

Dian sanguis draconis improves pulmonary fibrosis by activating autophagy to regulate the PA/PAI-1 balance

Jiayi Song^{1,2}, Qian Li³, Chunyan Yang², Qing Liu², Jianmei Li² and Yi Fu^{1,2}

¹The First Clinical Medical College, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, ²Pulmonary Disease/Respiratory and Critical Care Medicine, The Third Affiliated Hospital of Yunnan University of Traditional Chinese Medicine (Kunming Hospital of Traditional Chinese Medicine) and ³Internal Medicine Department, Yunnan Provincial Corps Hospital of Chinese People's Armed Police Force CN, Kunming, Yunnan, PR China

Summary. Background. Pulmonary fibrosis (PF) is a severe lung disease that manifests as lung tissue destruction and collagen deposition and easily leads to respiratory failure. It is difficult to reverse these conditions using current treatment methods. This study focused on the exploration and development of novel drugs for the treatment of PF.

Methods. This study simulated the pathological process of PF by using a bleomycin (BLM)-induced rat model and a TGF- β 1-induced *in vitro* cell model. Dian sanguis draconis (DSD) was used for intervention, and the effects on the lung tissue structure, collagen fiber deposition, autophagy level and PA/PAI-1 balance were evaluated via pathological tissue staining, western blotting, and ELISA.

Results. In untreated PF rats, severely disordered lung tissue, thickened alveolar septa, and excessive deposition of collagen fibers were observed. In addition, the level of autophagy was inhibited, and the balance of PA/PAI-1 in the lung tissue was disrupted. After treatment with DSD, these pathological injuries improved, as demonstrated by the restoration of lung tissue structure, reduction in collagen fiber deposition, recovery of autophagy levels, and remodeling of the PA/PAI-1 balance. In addition, mechanistically, DSD improves PF by increasing the level of autophagy-related proteins and regulating the PA/PAI-1 balance.

Conclusion. This study confirmed the significant effect of DSD in alleviating PF. These findings provide new drug candidates for the treatment of PF.

Key words: Dian sanguis draconis, Pulmonary fibrosis, Fibrinolytic mechanism, PA/PAI-1 balance, Autophagy

Corresponding Author: Yi Fu, Pulmonary Disease/Respiratory and Critical Care Medicine, The Third Affiliated Hospital of Yunnan University of Traditional Chinese Medicine (Kunming Hospital of Traditional Chinese Medicine), Kunming 650011, Yunnan, PR China. e-mail: 13708797909@163.com
www.hh.um.es. DOI: 10.14670/HH-18-886

Introduction

Pulmonary fibrosis (PF) is a severe lung disease caused by repetitive damage to lung tissue. This damage causes harm to lung epithelial cells and destruction of the alveolar-capillary basement membrane. Furthermore, PF stimulates abnormal fibroblast proliferation and infiltration, resulting in excessive ECM deposition and persistent inflammatory damage to the lung tissue structure. These consecutive pathological events lead to a gradual decline in lung tissue function, posing a significant risk to patient survival (Wijsenbeek et al., 2022; Rajan et al., 2023). The typical progression of PF involves three key phases: initial injury, subsequent inflammation, and eventual tissue repair (Tanner et al., 2023). However, owing to atypical fibroblast activity, increased extracellular matrix buildup, ongoing inflammation, and destruction of lung tissue, patients experience a steady decline in lung function. As the clinical symptoms worsen, treatment becomes more complex, leading to a notable increase in mortality rates (Raghu et al., 2018; Yanagihara et al., 2019; George et al., 2020). PF is caused by a variety of causative factors, including autoimmune diseases, environmental or occupational exposures, and side effects of certain medications (Xie et al., 2023). However, it is important to note that the cause of some PF cases is unknown, and there is currently no effective treatment to cure the disease.

Fibrosis receptors and inhibitors play central roles in tissue remodeling and inflammation (Takabayashi and Schleimer 2020). Specifically, various phenotypes and endotypes are linked to tissue remodeling in upper respiratory conditions (Barham et al., 2015). Plasminogen activator inhibitor-1 (PAI-1), belonging to the serine protease inhibitor family, mainly targets tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA). PAI-1 facilitates the conversion of fibrinogen into fibrin, thus contributing to the fiber decomposition process (Huebner et al., 2018).



