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Original Article

Anti-fibrosis effect of Saikosaponin D on pulmonary fibrosis *in vivo* and *in vitro* via suppressing alveolar epithelial cell apoptosis and epithelium-mesenchymal transformation

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Abstract: Pulmonary fibrosis (PF) is a progressive, chronic, irreversible and life-threatening disease. In our research, we aimed to investigate the effects of Saikosaponin D (SSD) on pulmonary fibrosis by using bleomycin induced PF mice and human embryonic lung fibroblast (HELF). After successful preparation of BLM (5 mg/kg, intratracheal instillation) induced PF mice, SSD was administered to the BLM induced mice by intraperitoneal injection (2 mg/kg/d, *ip*) for 28 days. Then, lung tissues were collected for histological examination with H&E, Masson's trichrome and TUNEL staining. In addition, reverse transcription PCR assay was performed to determine the mRNA expression of Caspase-3, and western blotting was carried out to determine the protein expressions of E-cadherin (E-cad), fibronectin (FN), Wnt and β-catenin. Furthermore, we also determined the anti-proliferative effects of SSD on HELF cells and transforming growth factor (TGF)-β1 expressions in HELF cells. Our results showed that SSD alleviated pulmonary alveolitis ($P < 0.05$), pulmonary fibrosis ($P < 0.05$) and cell apoptosis ($P < 0.01$) in BLM induced mice. Furthermore, SSD down-regulated Caspase-3 ($P < 0.05$), FN ($P< 0.05$), Wnt ($P < 0.05$) and β-catenin ($P < 0.05$) in both 14 and 28 days, whereas the E-cad obviously up-regulated ($P < 0.05$). Besides, SSD also inhibited cell proliferation of HELF and the TGF-β1 ($P < 0.05$) expression. In conclusion, our research suggested that SSD possess notable anti-fibrosis effect of on PF via suppressing alveolar epithelial cell apoptosis and epithelium-mesenchymal transformation.

Keywords: Saikosaponin D, pulmonary fibrosis, therapeutic mechanism, alveolar epithelial cell apoptosis, epithelium-mesenchymal transformation

Introduction

Pulmonary fibrosis (PF), the most common ofinterstitial lung diseases (ILD), is a progressive, chronic, irreversible and life-threatening disease [1, 2]. PF is characterized by severe inflammatory cells infiltration, pulmonary alveolar structural damage, excessive collagen accumulation, and massive fibroblast proliferation [3, 4]. The progress of PF from asymptomatic to symptomatic disease might pass over decades; however, from the definite diagnosis of PF, the median survival time among IPF patients is only two to three years [5]. PF commonly results in pulmonary function decline and even finally respiratory failure, and nowadays the curative treatment for PF patients is lacking [6, 7]. Thus, it is urgent for finding some effective drugs for treating PF.

The traditional Chinese medicine (TCM) is a promising resource for finding some useful candidate drugs for various diseases [8, 9]. Saikosaponin D (SSD) is an active constituent isolated from the Radix Bupleuri which is a well-known herbal medicine in China with broad spectrum pharmacological effects [10]. Increasing researches have demonstrated that SSD possesses various pharmacological properties, such as anti-inflammatory, hepatoprotective, antioxidant, and immunomodulatory activities [11-13]. Currently, there are also some reports indicating that SSD could be used to treat hepatic fibrosis [14, 15]. Therefore, in our pres-

Table 1. Criteria for grading pulmonary inflammation and fibrosis scores

Grades	Scores	Lesion of total pulmonary area	
		Pulmonary alveolitis	Pulmonary fibrosis
I	0	No obvious inflammatory reactions was observed	No obvious fibrosis was observed
II	1	Inflammatory area presence less than 20%	Fibrosis area presence less than 20%
III	2	Inflammatory area presence between 20% and 50%	Fibrosis area presence between 20% and 50%
IV	3	Inflammatory area presence over 50%	Fibrosis area presence over 50%

ent work, we aimed to investigate the effects of SSD on pulmonary fibrosis *in vivo* and *in vitro* by using bleomycin induced pulmonary fibrosis mice and human embryonic lung fibroblast, which have significant reference value for future using SSD to treat PF in clinical.

