Oridonin Inhibits Myofibroblast Differentiation and Bleomycin-induced Pulmonary Fibrosis by Regulating Transforming Growth Factor β (TGFβ)/Smad Pathway

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Background:
Idiopathic pulmonary fibrosis (IPF) is a progressive disease with unknow. etiology and a high mortality rate. Oridonin is a diterpenoid isolated from the *Rabdosia rubesecens* with diverse biological functions. However, whether oridonin possess potential protective activity on IPF is still unclear.

Material/Methods:
The aim of the present study was to explore the therapeutic effects of oridonin on IPF. First, TGF-β1-induced MRC-5 cells were employed for the evaluation of inhibitory activity *in vitro*. Then, a bleomycin (BLM)-induced mice pulmonary fibrosis model was used to verify the activity of oridonin *in vivo*. Several pathological changes, including alveolar space collapse, emphysema, and infiltration of inflammatory cells, were observed in the BLM-treated mice.

Results:
Oridonin could significantly inhibit the mRNA and protein expression levels of α-SMA and COL1A1 in TGF-β1-induced MRC-5 cells. Oridonin could attenuate pathological changes, including alveolar space collapse, emphysema, and infiltration of inflammatory cells induced by BLM. In addition, oridonin could significantly inhibit BLM-induced upregulation of α-SMA and COL1A1 and the phosphorylation of Smad2/3 in lung tissues of mice.

Conclusions:
Oridonin could be used as a potential therapeutic agent in treatment for patients with IPF. The mechanisms of anti-fibrosis effect of oridonin might be inhibition of the TGF-β/Smad pathway.

MeSH Keywords:
Idiopathic Pulmonary Fibrosis • Smad Proteins • Transforming Growth Factor beta1

Full-text PDF:
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Background

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive, and irreversible lung disease caused by a variety of factors [1]. The annual incidence of IPF is estimated to range between 2.8 and 18 cases per 100 000 people in Europe and North America, and the prevalence rate has risen over time [2]. This disease is characterized by diffuse interstitial inflammation, excessive extracellular matrix deposition, and respiratory dysfunction. But the pathogenesis is still unclear. Current evidence indicates that IPF was associated with the differentiation of fibroblast to myofibroblast activated by various fibrosis-related mediators, and the myofibroblasts secrete excessive amounts of extracellular matrix molecules resulting of destruction of the lung architecture. Recently, 2 drugs, pirfenidone (PFD) and nintedanib, have received regulatory approval in the United States and elsewhere for the treatment of IPF [3]. However, none of these have prospectively shown a survival benefit in many trials [4]. Thus, there is no satisfactory drug for IPF, and lung transplantation is still the best treatment for prolonging the life of patients with IPF [5]. Therefore, searching for effective drugs to combat IPF is desperately needed.

In recent years, traditional Chinese medicines have attracted greater attention and became a source to explore potential drugs for the treatment of IPF [6]. Oridonin, a diterpenoid, is the main active compound isolated from the *Rabdosia rubesecens* which used for the treatment of inflammation and cancer in Asian countries for hundreds of years. A number of studies focus on the anti-cancer activity of oridonin, including inhibition cells proliferation and induction cells apoptosis [7]. Recently, several studies reported potential activity of oridonin on pulmonary diseases such as acute lung injury [8] and asthma [9]. Besides, a finding suggested the protective role of oridonin in liver fibrosis [10]. In the light of these research findings, we speculated that oridonin might have therapeutic effects on pulmonary fibrosis.

In this study, we determined the effects of oridonin on TGF-β1-induced human lung fibroblasts (MRC-5). Then, the antifibrotic of oridonin *in vivo* was investigated by a bleomycin (BLM)-induced pulmonary fibrosis mice model and the underlying regulatory mechanisms was also explored.

Material and Methods

Animals

Fifty Kunming (KM) mice (female, 18–20 g) were obtained from the Experimental Animal Center of Henan Province (Zhengzhou, China). All animals were housed in controlled ambient temperature (22±2°C), humidity (40–60%), and a 12-hour light/ dark cycle with free access to food and water. The animals were acclimated to the housing conditions for 2–3 days before experiments. All experiments and surgical procedures were approved by the Experimental Animal Care and Ethics Committee of the First Affiliated Hospital, Henan University of Chinese Medicine, which complies with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Chemicals and reagents