Importantly, t-PA is considered an essential activator of fibrinogen in the fibrinolysis process (Godtfredsen et al., 2022). PAI-1 is expressed ubiquitously in various essential organs, such as the skin, esophagus, lung, kidney, liver, eye, and heart, underscoring its broad impact on living beings (Liu et al., 2023). Research has indicated that PAI-1 plays a role in various disease pathways and is abundant in liver fibrosis, homogenized mouse tissues, and fibroblasts (Chen et al., 2019; Zanetto et al., 2022). Although these findings reveal the broad effects of PAI-1, its specific mechanism of action in PF still needs to be further explored.

Dian sanguis draconis (DSD), a powerful element found in ancient Chinese herbal remedies, has various pharmacological benefits, such as anti-inflammatory, pain-relieving, antithrombotic, microcirculation enhancement, antioxidant, lipid reduction, and hypoglycemic effects (Long, 2018; Zhang et al., 2018). Recent research has demonstrated that DSD has the potential to effectively decrease fibrosis severity in rats afflicted with pulmonary fibrosis. This effect is believed to stem from the suppression of transforming growth factor (TGF)- β 1 mRNA expression in pulmonary tissue, resulting in the excessive accumulation of type I collagen (Jing et al., 2019). The exact process through which DSD influences PF is not yet completely understood. Therefore, the purpose of this study was to explore the potential mechanism of action of DSD in PF to provide new strategies for the treatment of PF.

In summary, the aim of this study was to explore the potential mechanism of action of DSD in the treatment of PF. Given the demonstrated benefits of DSD in reducing fibrosis in rats with PF, we further explored whether the specific pathways of action and molecular mechanisms of DSD in the treatment of PF are related to the PA/PAI-1 balance. These results not only enhance our knowledge of PF development but also offer valuable experimental and theoretical groundwork for future PF treatment innovations.

Materials and methods

Cell culture and treatment

The fibroblast line derived from human fetal lung (MRC-5) was obtained from the ATCC Biological Standards Resource Center (CCL-171; Manassas, Virginia, USA) and maintained in a 5% CO₂ atmosphere at 37°C. MRC-5 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 IU/mL penicillin and 100 mg/mL streptomycin. A PF model was created *in vitro* by exposing MRC-5 cells to 10 ng/mL TGF- β 1 (eBioscience, CA, USA) for 48 hours (Hussein et al., 2024). Moreover, based on the specific experimental requirements, various intervention factors were used to stimulate cells. MRC-5 cells were exposed to 10 nM rapamycin, which acts as an autophagy activator (Rapa, 553210, Merck), for 2 hours. For the DSD treatment

group, MRC-5 cells were treated with 1, 10, 50, or 100 μ g/mL DSD for 48 hours while TGF- β 1 was added.

Animal modeling

Six- to eight-week-old SPF-grade Sprague-Dawley (SD) rats, weighing approximately 200-220 g, were obtained from the Animal Experiment Center at Kunming Medical University. The animal experimental protocols were supervised and approved by the Animal Ethics Committee of Kunming Medical University (approval number: R-062021LH-074). The rats were allowed to acclimate for one week and were provided tap water and standard feed in a controlled environment with a temperature of 23-25°C, humidity of 50-60%, and a 12-hour light/dark cycle. After acclimatization, the rats were randomly divided into two groups: the control group (n=10) and the BLM-induced model group (n=50). The control group of rats received 3 mL of distilled water, whereas the BLM-induced group received an intratracheal injection of 5 mg/kg BLM (Sigma-Aldrich, USA) to induce pulmonary fibrosis.

Next, the BLM-induced rats were randomly divided into 5 groups (n=10): the BLM group, positive control group treated with pirfenidone (PFD), low-dose DSD (DSD-L) group, medium-dose DSD (DSD-M) group, and high-dose DSD (DSD-H) group. In the BLM group, the rats were given 3 mL of distilled water orally daily. The rats in the PFD group were given 0.12 mg/kg PFD (dissolved in 3 mL of distilled water) by intragastric administration daily, whereas the high, medium, and low DSD dose groups were administered DSD powder at dosages of 2.4 g/kg, 1.2 g/kg, and 0.6 g/kg, respectively, dissolved in 3 mL of distilled water via intragastric administration. Rats in the treatment group received intragastric administration on day 2 after BLM induction. All groups of rats were euthanized on Days 14 and 28 after treatment (with five rats from each group being sacrificed at each time point). Lung tissue samples were collected for subsequent analysis.