Materials and methods

Chemicals and reagents

Saikosapoin-D (SSD) was purchased from the Jiangxi Herbfine Science and Technology Co. Ltd (Nanchang, China); Bleomycin (BLM) was purchased from the Nippon Kayaku Co., Ltd. (Tokyo, Japan); TUNEL apoptosis assay kit, tissues RIPA buffer, Hematoxylin and Eosin (H&E), Masson's Trichrome kit, primary antibodies for E-cadherin (E-cad, 1:300, cat No. BA0475-2), fibronectin (FN, 1:300, cat. No. BA1771), Wnt (1:300, cat. No. BA2628-2) and β-actin (1:500, cat. No. BA2305), and HRP-conjugated secondary antibody (1:1000, cat. No. BA1082) were purchased from Wuhan Boster Biotech Co. (Wuhan, China); primary antibodies for β-catenin (1:500, cat. Ab6302) and transforming growth factor (TGF)-β1 (1:500, cat. No. ab92486) were purchased from Abcam Co. (Cambridge, MA, USA); primary antibody for caspase-3 was purchased from the Santa Cruz biotechnology, Inc. (San Jose, CA, USA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS)were purchased from Gibco Co. (Grand Island, NY, USA); Methyl-thiazdyldiphenyl-tetrazolium bromide (MTT), ECL chemiluminescence kit, dimethyl sulfoxide (DMSO), and BCA reagent kit were purchased from Beyotime Co. (Haimen, China); PVDF membranes and skimmed milk powder were purchased from the Millipore Co. Ltd. (Billerica, MA, America); chloral hydrate was obtained from the pharmacy department of the third affiliated hospital of Soochow university hospital (Changzhou, China); Trizol reagents kit and Goldview Nucleic Acid Gel Stain kit were purchased from the Invitrogen/Life Technologies (Carlsbad, CA, USA).

Animals

SPF male ICR mice (6 weeks old, 18 ± 2 g) were purchased from the Laboratory Animal Center of Yangzhou University (Yangzhou, China). All animals were kept at a constant temperature controlled room (25°C, 50% humidity) with 12-hlight/dark cycle, and allowed to free access to food and water. All the animal protocol were performed in accordance with the research proposal for the Care and Use of Laboratory Animals and approved by the Laboratory Animal Committee of the Third Affiliated Hospital of Soochow University.

Animal model establishment and protocols

After a 7-day acclimation period, total 60 mice were randomly divided into 3 groups, including Control group, BLM group (BLM-induced mice), and SSD + BLM group (SSD treated BLM mice), and each group consisted of 20 mice. BLM induced pulmonary fibrosis mice were prepared according to previous reference with minor modifications [16]. Briefly, after anaesthetized by intraperitoneal injection of chloral hydrate (0.01 mg/kg, ip), mice in BLM and BLM + SSD groups were injected with BLM (5 mg/kg, body weight) by intratracheal instillation, while mice in control group were given the same volume of physiological saline instead of BLM. Since one day after BLM injection, SSD treated mice were continuously administered with SSD (2 mg/kg/d, ip) for 28 days, while mice in Control and BLM groups were given equal volume of physiological saline instead of SSD.

Lung tissues samples collection

On day 14 and 28 after BLM injection, 10 mice of each group were sacrificed by cervical dislocation and lung tissue samples were harvested. The left lung tissues were fixed with 4% paraformaldehyde for histological examination, while the right lung tissues were frozen in liquid nitrogen for 10 min and then stored at -70°C.

Table 2. Primers used for PCR analysis

Genes	Sequence (5'-3')
GAPDH	Forword-5'-ATCATCAGCAATGCCTCCTG-3'
	Reverse-5'-ATGGACTGTGGTCATGAGTC-3'
Caspase-3	Forword-5'-ATGGACAACAACGAAACCTC-3'
	Reverse-5'-TTAGTGATAAAGTACAGTTCTT-3'

Histological examination

The formaldehyde fixed lung tissues were embedded in paraffin and successively sliced at 4 μm. Sections were stained with H&E to detect the degrees of inflammation and pulmonary alveolitis, and Masson's trichrome to evaluate the degree of fibrosis, respectively. According to methods established by Szapiel *et al* [17, 18], pathological damages were determined and graded following the criteria described in **Table 1**.

TUNEL apoptosis assay

Lung tissue sections were stained with TUNEL apoptosis assay commercial kits according to the manufacturer's instructions to detect the degrees of cell apoptosis.