Oridonin was purchased from Xi'an Haoxuan Co., Ltd., and the purity was determined to be 99% by HPLC. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was obtained from USB Corporation. Oridonin was purified by our laboratory. BLM hydrochloride was obtained from the Nippon Kayaku Co. Ltd. (Tokyo, Japan). PFD was purchased from the Beijing Kangdini Pharmaceutical Co. Ltd. (lot 150603, Beijing, China). The human fetal lung MRC-5 fibroblasts were purchased from the Cell Resource Center, Institute of Basic Medical Science, Chinese Academy of Medical Science. Fetal bovine serum was purchased from Gibico (Grand Island, NY, USA). TGF-β1 was purchased from PeproTech (Rocky Hill, NJ, USA) and dissolved in DMEM medium before use. Small mother against decapentaplegic (Smad)-2/3, p-Smad2, and p-Smad3 antibodies were all purchased from Proteintech (Wuhan, China). Rabbit anti-mouse α-smooth muscle actin (SMA) polyclonal antibodies, collagen I alpha 1 (COL1A1) antibody was the product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hydroxyproline (HYP) assay kits were purchased from Jiancheng Biochemical Institute (Nanjing, China). DMEM medium was obtained from Nanjing Key Gen Biological Technology Development (Nanjing, China).

MTT assay

The MRC-5 cells were placed in 96-well plates at a density of 3×10^3 cells per well in 100 µL culture medium. The cells were incubated overnight and exposed to 2.5, 5, 10, 15, 20 µM of oridonin for 48 hours. MTT solution (5 mg/mL, 20 µL/well) was added to each well. Plates were incubated at 37°C for 4 hours. Then, 150 µL/well DMSO was added to dissolve formazan crystals. Finally, the absorbance was record at 570 nm with a Model 1500 Multiskan spectrum microplate Reader (Thermo, Waltham, MA, USA).

Cell culture and exposure to TGF-β1

MRC-5 cells were cultured at 37°C in a 5% CO_2 humidified environment and in DMEM supplemented with 10% fetal calf serum for 24 hours. Then, cells were placed into 6-well culture plates at a density of 2×10^5 cells per well for later gene and protein expression assays. After incubating for 12 hours in a serum-free media to induce serum starvation, fibroblasts were exposed to oridonin at concentrations of 5 and 10 µM and PFD
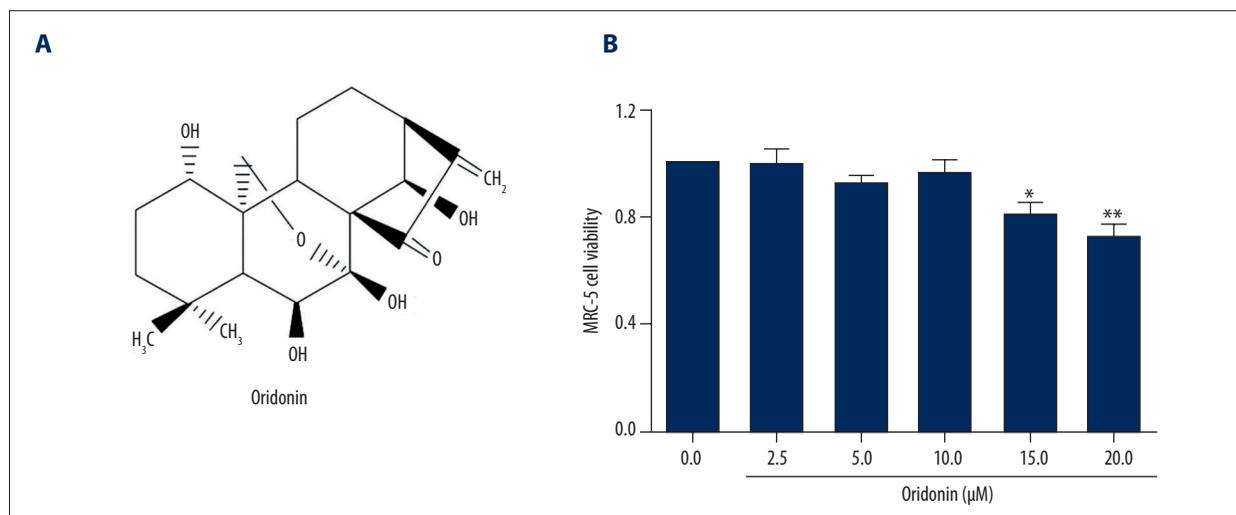
Figure 1. Effect of Oridonin on the cell viability of MRC-5 cells. (**A**) The structure of Oridonin. (**B**) MRC-5 cells were placed into the 96-well plate for 24 hours, then treated with different concentrations (2.5, 5, 10, 15, and 20 μM) of Orid for 48 hours. Finally, the cell viability was analyzed by using MTT assay. Data are presented as mean ±SEM, n=6 per group. * $P<0.05$, ** $P<0.01$ vs. group of Oridonin 0 μM. SEM – standard error of the mean.