Pathological tissue staining

H&E staining: Deparaffinized lung tissue sections were stained with hematoxylin for 5 minutes. Following a wash with ammonia water, the sections were stained with eosin staining solution for 1 minute. Ethanol dehydration was performed before the sections were clarified and sealed with neutral glue. The degree of pulmonary fibrosis was observed. Sections were stained using a Masson staining kit (Solarbio, Beijing, China). A light microscope was used to observe the results.

Determination of hydroxyproline (Hyp) content

To evaluate the collagen levels in the lung tissue, Hyp was analyzed according to the protocol provided by the manufacturer using a kit (ab222941; Abcam, UK). A total of 10 mg of tissue was homogenized in 100 μ L of distilled

Dian sanguis draconis improves pulmonary fibrosis by activating autophagy

water. Following this, hydrolysis was performed overnight at 120°C by adding 100 µL of 12 M HCl to the sample. The resulting mixture was then subjected to centrifugation at 14000 rpm for 20 minutes, and 10 µL of the obtained supernatant was transferred to a 96-well plate. Each sample was subsequently treated with 100 µL of chloramine-T reagent, which was allowed to incubate at room temperature for 5 minutes. To this mixture, 100 µL of dimethylaminobenzaldehyde reagent was added, and the mixture was incubated at 60°C for 90 minutes. Finally, the absorbance of each sample was measured, and the Hyp content was quantified as µg/mg of lung tissue.

Immunohistochemistry

Paraffin sections of lung tissue were dewaxed and hydrated. Then, antigen retrieval was performed, and any inherent peroxidase activity was determined using a 5% hydrogen peroxide (H₂O₂) solution. The tissue samples were then incubated overnight with primary antibodies targeting α-SMA (1/200, ab7817, Abcam, UK), collagen I (1/150, ab34710, Abcam, UK), and LC3B (1/100, ab232940, Abcam, UK). Next, the sections were treated with the corresponding secondary antibody for 2 hours and then washed with PBS. Finally, protein expression was observed with a light microscope after the samples were stained with DAB.

Western blot

Proteins were extracted from lung tissue and MRC-5 cells using RIPA buffer (Sigma-Aldrich, USA) containing 1% protease inhibitors and phosphatase inhibitors. The protein concentration was assessed using a bicinchoninic acid (BCA) detection kit (Thermo Scientific, USA). Following SDS-PAGE separation, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, USA) and blocked with 5% skim milk at room temperature for 1.5 hours. The primary antibody (Table 1) was diluted and incubated overnight at 4°C. The membrane was subsequently incubated with a secondary antibody (1:4000, ab97051, Abcam, UK) at room temperature for 1 hour and then visualized with an enhanced chemiluminescence (ECL) kit (Millipore, USA). The bands were semiquantitatively

analyzed using ImageJ software.

ELISA

Briefly, t-PA (CSB-E07917r, CSB-E07916h), uPA (CSB-E07368r, CSB-E04751h) and PAI-1 (CSB-E07948r, CSB-E07946h) ELISA kits (CUSABIO, Wuhan, China) were used to detect t-PA, uPA and PAI-1 expression in rat lung tissues and cells. For each experimental group, rat lung tissue homogenate or MRC-5 cell medium was collected, transferred to sterile centrifuge tubes, and centrifuged at 1,000 × g for 10 minutes at 4°C. The resulting supernatants were aliquoted into small EP tubes. In the next step, each necessary working solution was added, and the optical density (OD) value was determined using a microplate reader (Thermo Fisher Scientific, MA, USA).

RT-qPCR

Rat lung tissue and MRC-5 cells were used to extract total RNA using TRIzol reagent (15596026CN, Invitrogen) following experimental protocols. After the RNA was reverse transcribed into cDNA, real-time PCR analysis was conducted using the SYBR Green Real-Time PCR Kit (Solarbio, China), with GAPDH as the internal reference. The data were then analyzed using the 2^{-ΔΔCt} method. For the specific primer sequences, please refer to Table 2.

Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS. The cells were blocked with 10% BSA at room temperature for 1 hour, after which primary antibodies targeting α-SMA (1/100, ab7817, Abcam, UK), collagen I (1/150, ab34710, Abcam, UK), and LC3B (1/100, ab232940, Abcam, UK) were added and incubated overnight at 4°C. Following PBS washes, the fluorescent secondary antibody was applied, and the cells were incubated at 37°C for 2 hours in the dark. After being washed with PBS, the cells were then stained with DAPI solution for 5 minutes at room temperature, followed by another round of washing with PBS. Finally, an anti-fluorescence quencher was used to

Table 1. Primary antibodies used for western blotting.

Antibody	Dilution	Article No.	Company
α-SMA	1/1000	ab108424	Abcam
Collagen I	1/1000	ab316222	Abcam
Collagen III	1/1000	ab184993	Abcam
LC3 I/II	1/2000	ab192890	Abcam
Beclin1	1/2000	ab207612	Abcam
p62	1/1000	ab109012	Abcam
Fibronectin	1/1000	ab268020	Abcam
TGF-β1	1/1000	ab315254	Abcam
GAPDH	1/2000	ab8245	Abcam

Table 2. Primer sequences.

Genes	Sequences
<i>t-PA</i>	Forward 5'-TTGTGGAGTGGCGTTCA-3' Reverse 5'-GGCACTGGCAGACGAA-3'
<i>uPA</i>	Forward 5'-AGGCGACCCCTGGTGCTA-3' Reverse 5'-GGGCTTCCTCCCTTATTCT-3'
<i>PAI-1</i>	Forward 5'-ACGACATCCTGGAAGTGC-3' Reverse 5'-TCACCTCGATCTTGACCTT-3'
<i>GAPDH</i>	Forward 5'-ACAGCAACAGGGTGGTGGAC-3' Reverse 5'-TTTGAGGGTGCAGCGAAGCTT-3'

mount the cell sheets, which were subsequently observed and imaged using a fluorescence microscope.

CCK-8 assay

MRC-5 cells were seeded in 96-well plates at a density of 3×10^3 cells per well. After 6 hours, the cells were treated with TGF- β 1 and DSD for 48 hours. Then, 10 μ L of CCK-8 solution was added to each well, and the cells were further incubated for 4 hours. Finally, the absorbance of the cells at 450 nm was measured with a microplate reader.

Statistical analysis

GraphPad Prism 8.0 (GraphPad Software Inc., San

Diego, CA, USA) was used to conduct the statistical analyses. All the experiments were repeated at least three times. The results are displayed as the means $\pm$ standard deviations. Group comparisons were assessed with a t test, and multiple groups were compared via one- or two-way analysis of variance (ANOVA) followed by Fisher's LSD multiple comparisons test. Statistical significance was defined as $p < 0.05$.

Results

DSD relieves lung histopathological injury in PF rats

To explore the impact of DSD on lung histopathological damage in PF rats, the BLM-induced PF rat model was treated with varying doses of DSD. Lung

