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Original Article

Saikosaponin-d attenuates ventilator-induced lung injury in rats

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Abstract: Saikosaponin-d is one of the main bioactive components in the traditional Chinese medicine *Bupleurum falcatum* L and possesses anti-inflammatory and immune-modulatory properties. The current study aimed to investigate the protective effects of saikosaponin-d on ventilator-induced lung injury (VILI) in rats. We found that saikosaponin-d treatment significantly attenuated the pathological changes of lungs induced by mechanical ventilation. Administration of saikosaponin-d reduced the pulmonary neutrophil infiltration as well as the MPO concentrations. Saikosaponin-d also decreased the expression of pro-inflammatory cytokines including MIP-2, IL-6 and TNF-α. Meanwhile, the expression of anti-inflammatory mediators, such as TGF-β1 and IL-10, was obviously elevated after saikosaponin-d administration. Saikosaponin-d remarkably reduced the oxidative stress and apoptosis rate in lung tissues. On the molecular level, saikosaponin-d treatment obviously downregulated the expression of caspases-3 and the pro-apoptotic protein bax, and promoted the expression level of anti-apoptotic protein bcl-2. Collectively, our study demonstrated that saikosaponin-d may attenuate ventilator induced lung injury through inhibition of inflammatory responses, oxidative stress and apoptosis.

Keywords: Saikosaponin-d, ventilator induced lung injury, inflammation, apoptosis

Introduction

Acute lung injury and its most severe manifestation, acute respiratory distress syndrome, are characterized by increased capillary leakage and microvascular permeability due to the epithelial and endothelial injury [1, 2]. Mechanical ventilation has been part of basic life support in traditional medical practice [3]. However, it will cause ventilator-induced lung injury (VILI) manifested by noncardiogenic pulmonary edema, disturbance of the alveolar-capillary barrier as well as enhanced production of cytokines and chemokines [4]. Accumulating evidences indicate that many pro- and anti-inflammatory cytokines, including interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin 10 (IL-10) and transforming growth factor beta 1 (TGF-β1), play critical roles in the development of VILI [5-7].

Many natural products possess anti-inflammotary and immunomodulatory properties, which have long been used for treatment of cancers, cardiovascular diseases, autoimmune diseases, etc. Saikosaponin-d, one of triterpenoid saponins derived from *Bupleurum falcatum* L, is a commonly prescribed agent against inflammatory diseases in China, Japan and other Asian countries [8, 9]. Several studies show saikosaponin-d has anti-inflammatory, immunomodulatory, antiviral and anticancer activities. For instance, saikosaponin d has been shown to promote apoptosis and inhibit proliferation of rat hepatic stellate cells via regulation of phosphorylation levels ofp38 and extracellular matrix-regulated kinase 1/2 [10]. Other researches found that saikosaponin-d exhibited anti-proliferative effect on the activated T lymphocyte via suppression of NF-κB, NF-AT, and AP-1 signaling pathways [11, 12]. In the current study, we explored the protective effects of saikosaponin-d on ventilator-induced lung injury in rats. Our results showed that administration of saikosaponin-d attenuated the lung injury induced by ventilator through suppression of inflammatory responses, oxidative stress and cell apoptosis. In summary, our data suggested

that saikosaponin-d has a therapeutic effect on ventilator-induced lung injury.

Materials and methods

Animals

This study was approved by the institutional animal care and use committee of the School of Medicine, Zhejiang University. Animal procedures were carried out in compliance with Institutional Standards for Use of Animal Laboratory Animals. Male Sprague-Dawley rats were purchased from Chinese academy of sciences (Shanghai, China). Rats were housed in specific pathogen free laboratory for 72 h with free access to water and food. The rats were randomized into three groups: 1) a control group (n = 8), in which rats were tracheostomized and instilled with saline; 2) VILI group (n = 8), in which rats were ventilated with a high tidal volume; and 3) saikosaponin-d -treated group (n = 8), in which rats were pretreated with the saikosaponin-d and ventilated with the same settings as in the VILI group.