Reverse transcription PCR (RT-PCR) assay

Total RNA was isolated from the frozen lung tissues by using Trizol reagents kit according to the commercial manufacturer's instruction. Total RNA was used for cDNA synthesis by reverse transcription, and caspase-3 and GAPDH were amplified by PCR. The mRNA primers of caspase-3 and GAPDH were designed using Primer Premier 5.0 and synthesized by Shanghai Sangon Genomics Institute (Shanghai, China) as shown in **Table 2**. After denaturation at 94°C for 5 min, PCR amplification was performed under the following program: 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and a final extension at 72°C for 8 min [19]. Amplification products were analyzed on agarose gel electrophoresis, and were subsequently visualized by Goldview staining with a gel imaging system (Bio-Rad, Hercules, CA, USA) and quantitatively determined by Quantity One software, respectively.

Cell culture and cell viability assay by MTT

Human embryonic lung fibroblasts (HELF) were purchased from the Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China). HELF cells were cultured in DMEM medium supplemented with 10% FBS, antibiotics (penicillin 200 U/ml, streptomycin 100 μg/ml) at 37°C in a 5% CO_2 humidified atmosphere.

Cells were seeded in 96-well plates at 5×10^4 cells/well (200 μL) and grown for 24 h. Then, cells were treated with SSD at final concentrations (2.5, 5 and 10 μg/ml) for 24, 48, 72 and 96 h, respectively, and subsequently the cell viability of HELF cells was determined by MTT following previously described method [9]. Optical density values (OD) were measured at 490 nm using a 96-well plate reader (Bio-Rad, Hercules, CA, USA).

Western blotting assay

Tissues or cells were homogenized and lysed with RIPA buffer, and the total proteins were extracted. After determination of the protein concentration by using BCA reagents, equally 30 μg proteins were separated by 10% SDS-PAGE and subsequently blotted to PVDF membranes. The PVDF membranes were blocked by 5% skimmed milk powder and subsequently incubated with primary polyclonal antibodies respectively, then re-incubated with HRP-conjugated secondary antibody. Specific protein bands were visualized using ECL chemiluminescence kit, and scanned and quantitatively determined using gel imaging system and Quantity One software, respectively. To normalize the loading proteins, β-actin was used as internal control.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The statistical significances of differences between groups were evaluated by using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance (ANOVA) followed by LSD-*t* test (Equal Variances assumed) or Games-Howell test (Equal Variances not assumed). *P* value less than 0.05 was recognized as statistically significant.

Results

Effects of SSD on histopathological changes of lung tissue

As can be seen from **Figures 1**, **2**, for control mice, lung tissues are normal and no obvious

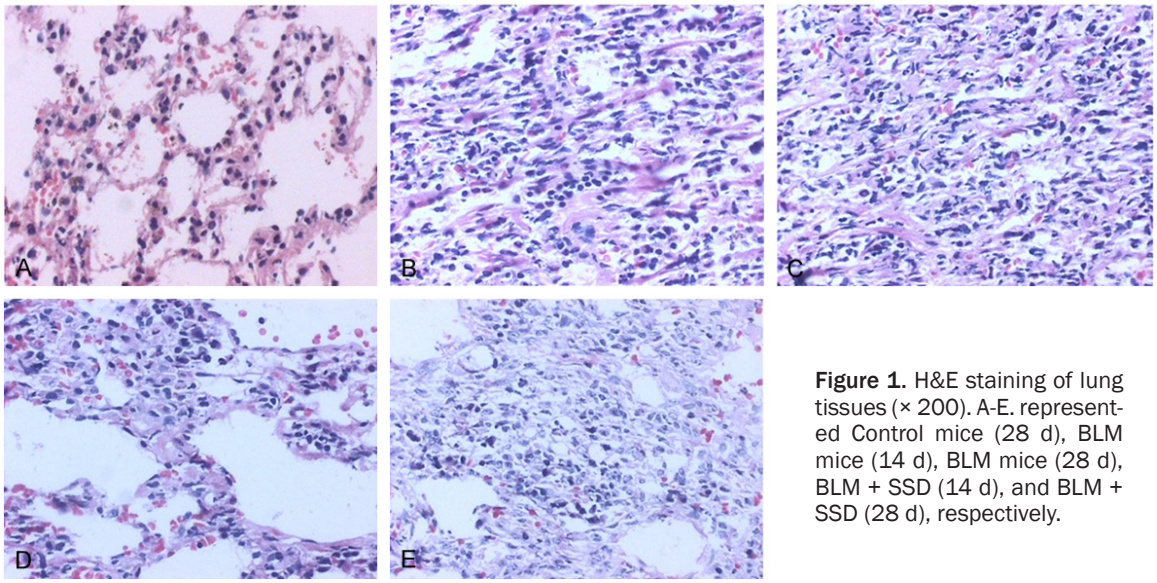

Figure 1. H&E staining of lung tissues (× 200). A-E. represented Control mice (28 d), BLM mice (14 d), BLM mice (28 d), BLM + SSD (14 d), and BLM + SSD (28 d), respectively.

