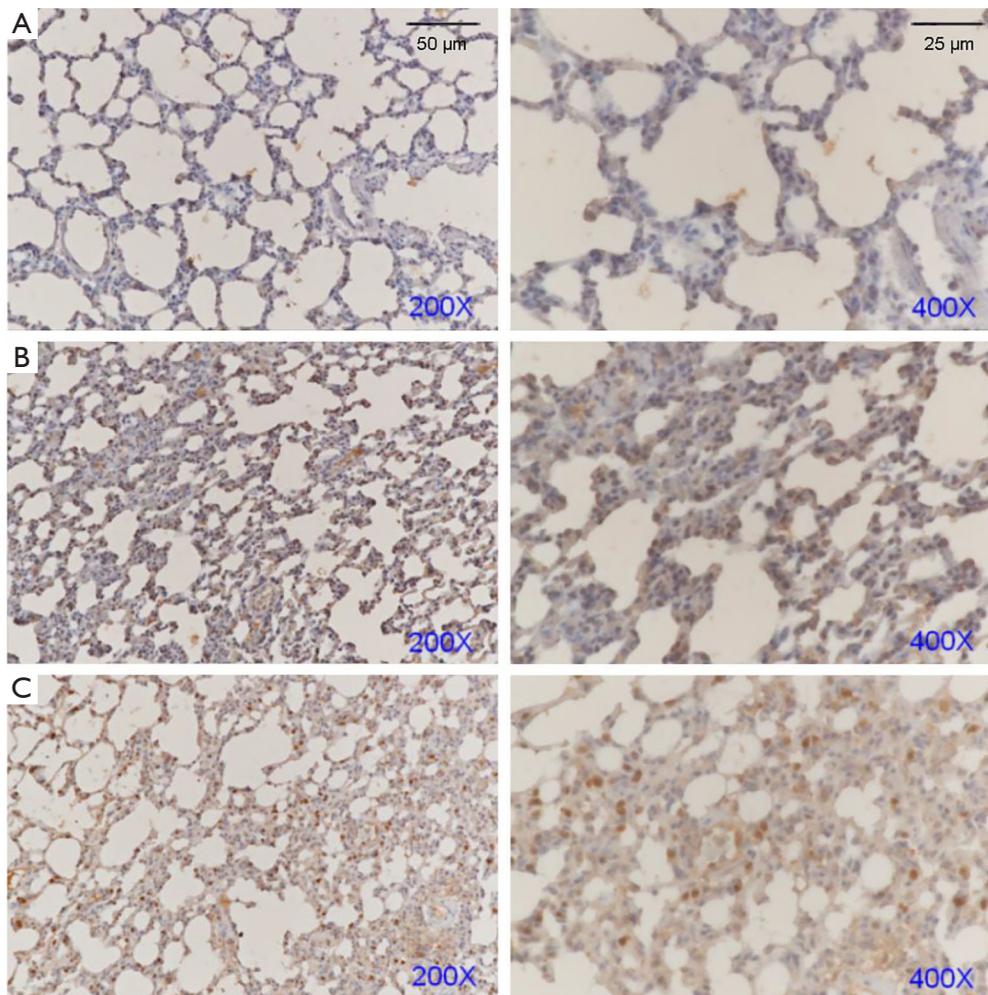
Figure 6 Immunohistochemical assays revealed that FSM increased Notch1 in lung tissues of ARDS mice. (A-C) Immunohistochemical assays results of Notch1 in Control, Model, and Treatment groups, respectively. FSM, Fusu mixture; ARDS, acute respiratory distress syndrome.
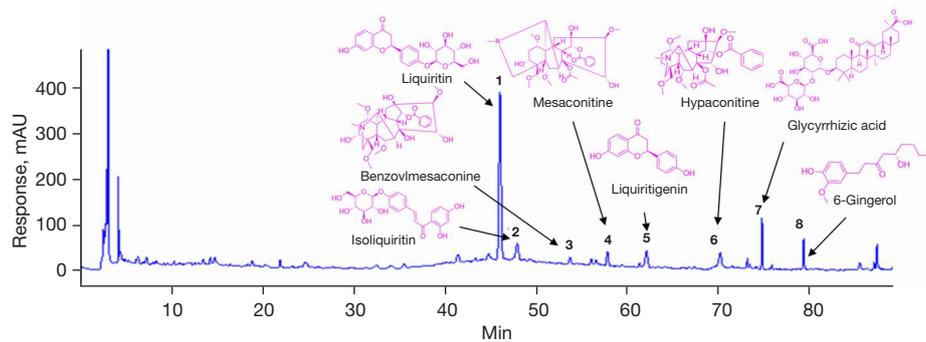

Figure 7 Chemical analysis of FSM using HPLC. FSM, Fusu mixture; HPLC, high-performance liquid chromatography.

LPS-induced ARDS mice via regulation of SP-C, AQP-5, and Notch1 in lung tissues, providing basic evidence for the clinical treatment.

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-367/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-367/dss

Peer Review File: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-367/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-367/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2021DL-003) granted by the Experimental Animal Ethics Committee of the Hospital of Chengdu University of Traditional Chinese Medicine, in compliance with institutional guidelines for the care and use of animals.

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