Mechanical ventilation

Male Sprague-Dawley rats, weighing 350-400 grams were anaesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (10 mg/kg body weight). After anesthesia, a well established model of VILI was performed as previously described [13]. In brief, a tracheotomy was performed and a metal cannula was inserted in the trachea. Two sutures were placed around the exposed part of the trachea into which the cannula was inserted and tied down thoroughly. The rats were ventilated with a small animal rodent ventilator (Harvard Apparatus, Holliston, MA, USA) by using the following settings: respiratory rate, 40 breaths/minute; tidal volume, 8 ml/kg body weight; inspiratory: expiratory ratio of 1:1; positive end-expiratory pressure (PEEP), 3 cm H_2O; and FiO_2, 0.21 for 30 minutes. Later the tidal volume increased slowly to 40 ml/kg body weight with a PEEP of 0 cm H_2O for an hour. Temperature was monitored rectally and maintained at 37°C by a thermo mattress.

Myeloperoxidase and thiobarbituric acid reactive substances (TBARS) assay

Concentration of myeloperoxidase (MPO), an index of neutrophil sequestration in the lungs, was measured as previously described [14]. TBARS level in serum was measured using OxiSelect TBARS assay kit (Geneteks Biosciences, Inc.).

Lung wet/dry weight ratio

The superior lobe from the right lung was weighed to obtain the wet weight, and then placed in an oven at 80 for measurement of the dry weight. The ratio of the wet weight to dry weight was calculated to assess the tissue edema.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed to determine the contents of IL-6, TNF-α, TGF-β1 and IL-10 in the bronchoalveolar lavage fluid (BALF) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Absorbance was measured at 450 nm by microplate assay.

Lung histopathology

The lungs were fixed immediately in 10% formalin. The right middle lobes were dehydrated in alcohol, embedded in paraffin, and stained with hematoxylin/eosin. Lung injury was scored using an average score of the following items: alveolar congestion, hemorrhage, infiltration of neutrophils into airspace or the vessel wall, and thickness of the alveolar wall [15]. Lung injury scores were scaled from 1, mild; 2, moderate; 3, severe; and 4, very severe. The lung injury scores were evaluated by two pathologists who were blinded to the experimental conditions.

Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay

Paraffin-embedded lung tissues were labeled using a TUNEL assay kit (Roche Diagnostics, Basel, Switzerland). The number of TUNEL-positive (apoptotic) cells on three sections per rat was counted under a fluorescence microscope at x 400 (Carl Zeiss Microsystems, Thornwood, NY, USA).

Western blot analysis

Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, UK). Immunodetection was performed with anti-caspase-3, bax, bcl-2 and β-actin anti-