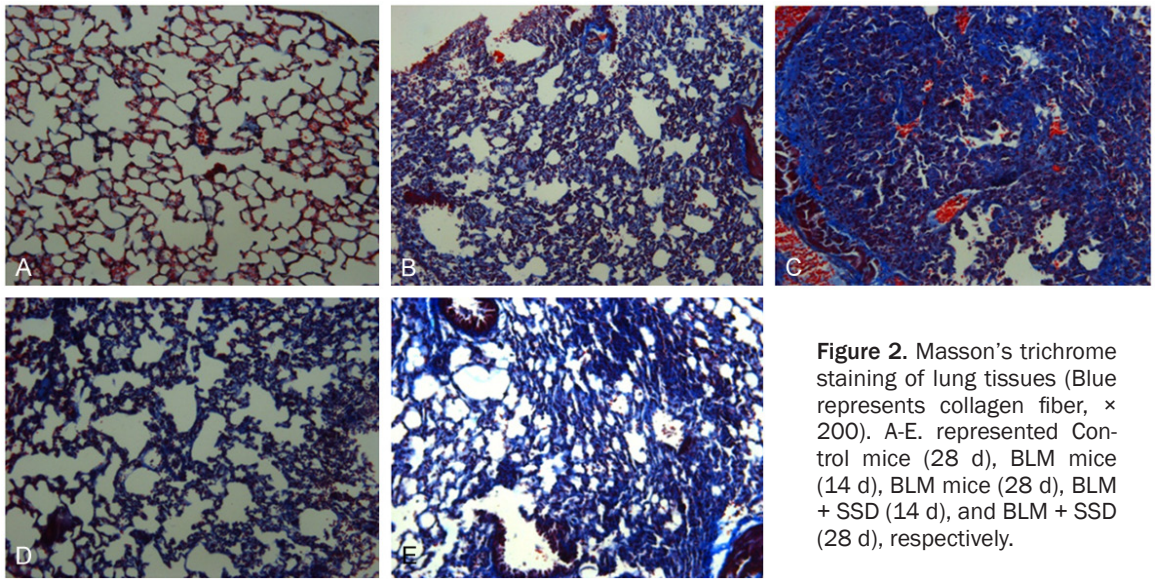

Figure 2. Masson's trichrome staining of lung tissues (Blue represents collagen fiber, × 200). A-E. represented Control mice (28 d), BLM mice (14 d), BLM mice (28 d), BLM + SSD (14 d), and BLM + SSD (28 d), respectively.

Table 3. Effects of SSD on pulmonary inflammation and fibrosis scores of the BLM induced pulmonary fibrosis mice

	14 day		28 day	
	Alveolitis	Fibrosis	Alveolitis	Fibrosis
Control	1.00 ± 0.00**	0.30 ± 0.48**	1.00 ± 0.00**	0.50 ± 0.53**
BLM	3.84 ± 0.81	4.20 ± 0.79	3.27 ± 0.57	7.30 ± 0.67
BLM + SSD	2.76 ± 0.69 *	3.60 ± 0.52*	2.39 ± 0.45*	5.00 ± 0.82**

Data were presented as mean ± SD (n = 10),* $P < 0.05$, **$P < 0.01$, compared with BLM mice.

inflammatory cell infiltration was observed (**Figures 1A**, **2A**). At 14 day after BLM injection, we found clear inflammatory cell infiltration, thickened alveolar wall, and collagen fibers (**Figures 1B**, **2B**). At 28 day after BLM injection, severe tissues damage, inflammatory cell infiltration (pulmonary alveolitis), collapsed partial alveolar and extensive collagen fibers (pulmonary fibrosis) were observed (**Figures 1D**, **2D**), presenting diffuse interstitial pulmonary fibrosis. However, for the SSD treated BLM mice, these abnormal pathological changes above, including pulmonary