(2 mM) with 5 ng/mL of TGF-β1 for 48 hours and then harvested for subsequent analysis.

Induction of pulmonary fibrosis and drug administration

Fifty mice were randomly divided into the following 5 groups: normal group, model group, oridonin (10 and 20 mg/kg) groups and PFD (300 mg/kg) group. The pulmonary fibrosis model was induced by a single intratracheal instillation of 5 mg/kg BLM within 0.9% saline, except for the normal group on day 0. After the instillation, mice were immediately rotated for 2 minutes to ensure uniform distribution of BLM in the lung. Rats in oridonin groups and PFD group were intragastrically administrated with oridonin (10 and 20 mg/kg) and PFD (300 mg/kg) once per day from day 1 to 28, respectively. Mice from normal and model group were given an equal volume of vehicle. On day 28, mice were anesthetized with 300 mg/kg chloral hydrate (monitoring of anesthesia: corneal reflex, respiratory rate) and then sacrificed with dislocation of the neck. Animal death was verified with permanent cessation of the circulation and onset of rigor mortis.

Histological examination

Left lung samples were fixed in 10% neutral formalin, embedded in paraffin, and sectioned. Sections were stained with hematoxylin and eosin (H&E) or Masson's trichrome according to conventional methods. The stained sections were evaluated under a light microscope.

Hydroxyproline (HYP) assay

On day 28, mice were sacrificed, and the right upper lung lobes were harvested. The total content of HYP in lung lobe was measured by assay kit instruction of the manufacturer (Jiancheng, Nanjing, Jiangsu, China).

Real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from lung tissues was extracted by using TRIzol reagent (Ambion, California, US) according to the manufacturer's protocol, the concentration and integrity of total RNA were verified by a NanoDrop2000 nano-spectrophotometer (Thermo, MA, USA). A total of 2 μg RNA was converted to cDNA by using a first-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). TaqMan Master Mix (Biosystems, Foster City, CA, USA) was used to amplify 2 μg cDNA. The expressions of target genes were normalized to β-actin levels. The data was analyzed with using the comparative threshold cycle ($2^{-\Delta CT}$) method.