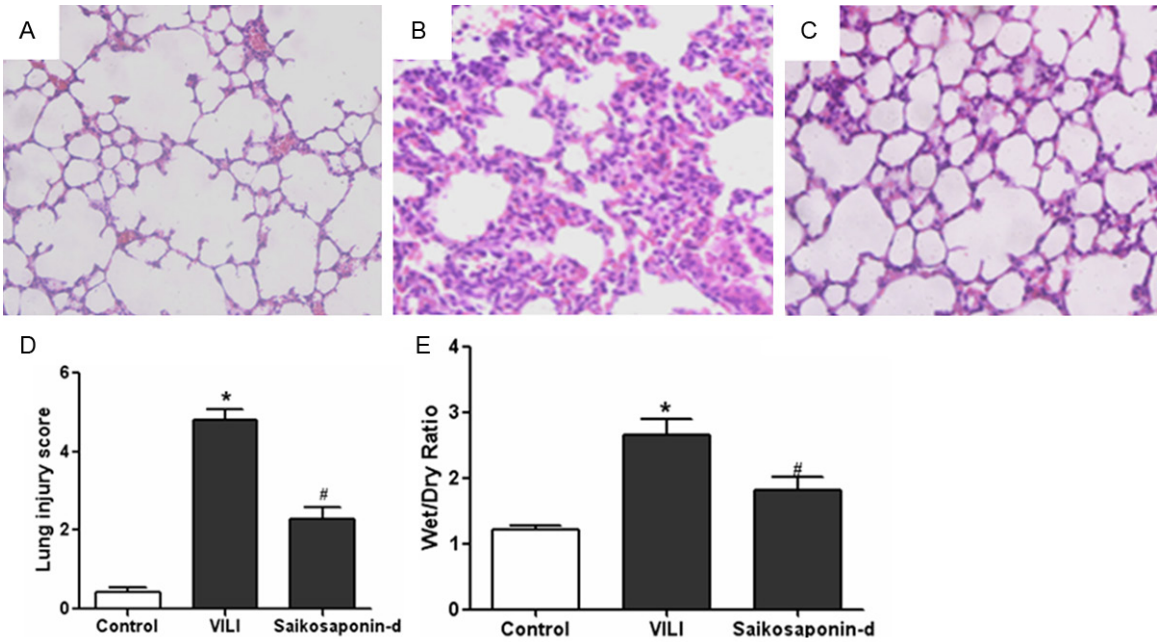

Figure 1. Effect of saikosaponin-d on ventilator-induced pathological changes in lung tissue. A-D. Rats were anesthetized and ventilated at high tidal volumes for 60 minutes. At the end of ventilation, the lung tissue was stained with hematoxylin and eosin (H&E) to determine the pathological changes. The figures demonstrated representative views (× 200) from each group. E. The wet/dry weight ratio of lung tissues from rats with indicated treatment was measured. *P < 0.05 vs. Control.

body (Abcam, Cambridge, UK) overnight at 4°C. Then the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (Abcam, Cambridge, UK) for 1 h at room temperature. Protein expression was visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL). β-actin was used as a loading control.

Statistical analysis

Each experiment was performed in triplicate, and repeated at least three times. All the data were presented as means ± SD and treated for statistics analysis by SPSS 13.0 program. Comparison between groups was made using ANOVA and statistically significant difference was defined as $P < 0.05$.

Results

Effect of saikosaponin-d on ventilator-induced pathological changes in lung tissue

VILI model was established by application of high tidal volumes, which is characterized by perivascular edema, interstitial and intra-alveolar leukocyte infiltration and heterogeneity in alveolar inflation. In contrast, pretreatment with saikosaponin-d significantly attenuated such pathological changes (**Figure 1A-C**) and lung injury scores (**Figure 1D**). We then assess the pulmonary edema by determination of lung wet/dry weight ratio (W/D). As shown in **Figure 1E**, pretreatment with saikosaponin-d significantly reduced the W/D (**Figure 1E**). Taken together, these results demonstrated that saikosaponin-d pretreatment alleviated the ventilator-induced pathological changes in lung tissue.

Saikosaponin-d attenuated inflammatory responses in VILI model

Concentration of MPO was detected to reflect the effect of saikosaponin-d on pulmonary neutrophil infiltration. We found that the saikosaponin-d treatment reduced the levels of MPO compared with the VILI group (**Figure 2A**). The expression levels of TNF-α, IL-1β and MIP-2 in BALF were higher in the VILI group than in the control group. However, administration of saikosaponin-d obviously reduced these cytokines production (**Figure 2B-D**). Meanwhile, the anti-inflammatory mediator TGF-β1 and IL-10