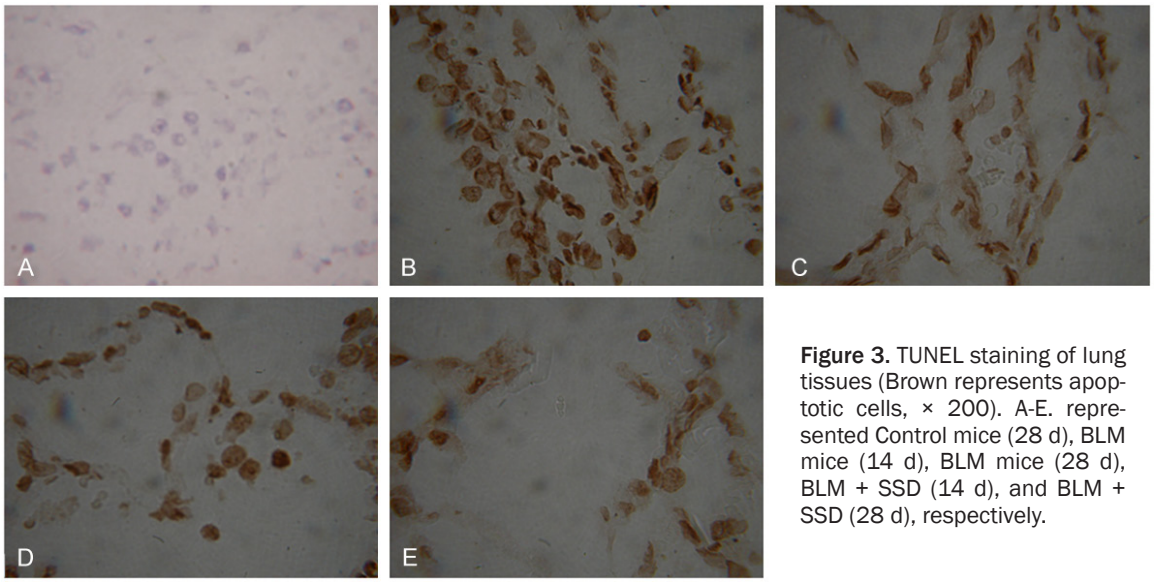

Figure 3. TUNEL staining of lung tissues (Brown represents apoptotic cells, × 200). A-E. represented Control mice (28 d), BLM mice (14 d), BLM mice (28 d), BLM + SSD (14 d), and BLM + SSD (28 d), respectively.

Table 4. Effects of SSD on Pulmonary apoptotic indexes of the BLM induced pulmonary fibrosis mice

	14 day	28 day
Control	12.91 ± 1.99**	12.49 ± 2.25**
BLM	81.26 ± 5.89	49.06 ± 5.74
BLM + SSD	58.67 ± 6.05**	26.09 ± 4.20**

Data were presented as mean ± SD (n = 10), *P < 0.05, **P < 0.01, compared with BLM mice.

alveolitis and pulmonary fibrosis, were notably alleviated both at 14 d (**Figures 1C**, **2C**) and at 28 d (**Figures 1E**, **2E**) after BLM injection.

The scores of pulmonary alveolitis and fibrosis were showed in **Table 3**. Compared with the normal mice, BLM resulted in higher scores of pulmonary alveolitis and fibrosis both in 14 d and in 28 d after BLM injection (P < 0.01). However, SSD treatment could significantly decreased the scores of pulmonary alveolitis and fibrosis both in 14 d and in 28 d after BLM injection (P < 0.05), compared with the BLM mice. These results above suggested that SSD could obviously suppress the BLM-induced pulmonary fibrosis in mice.

Results of TUNEL apoptosis assay

As shown in **Figure 3**, results of TUNEL staining apoptosis assay were depicted. After 28 days of BLM injection, no obvious cell apoptosis was observed in control mice (**Figure 3A**). In contrary, BLM mice in 14 d (**Figure 3B**) and 28 d (**Figure 3D**) after BLM injection showed notable cell apoptosis phenomenon compared with the control mice, and extensive apoptotic epithelial cells with brown nucleus distributed in alveolar ducts and bronchioles were also observed. Interestingly, SSD treatment could obviously alleviate the apoptosis induced by BLM both at 14 d (**Figure 3C**) and at 28 d (**Figure 3E**) after BLM injection. Furthermore, pulmonary apoptotic indexes of the 3 groups were showed in **Table 4**. Similar to the above results, the results indicated that SSD treatment significantly decreased the pulmonary apoptotic indexes in both at 14 d (P < 0.01) and at 28 d (P < 0.01) after BLM injection, compared with BLM mice.