Western blot analysis

The cultured cells and lung tissue were used for subsequent immunoblot analyses. The protein of MRC5 cells and lung tissue was extracted by lysis buffer (150 mM NaCl, 50mM Tris-HCl, 0.02% NaN3, 1% NP40, and 1mM PMSF) for 10 minutes on ice. The lysate was centrifuged at 12 000 rpm for 10 minutes, and the supernatant was denatured with loading buffer at 100°C for 10 minutes. Total protein concentration was measured using a BCA protein kit (Vazyme, China). Protein was separated by 10% SDS PAGE gels and transferred to PVDF membrane. Membranes
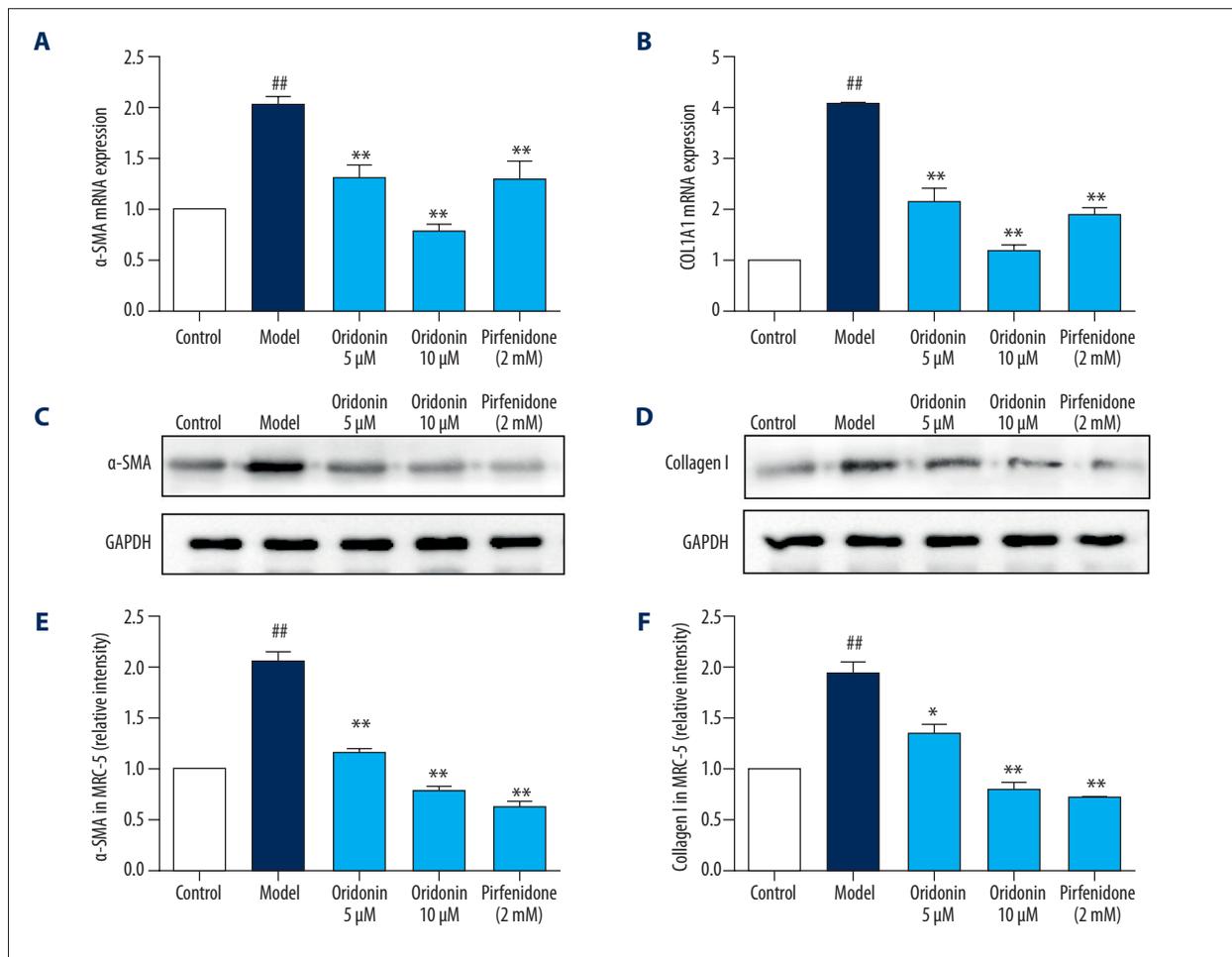
Figure 2. Effect of Oridonin on α-SMA and COL1A1 expression in TGF-β1 induced MRC-5 cells. MRC-5 cells were exposed to Oridonin at concentrations of 5 and 10 μM and Pirfenidone (2 mM) with 5 ng/mL of TGF-β1 for 48 hours. The mRNA expression of α-SMA (**A**) and COL1A1 (**B**) was measured by real-time PCR. The relative quantification was determined using the ($2^{-\Delta CT}$) method normalized to β-actin. Protein levels of α-SMA (**C**) and COL1A1 (**D**) were determined by western blot. GAPDH was used as the loading control. Densitometric analyses of α-SMA (**E**) and COL1A1 (**F**). Data are presented as mean ±SEM, n=6 per group. ## $P<0.01$ *vs.* control group, * $P<0.05$, ** $P<0.01$ *vs.* model group. PCR – polymerase chain reaction; SEM – standard error of the mean.

were blocked in 5% milk in TBST at room temperature for 1 hour and then incubated with the first antibodies. Membranes were washed 3 times with TBST for a total of 30 minutes and then incubated with the horse-radish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The protein bands were visualized with ECL solution.

Statistical analysis

An independent 2 group *t*-test were used for the evaluation of significance between different groups. Date were presented as mean ± standard error of the mean (SEM). A *P* value of less than 0.05 was considered statistically significant.

Results

Effect of oridonin on α-SMA and collagen I expression on MRC5 cell induced by TGF-β