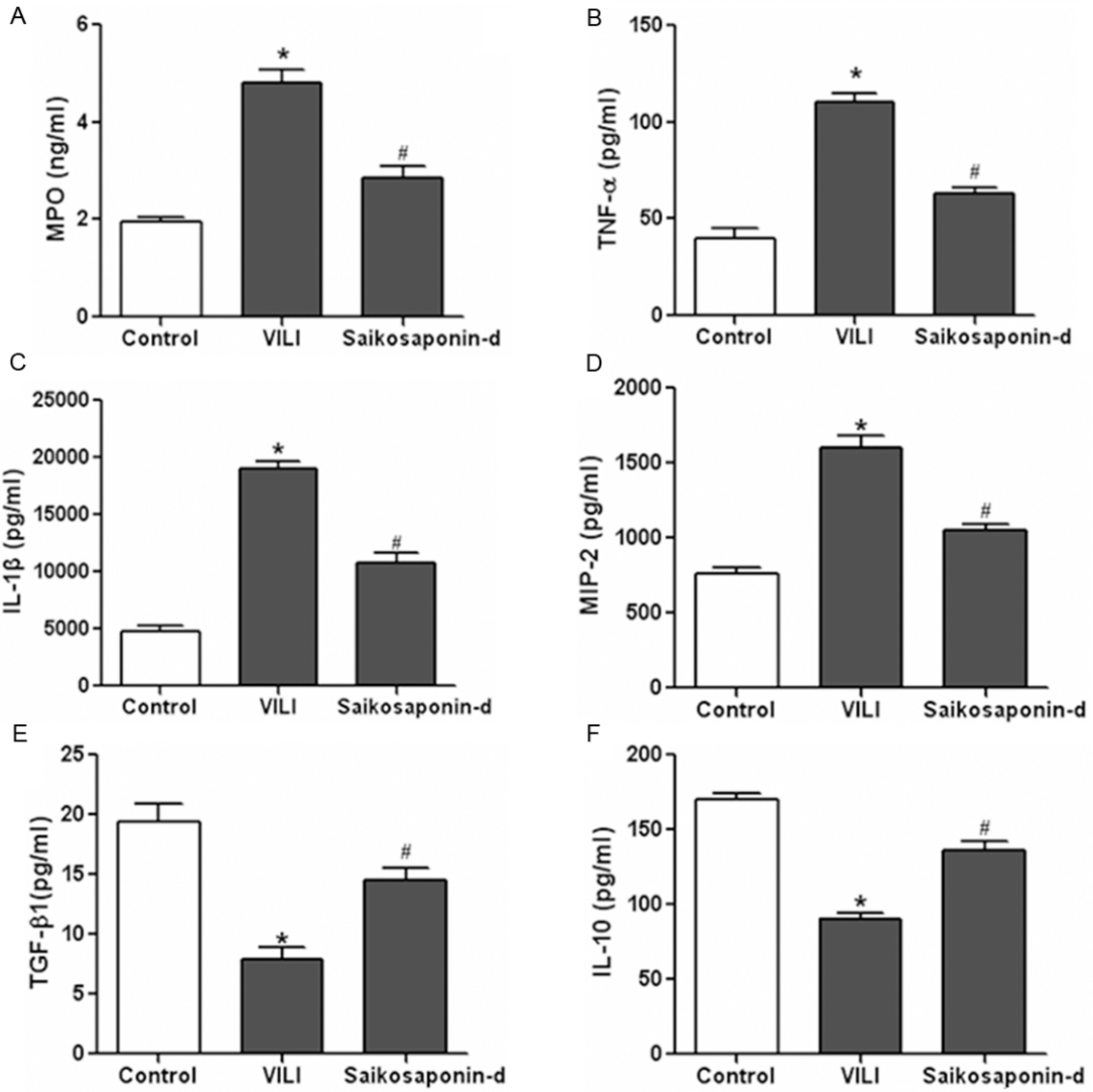

Figure 2. Saikosaponin-d attenuated inflammatory responses in VILI model. The concentration of MPO was determined in VILI group with or without saikosaponin-d pretreatment (A). ELISA was performed to determine the expression of TNF-α (B), IL-1β (C), MIP-2 (D), TGF-β1 (E) and IL-10 (F) in BALF in the rats. *P < 0.05 vs. Control. #P < 0.05 vs. VILI.

were decreased in the VILI group, which were reversed in the presence of saikosaponin-d pretreatment (**Figure 2E**, **2F**).

Saikosaponin-d decreased oxidative stress in VILI model

Oxidative stress is associated with mechanical ventilation-induced lung injury. We then measured the concentrations of TBARS and H_2O_2 in BALF to reflect the oxidative status. As shown in the **Figure 3A** and **3B**, the concentrations of TBARS and H_2O_2 in the VILI group were significantly increased than that in the control group. By contrast, pretreatment with saikosaponin-d remarkably suppressed the levels of TBARS and H_2O_2.

Effect of saikosaponin-d on apoptosis in lung tissues

To determine the effect of saikosaponin-d on apoptotic death, we performed TUNEL assay on lung sections from rats in different groups (**Figure 4A**). We found that the apoptosis rate in VILI group was significantly increased com-