Results of the RT-PCR assay on Caspase-3 expression in lung tissues

As can be seen from the **Figure 4**, mRNA expression of Caspase-3 was presented. Caspase-3 mRNA expression was obviously up-regulated at both 14 d (P < 0.01) and 28 d (P < 0.01) after BLM injection, compared with the control mice. However, SSD treatment could significantly reversed the up-regulative mRNA expression of Caspase-3 at both 14 d (P < 0.05) and 28 d (P < 0.05), compared with BLM mice.

Results of the western blot assay on protein expressions of E-cad, FN, Wnt and β-catenin in lung tissues

In our present study, we also determined the protein expressions of E-cad, FN, Wnt and

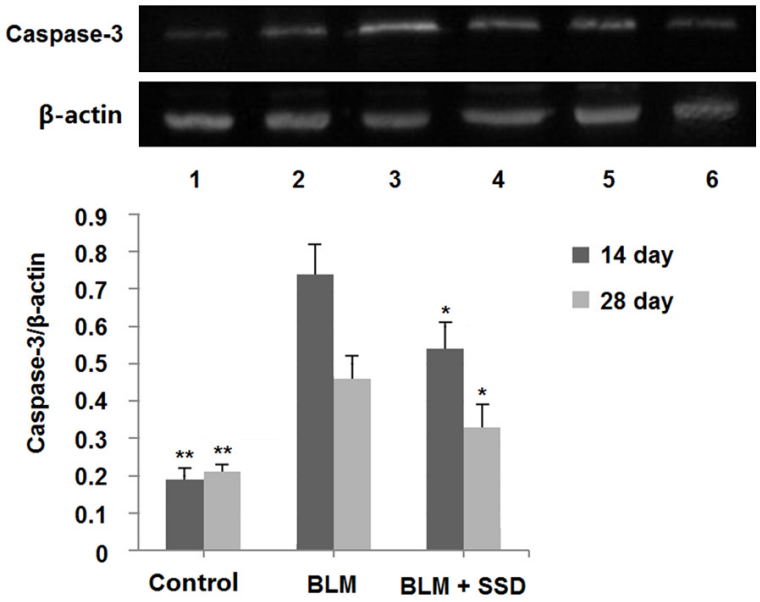

Figure 4. mRNA expression of Caspase-3 in lung tissues. 1-6 represented Control mice (14 d), Control mice (28 d), BLM mice (14 d), BLM mice (28 d), BLM + SSD (14 d), and BLM + SSD (28 d), respectively. Data were expressed as mean ± SD (n = 6), *P < 0.05, **P < 0.01, compared with BLM mice.