MRC-5 cells were used to assess the effect of oridonin on collagen production. α-SMA, a marker of myofibroblasts, and collagen I, the major components of extracellular matrix were measured by real-time PCR and Western blot. First, we detect the effect of oridonin on the cell viability of MRC-5 cell to find the non-toxic concentrations of oridonin for subsequence experiments. As shown in Figure 1, we found that 2.5–10 μM of oridonin have no effect on the cell viability. While 15 μM and 20 μM of oridonin significantly decreased the cell viability. Thus, we selected 5 μM and 10 μM of oridonin for subsequence
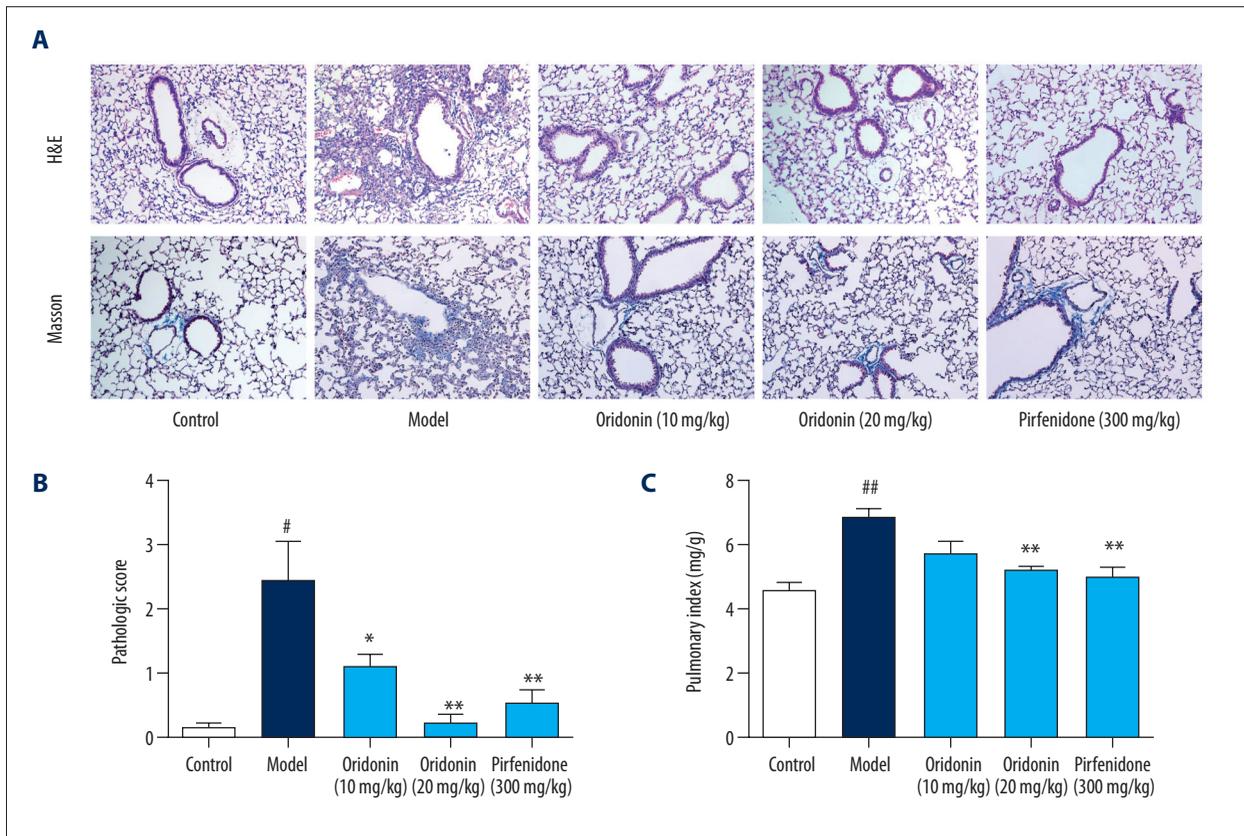
Figure 3. Effects of Oridonin on PF in BLM-exposed mice. From day 1 after the instillation of BLM (5 mg/kg), mice were orally administered with Oridonin (10, 20 mg/kg) or Pirfenidone (300 mg/kg) for 28 days. (**A**) Photomicrograph of mouse lung tissues stained with H&E and Masson, respectively (original magnification 200×). (**B**) Pathologic score of 5 group. The pathologic scores were evaluated according to intra-alveolar congestion, emphysema, intra-alveolar epithelial hyperplasia, interstitial and intra-alveolar infiltration of inflammatory cells and collagen deposition. (**C**) The lung index of 5 group. Data are presented as mean ±SEM, n=6. ## $P<0.01$, # $P<0.05$ *vs.* control group, * $P<0.05$, ** $P<0.01$ *vs.* model group. PF – pulmonary fibrosis; BLM – bleomycin; H&E – hematoxylin and eosin; SEM – standard error of the mean.

experiments. As shown in Figure 2, after induced of TGF-β1, the mRNA and protein expression of α-SMA and collagen I were markedly upregulated compared with the normal group. However, TGF-β1 combined with 5 μM and 10 μM oridonin for 48 hours blocked this effect. The mRNA and protein of α-SMA and collagen I expression were also attenuated in the PFD-treated groups. The results showed that both oridonin and PFD could reduce TGF-β1-induced fibroblast differentiation and, excessive extracellular matrix (ECM) deposition.

Effect of oridonin on morphological changes in mice with pulmonary fibrosis

The lung index was calculated from the wet lung tissue divided by the body weight. As shown in Figure 3, the model group possessed a higher lung index than other groups. A significant depletion in lung index was observed in the oridonin-treated group in 20 mg/kg.