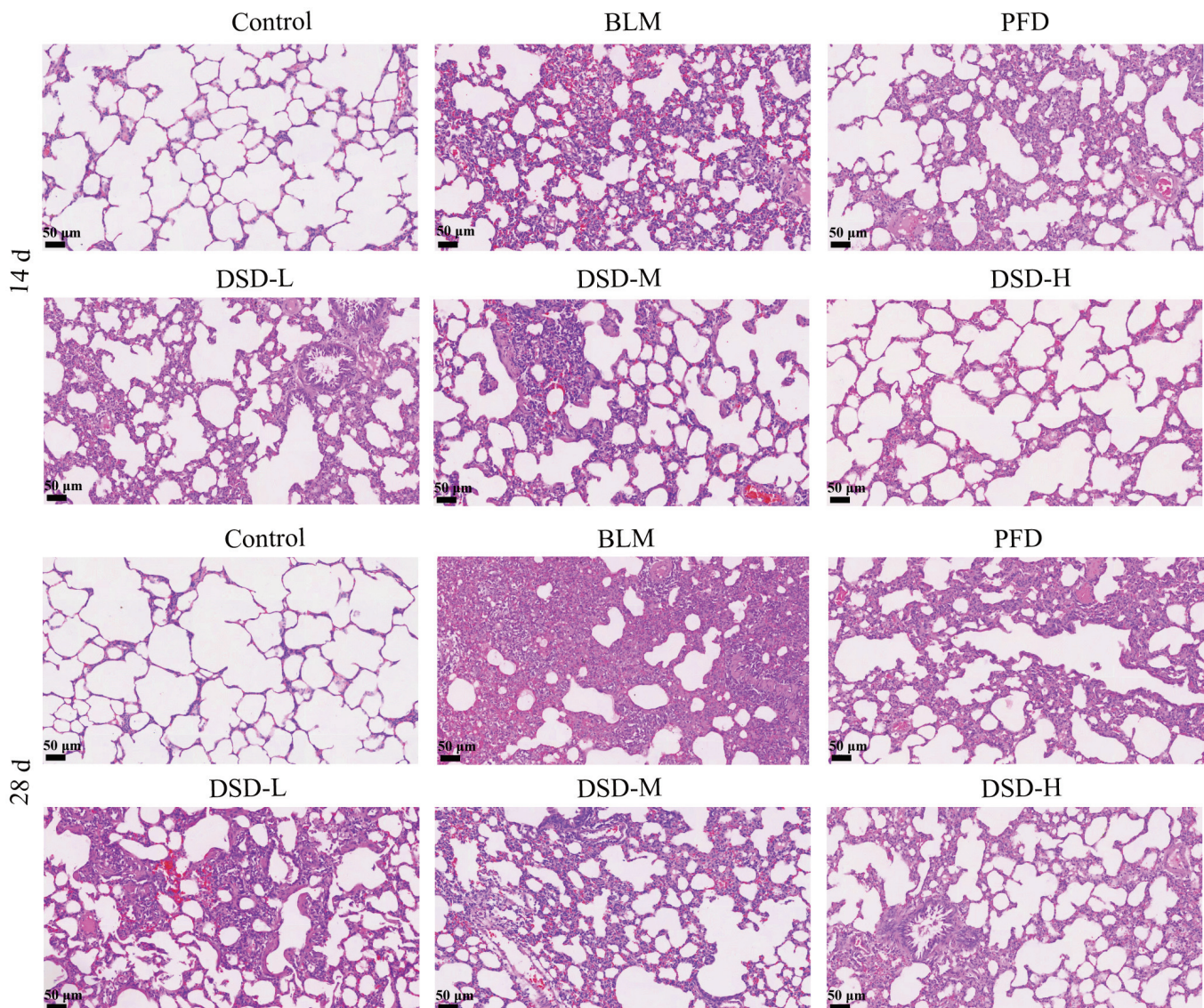


Fig. 1. DSD relieves histopathological lung injury in PF rats, pathological changes in the lung tissue were observed by HE staining. $n=5$; n represents the number of animals in each group.

samples were collected at 14 and 28 days posttreatment. The tissues were examined using HE staining to assess the degree of alveolar inflammation. Compared with those in the control group, BLM-induced lung tissue inflammation was more severe, and the alveolar septum was significantly thickened. The pathological status of the lung tissue improved after treatment with PFD and DSD, the therapeutic effect increased with increasing DSD dose, and the effect of 14 days of treatment was greater than that of 28 days of treatment (Fig. 1). Compared with the control group, the BLM-induced group presented extensive blue staining and increased collagen fibers in the extracellular matrix. Treatment with PFD and DSD alleviated these effects, with the degree of remission correlated with the DSD dosage (Fig. 2). Overall, the results of the tissue staining indicated that DSD alleviates lung tissue damage in PF

rats, with better outcomes observed with early treatment.

DSD decreased the Hyp content in the lung tissues of PF rats

Hyp content was measured to evaluate collagen levels in lung tissue. Compared with the control group. Following BLM induction, the Hyp content in rat lung tissue significantly increased but subsequently decreased with PFD and DSD treatment. DSD treatment caused a dose-dependent decrease (Fig. 3A,B).