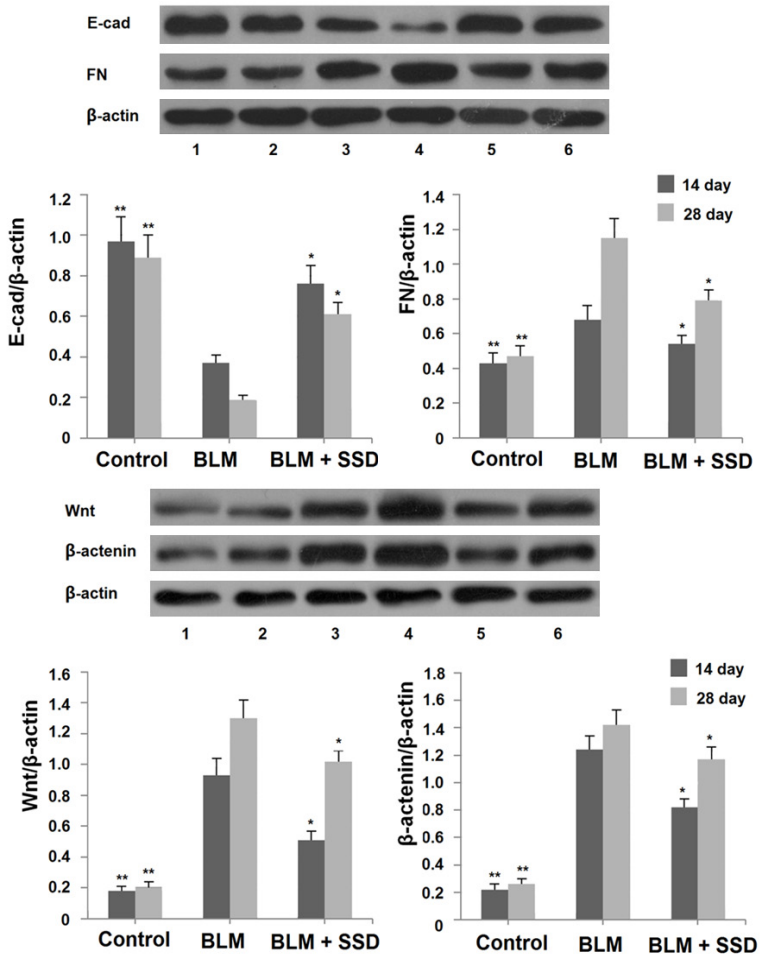

Figure 5. Protein expressions of E-cad, FN, Wnt and β-catenin in lung tissues. 1-6 represented Control mice (14 d), Control mice (28 d), BLM mice (14 d), BLM mice (28 d), BLM + SSD (14 d), and BLM + SSD (28 d), respectively. Data were expressed as mean ± SD (n = 6), *P < 0.05, **P < 0.01, compared with BLM mice.

β-catenin in lung tissues of BLM mice at 14 and 28 days after BLM injection. As shown in **Figure 5**, expressions of FN (P < 0.01), Wnt (P < 0.01) and β-catenin (P < 0.01) significantly up-regulated after BLM treatment, whereas the E-cad obviously down-regulated (P < 0.01), compared with control mice. However, it's interesting that all these abnormal changes could be reversed by treatment with SSD. Results showed that in SSD mice, expressions of FN (P < 0.05), Wnt (P < 0.05) and β-catenin (P < 0.05) significantly down-regulated, whereas the E-cad obviously up-regulated (P < 0.05), compared with BLM mice.

Results of MTT assay

According to the MTT assay, our present results indicated that SSD (2.5, 5 and 10 μg/ml) could significantly inhibit the cell proliferation of HELF (P < 0.01) with a concentration-dependent manner (**Figure 6**). Further, we also investigated the anti-proliferative effects of SSD (2.5, 5 and 10 μg/ml) at different time-points (24, 48, 72 and 96 h), and our results showed a there presented an obvious time-dependent manner.

Results of the western blot assay on protein expressions of TGF-β1 in HELF cells

As shown in **Figure 7**, after treatment with SSD (2.5, 5

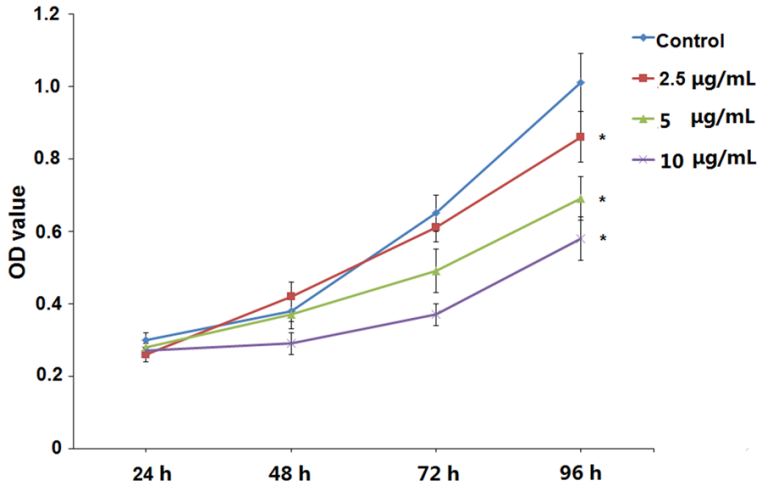

Figure 6. Results of HELF cells viability assay by MTT. Data were expressed as mean ± SD (n = 4), *P < 0.05, **P < 0.01, compared with Control.