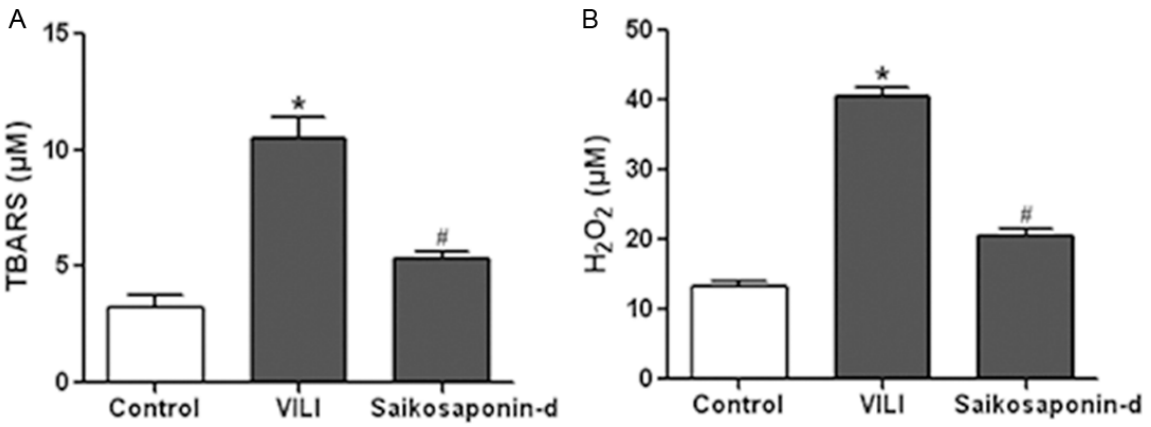

Figure 3. Saikosaponin-d decreased oxidative stress in VILI model. The concentrations of TBARS (A) and H_2O_2 (B) were measured in VILI group in the present or absence of saikosaponin-d. *P < 0.05 vs. Control. #P < 0.05 vs. VILI.

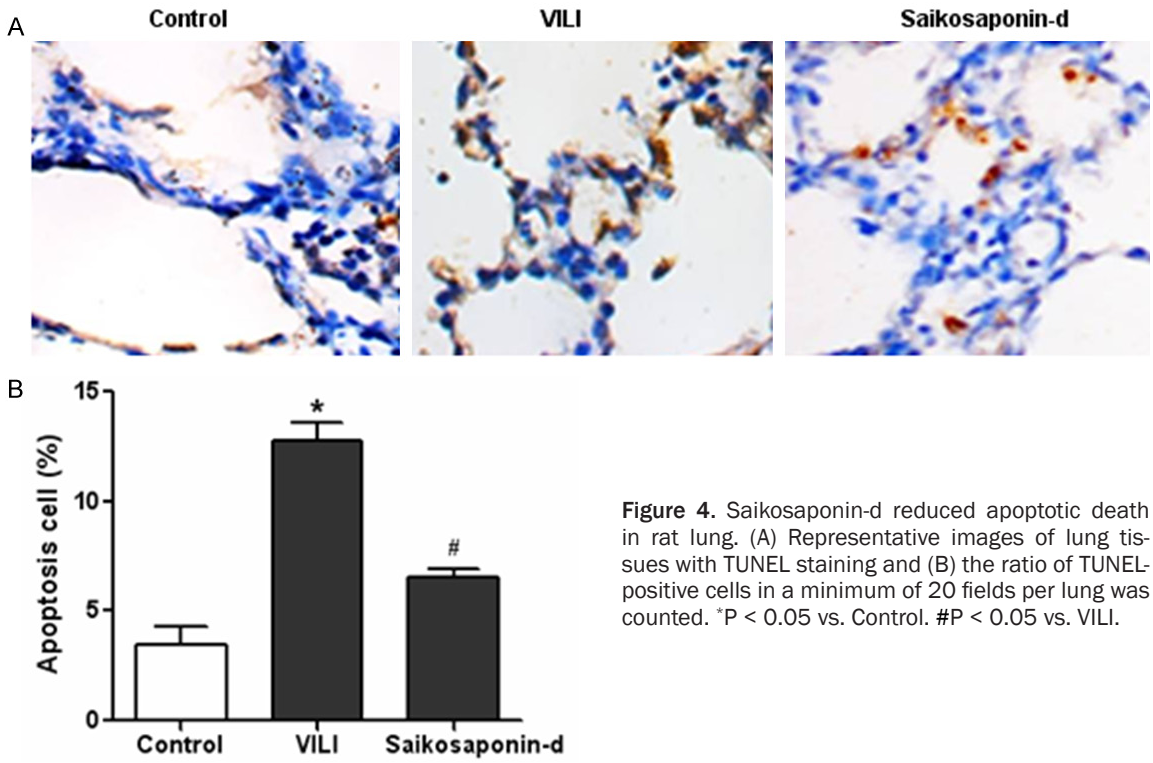

Figure 4. Saikosaponin-d reduced apoptotic death in rat lung. (A) Representative images of lung tissues with TUNEL staining and (B) the ratio of TUNEL-positive cells in a minimum of 20 fields per lung was counted. *P < 0.05 vs. Control. #P < 0.05 vs. VILI.

pared with the control group. However, treatment with saikosaponin-d obviously reversed the apoptotic death in the lung tissues (**Figure 4B**). In addition, we measured the expression levels of apoptosis-related proteins in the lungs. Western blot analysis showed that levels of caspase-3 protein were increased in the VILI group compared with the controls. However, the caspase-3 expression was significantly decreased after treatment with saikosaponin-d (**Figure 5A**, **5B**). Moreover, administration of saikosaponin-d led to the obvious downregulation of pro-apoptotic protein bax (**Figure 5C**, **5D**) and upregulation of anti-apoptotic protein bcl-2 (**Figure 5E**, **5F**). Collectively, these data indicated that saikosaponin-d protected pulmonary cells against apoptosis induced by mechanical ventilation.