To determine the effect of oridonin on the BLM-induced pulmonary fibrosis in mice, the histopathological analysis of pulmonary tissue by H&E and Masson's trichrome staining were employed. As shown in Figure 2, the lungs of mice in control group displayed no pathological changes. The tissue structure was intact and without inflammatory cell infiltration or interstitial thicken. Whereas, the tissue in the model group shows collapse alveolar space, emphysema, and infiltration of inflammatory cells. The pathology score was significant increased compared with control group. Masson's trichrome staining also displayed that the area of collagen fibers was increased remarkably and distributed in areas of alveolar wall and pulmonary vessels. Compared with the model group, the structure destruction, inflammation and collagen deposition in mouse lungs of oridonin (10 and 20 mg/kg) and PFD-treated groups were apparently extenuated. The pathology scores were decreased in oridonin-treated group compared with model group.
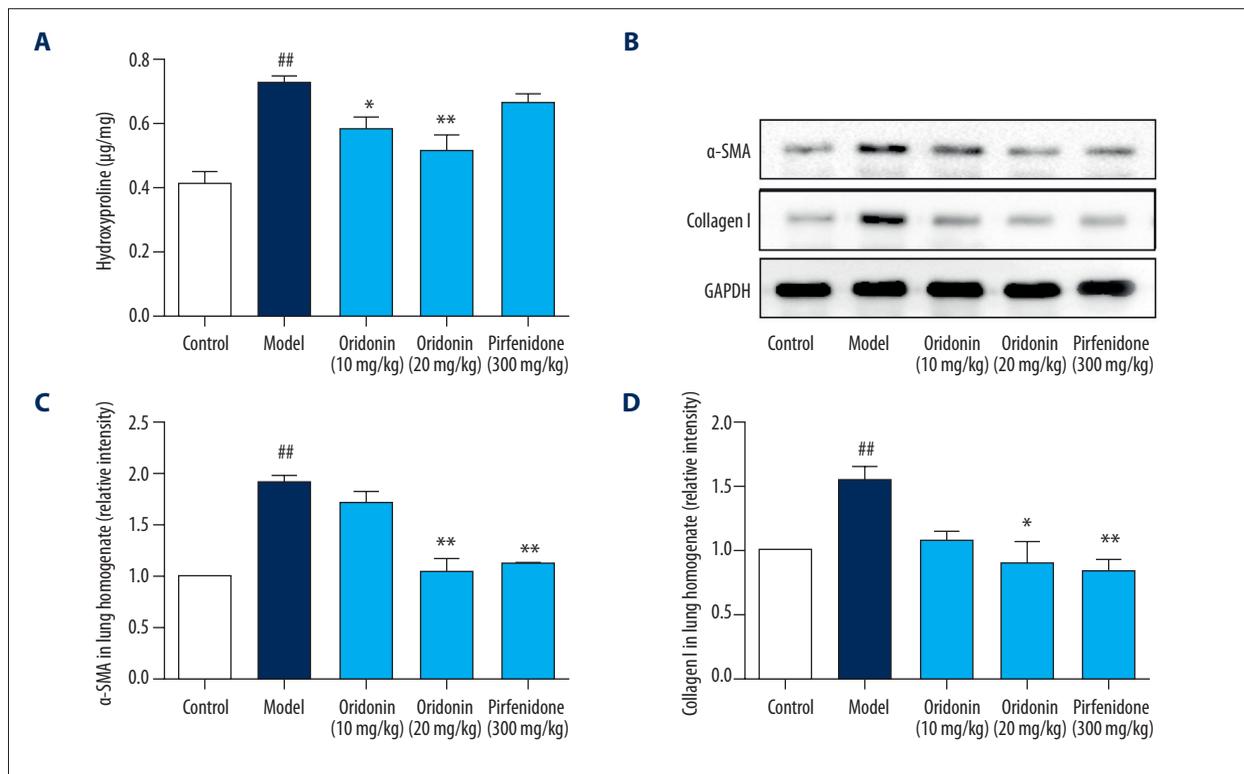
Figure 4. Effects of Oridonin on HP, α-SMA and COL1A1 expression in BLM-induced mice. (**A**) The concentration of HP was measured with by a commercial kit. (**B**) Protein levels of α-SMA and COL1A1 were determined by western blot. GAPDH was used as the loading control. Densitometric analyses of α-SMA (**C**) and COL1A1 (**D**). Data are presented as mean ±SEM, n=6 per group. ## *P*<0.01 *vs.* control group, * *P*<0.05, ** *P*<0.01 *vs.* model group. HYP – hydroxyproline, SEM – standard error of the mean.