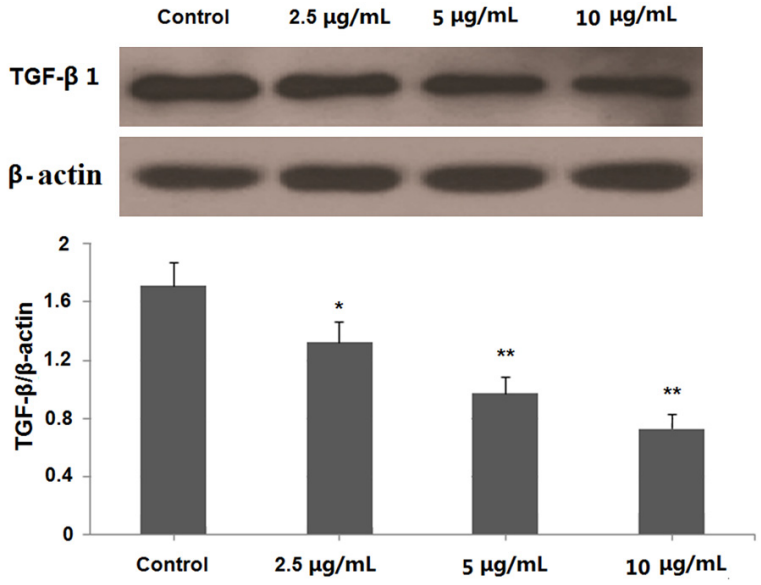

Figure 7. Protein expressions of TGF-β1 in HELF cells. HELF cells were cultured with SSD for 96 h. Data were expressed as mean ± SD (n = 4), *P < 0.05, **P < 0.01, compared with Control.

and 10 μg/ml) for 96 h, protein expressions of TGF-β1 significantly down-regulated (*P < 0.05, **P < 0.01, **P < 0.01, respectively) compared with the Control cells.

Discussion

Although great improvements have been achieved, pulmonary fibrosis (PF) is still an intractable disease so far. Bleomycin (BLM) induced pulmonary fibrosis animal model is commonly used to evaluate candidate drugs against PF [20]. In our research, after intratracheal instillation of BLM at the doses of 5 mg/kg, obvious cell inflammatory infiltration and fibrotic changes were observed, indicating successfully preparation of PF mice. Then, we evaluated the effects of saikosaponin D (SSD) on pulmonary fibrosis by using this animal model above. Interestingly, the animal experimental results indicated that the saikosaponin D (SSD) might be a feasible candidate drug for treating PF.

Currently, the pathogenesis of PF is still not clear, and PF is commonly considered to be related to inflammatory reactions, tissue damage, cell apoptosis and continuous superposed tissue repair in lung [1-4]. Excessive apoptosis of alveolar epithelial cells is considered as the initial damage of PF, and could induce the integrity damage of alveolar capillary membrane (ACM), and then abnormal repair could be resulted in, leading to the fibroblasts' formation, extracellular matrix hyperplasia, vascular remodeling, and finally formation of irreversible pulmonary fibrosis [21]. TUNEL assay is an *in situ* end labeling technique and also a classical method for determining apoptosis [22]. Cell apoptosis is a main type of cell programmed death way in body, and caspase-3 is the most important executor and regulative gene in apoptosis process. Therefore, mRNA expression level of caspase-3 could reflect the apoptosis extent in lung tissues [23, 24]. Our results demonstrated that SSD could significantly decrease the apoptotic extent in lung tissues of BLM induced mice as well as the mRNA expression of caspase-3. In the process of PF, it's reported that epithelial-mesenchymal transition (EMT) plays a crucial role, which is related to the loss of E-cadherin (E-cad) and over-

expression of fibronectin (FN) [25-27]. In the EMT pathway, current research indicated that Wnt/β-catenin signal is another important mechanism for the development of PF [28, 29]. Our results also suggested that SSD treatment could down-regulated expressions of FN, Wnt and β-catenin, whereas up-regulated the E-cad, indicating SSD could suppress the MET pathway in the process of PF. Besides, fibroblasts' excessive proliferation is also very important for the development of PF, and inhibiting the excessive proliferation of fibroblasts is considered as a strategy for treating PF [2-4]. In our results, SSD could significant inhibit the cell proliferation of HELF with a concentration and time-dependent manner. Transforming growth factor (TGF)-β1 belongs to the TGF-β family and is correlated to various aspects of physiological effects, such as promoting of cell apoptosis, EMT and fibrosis formation [30, 31]. Furthermore, TGF-β1 is also recognized as a key of organ fibrosis, and is a target for treating fibrosis [32]. Our results showed that SSD could inhibit the protein expression of TGF-β1 in HELF, which is another important possible mechanism of the anti-fibrosis effect of SSD on pulmonary fibrosis.

In conclusion, our research revealed that Saikosaponin D possess notable anti-fibrosis effect on pulmonary fibrosis *in vivo* and *in vitro* via suppressing alveolar epithelial cell apoptosis and epithelium-mesenchymal transformation.

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Disclosure of conflict of interest

None.

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