Discussion

Currently, mechanical ventilators are increasingly used for life-saving procedures. However, accumulating studies reported the lung injury

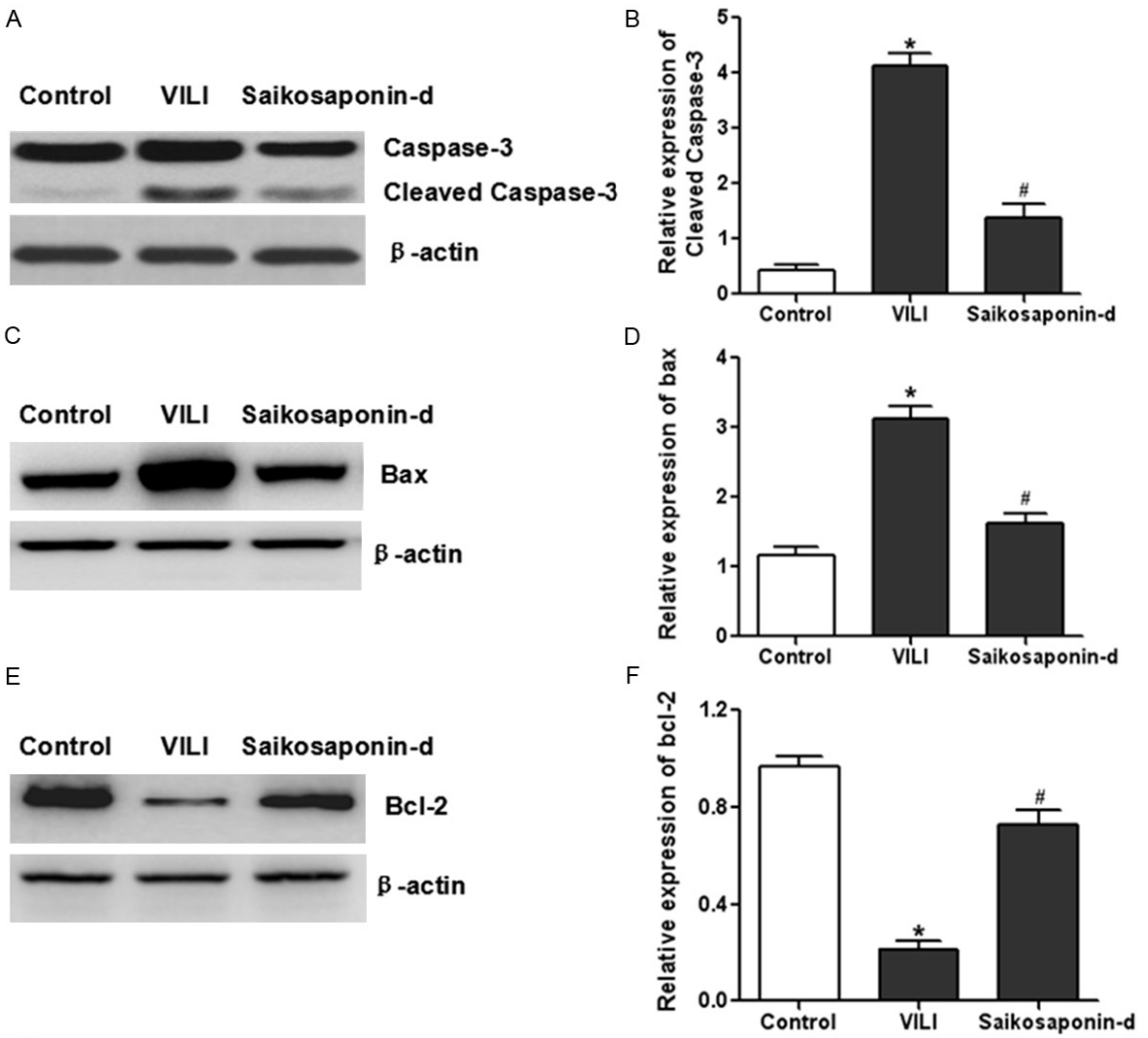

Figure 5. Effect of saikosaponin-d on the expression of apoptosis-related proteins in lung tissues. Western blot was performed to measure the protein expression of caspase-3 (A and B), bax (C and D) and bcl-2 (E and F). The expression of each protein was measured by relative band intensities. β-actin was used an an internal control. *P < 0.05 vs. Control. #P < 0.05 vs. VILI.

induced by mechanical ventilation, which is characterized by pulmonary edema and inflammation [4]. Number of studies showed that saikosaponin-d has antimicrobial, antioxidant and immunoregulatory properties [10-12]. In the present study, we demonstrated the protective effects of saikosaponin-d on ventilator induced lung injury with a rat model.

Pulmonary edema is the typical pathological changes resulted from ventilator induced lung injury, which leads to the reduction of lung compliance and deterioration of pulmonary gas exchange [16]. In the present study, histopathological features were relatively less severe after the instillation of saikosaponin-d when compared with the VILI group. Lung wet/dry weight ratio is often used to evaluate pulmonary edema [17]. We also found that saikosaponin-d treatment remarkably decreased the wet/dry weight ratio, suggesting saikosaponin-d might alleviate ventilator-induced lung injury.

Activation of inflammatory mediators is considered to play a critical role in the pathogenesis of injurious ventilation [18]. During this process, the infiltration of pathogenic lymphocytes into the lung tissue is a major event. Our data showed that as a marker of neutrophil influx [19], the MPO activity was decreased in the saikosaponin-d-treated rats compared to the VILI group. The macrophage inflammatory pro-

tein 2 (MIP-2) belongs to the family of chemotactic cytokines and it can induce the synthesis and release of several pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α from fibroblasts and macrophages [20, 21]. Moreover, TNF-α and IL-6 were reported to be the most important pro-inflammatory mediators in innate immune response [22]. Meanwhile, transforming growth factor β (TGF-β) and IL-10 play an anti-inflammatory role in immunity and autoimmunity [23]. We found that pretreatment with saikosaponin-d reduced the levels of MIP-2, TNF-α and IL-6 in BALF compared to that from the VILI group. By contrast, the anti-inflammatory cytokines TGF-β and IL-10 were increased after saikosaponin-d administration. Together, these data suggested that saikosaponin-d treatment suppressed inflammatory response in the lungs induced by mechanical ventilator.