Effect of oridonin on collagen biomarkers in BLM-induced pulmonary fibrotic mice

To further confirm the therapeutic effect of oridonin on pulmonary fibrotic mice, we detected the level of HYP, α-SMA, and collagen I. HYP is the main component in extracellular collagen, which is an indicator of pulmonary fibrosis. To evaluate the effects of oridonin on the accumulation of collagen, we quantified the HYP in the lung of mice. As shown in Figure 4, the HYP content of model group was significantly increased compared with the control group. Oridonin showed significant inhibition on HYP level on 10 mg/kg and 20 mg/kg, and PFD exhibited slight decrease in the HYP contents.

α-SMA and collagen I were also the important biomarkers *in vivo* and *in vitro*. Therefore, we detected the level of α-SMA and collagen I in lung tissues. As shown in Figure 4, The level α-SMA and collagen I of the lung tissue in BLM group greatly increased compared with the control group. The oridonin (20 mg/kg) and PFD could significantly inhibit BLM-induced upregulation of α-SMA and collagen I in lung tissues of mice. These results suggested that oridonin could prevent BLM-induced pulmonary damage and fibrosis.

Oridonin blocks TGF-β/Smad-2/-3 signaling transduction in lung tissues of BLM-induced pulmonary fibrotic mice

The aforementioned results suggested that oridonin could regulate collagen deposition in pulmonary fibrotic mice. Since the TGF-β/Smad signaling is commonly observed in collagen synthesis. To further investigate the mechanism of the effect of oridonin on collagen synthesis, we analyzed the protein expression phosphorylation of Smad2 (p-Smad2) and p-Smad3, the key members of TGF-β/Smad signaling pathway. As depicted in Figure 5, BLM-treated model group had a dramatic increase in the levels of p-Smad2 and p-Smad3. Oridonin and PFD significantly inhibited the phosphorylation of Smad2 and Smad3, while no detectable expression alterations of their total proteins were observed between different groups. The result indicated that oridonin was able to restrain BLM-induced TGF-β/Smad signaling transduction in lung tissues.

Discussion

In the current study, inspired by previous research, the TGF-β1 induced MRC-5 cells were first used to assay the anti-fibrotic activity of oridonin *in vitro*. Then, the BLM-induced mice
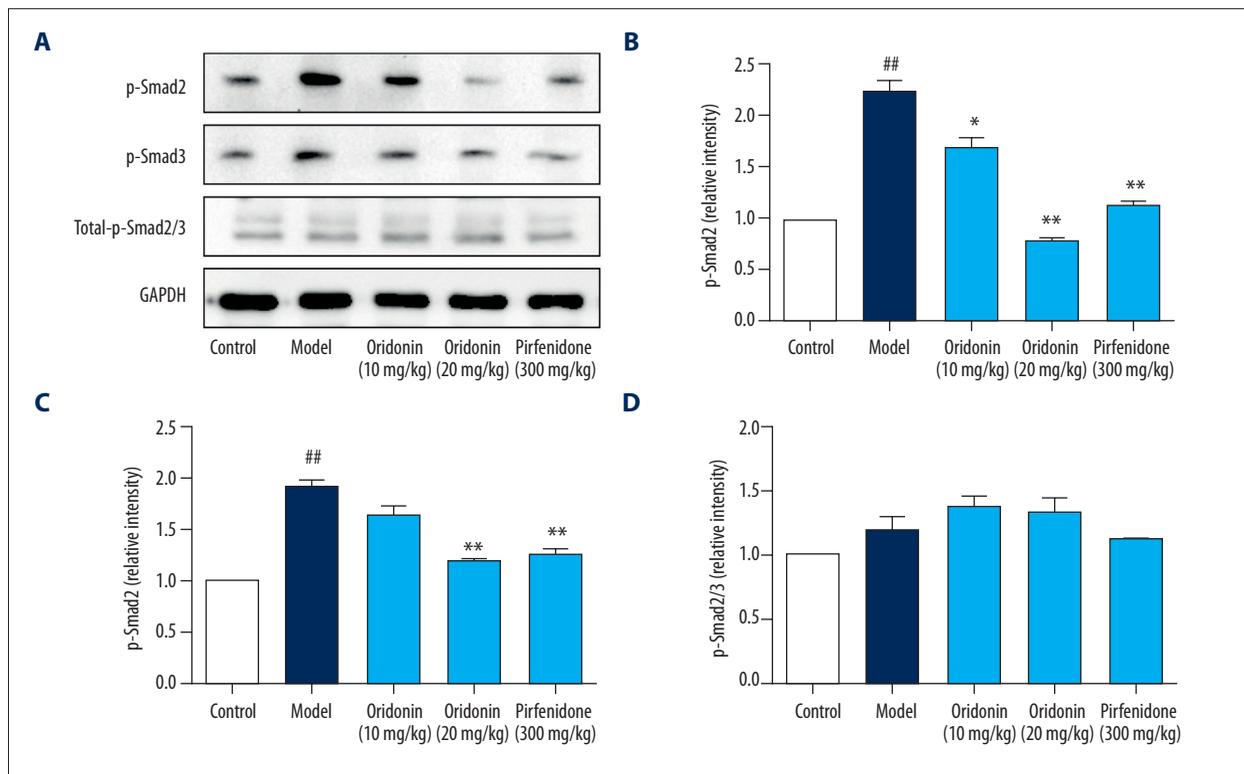
Figure 5. Effects of Oridonin on TGF-β/Smad signaling pathway in BLM-induced pulmonary fibrosis mice. (**A**) The protein expressions of total Smad2/3 and p-Smad 2 and p-Smad3 in lungs of pulmonary fibrosis mice were detected by western blot. Densitometric analyses of p-Smad 2 (**B**), p-Smad3 (**C**) and total Smad2/3 (**D**). Data are presented as mean ±SEM, n=6 per group. ## $P<0.01$ *vs.* control group, * $P<0.05$, ** $P<0.01$ *vs.* model group. BLM – bleomycin; SEm – standard error of the mean.

pulmonary fibrosis model was used to verify the activity of oridonin *in vitro*.

The pathogenic pathways involved in IPF are believed to be uncontrolled differentiation of lung fibroblasts into myofibroblasts, which induced by some profibrotic mediators, including TGF-β1 [11]. Meanwhile, excessive ECM deposit in the interstitial space resulting in loss of parenchymal architecture and lung function [12]. Therefore, inhibition of lung fibroblasts differentiation may yield potential therapeutic benefits for IPF. MRC-5 cells derived from human fetal lung fibroblasts, could be activated by TGF-β1 to differentiate into myofibroblasts [13]. Firstly, the TGF-β1 induced MRC-5 cells were used to assess the effect of oridonin on fibroblasts differentiation *in vitro*. In our study, TGF-β1 could stimulate α-SMA expression as well as increase COL1A1 deposition in MRC-5 cells, which are two important markers of myofibroblast differentiation. The results reveal that oridonin could suppress the mRNA and protein expression levels of α-SMA and COL1A1 in TGF-β1 induced MRC-5 cells. The finding suggested that oridonin could inhibit the differentiation of fibroblasts. Therefore, oridonin was further applied for BLM-induced pulmonary fibrosis model to clarify its effect on the fibrosis *in vivo*.

The BLM-induced animal model is commonly used in the assessment of potential anti-fibrotic agents [14,15]. After BLM instillation, alveolar epithelial cells were damaged at first, while alveolus inflammation was prominent within the first week. The development of fibrosis can be seen biochemically and histologically by day 14 and significantly appeared in the day 28. Therefore, the present animal experiment period was set to 28 days [16]. Our data in keeping with previous studies showed significant histopathological changes in lung, including collapse alveolar space, emphysema and infiltration of inflammatory cells and fibroblast proliferation, in mice delivered with BLM. However, these pathological alterations were attenuated by oridonin in a dose dependent way within the indicated doses. Meanwhile, three major biomarkers of fibrosis, including HYP, α-SMA and COL1A1, were decreased markedly. The above results clearly revealed that oridonin could improve fibrogenic changes through modifying deposition of collagen and ECM in pulmonary fibrosis.

Although the pathogenesis of IPF has not been fully clarified, it has been affirmed that various profibrotic cytokines are involved. Among these cytokines, TGF-β1 plays an important role in the fibrosis by stimulating fibroblast proliferation and differentiation [17]. TGF-β1 has its pleiotropic effects mainly through

TGF-β/Smad pathway. TGF-β1 binds to TbR-II and then triggers heteromerization with and transphosphorylation of TbR-I. The signal is propagated through phosphorylation of receptor associated Smads (Smad2 and Smad3). The phosphorylated Smad2 and Smad3 combine with Smad4, and the complex translate into the nucleus and promotes the expression of pro-fibrotic genes [18,19]. In the present study, oridonin could reduce the expression of phosphorylation Smad2 and Smad3. This suggested that oridonin might attenuate ECM secretion by blocking TGF-β/Smad signal transduction.

Conclusions

In summary, our results revealed that oridonin exhibited inhibitory effects on pulmonary fibrosis *in vitro* and *in vivo*. The mechanism might be due to the inhibition of the TGF-β/Smad pathway. These findings suggest that oridonin can be used as a potential therapeutic agent in treatment for patients with IPF.

Conflict of interest

None.

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