Oxidative stress reflects an imbalance between the systemic manifestation of ROS and the ability to repair the resulting damage. Hyperoxia contributes to acute lung injury in diseases such as acute respiratory distress syndrome in adults and bronchopulmonary dysplasia in premature infants [24, 25]. It has been suggested that oxidative stress is implicated in mechanical ventilation-induced lung injury and serves a significant therapeutic target in the context of VILI [26]. We found that the concentrations of TBARS and H_2O_2 in the saikosaponin-d-treated group were significantly reduced, indicating that saikosaponin-d could attenuate the oxidative stress. Moreover, ROS is involved in the excessive production of proinflammatory cytokines, leading to apoptotic cell death in the pathogenesis of VILI [27]. Apoptosis is the programmed cell death modulated by a series of molecules such as bax, bcl-2 and caspases-3 [28]. TUNEL assay showed that the number of apoptosis cell was obviously decreased following saikosaponin-d treatment. Caspase-3 is a member of the cysteine-aspartic acid protease family and its activation plays an important role in the apoptotic cell death [29]. Consistent with data from TUNEL assay, the caspase-3 expression was observed down-regulated after treatment with saikosaponin-d. Moreover, administration of saikosaponin-d led to the obvious downregulation of pro-apoptotic protein bax and upregulation of anti-apoptotic protein bcl-2, indicating that saikosaponin-d protected against apoptosis induced by mechanical ventilation.

In summary, our results demonstrated that application of saikosaponin-d can effectively attenuate ventilator induced lung injury through inhibition of inflammatory responses, oxidative stress and apoptosis. These results suggested that saikosaponin-d may be considered as an effective candidate drug for the potential treatment of lung injury induced by mechanical ventilation.

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Disclosure of conflict of interest

None.

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