DSD inhibits the expression of fibrosis-associated proteins in the lung tissue of PF rats

To explore whether the therapeutic effects of DSD on PF are associated with the regulation of fibrosis, fibrosis-

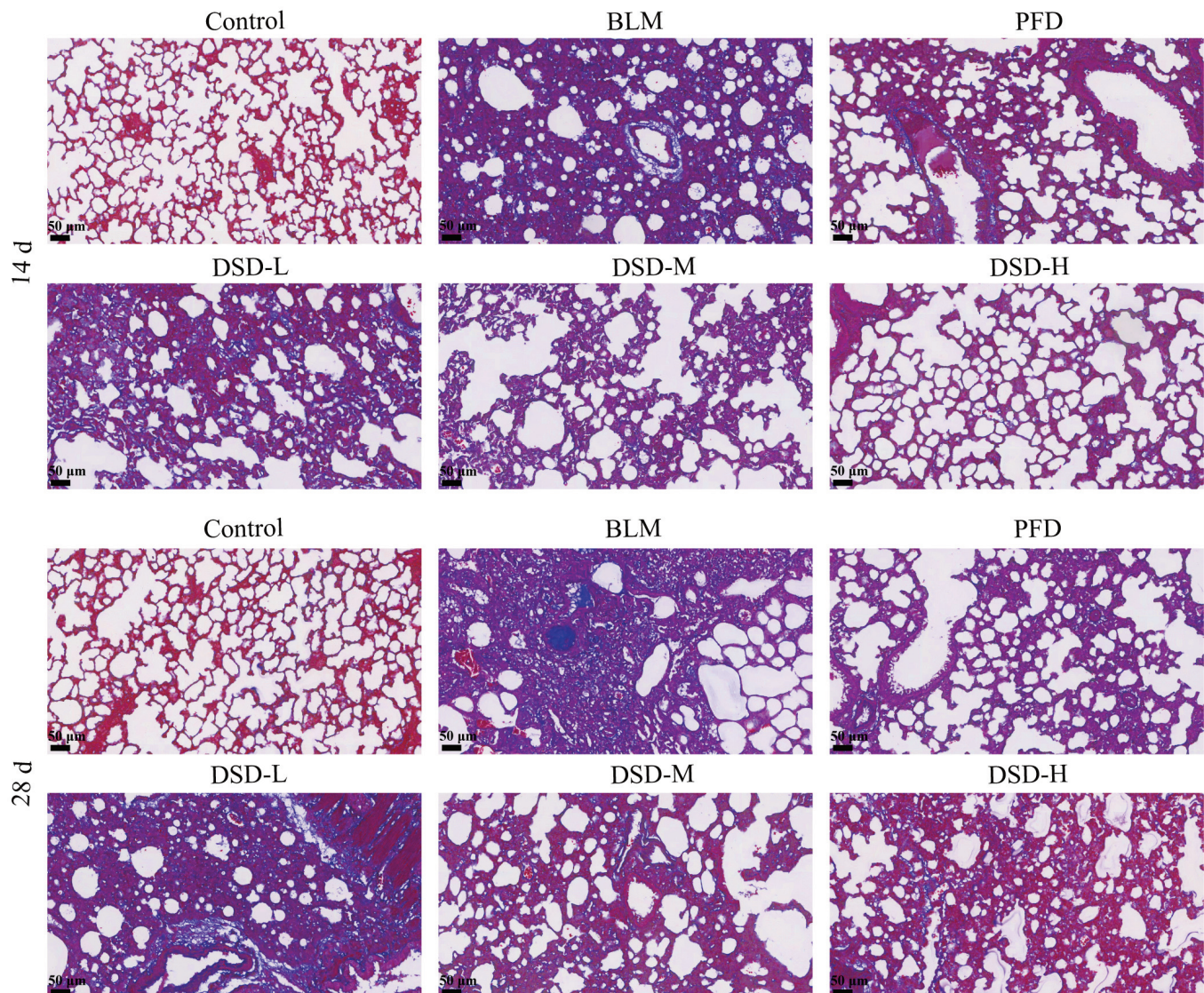


Fig. 2. DSD relieves histopathological lung injury in PF rats, masson staining was used to observe PF in the rats. n=5.

related protein levels in rat lung tissue were examined. The western blot results revealed significant increases in TGF- β 1, α -SMA, collagen I, collagen III, and fibronectin levels

in rat lung tissue after BLM induction compared with those in the control group. However, these elevated levels were reduced after DSD treatment, with the mitigation effect becoming more pronounced as the dosage of the drug increased (Fig. 4). Similarly, the immunohistochemical results also revealed that BLM induction increased α -SMA and collagen I protein expression levels and that DSD alleviated the effect of BLM in a dose-dependent manner (Fig. 5A,B). These findings suggest that DSD has the ability to suppress lung tissue fibrosis in PF rats.

DSD improved the PA/PAI-1 balance in the lung tissue of PF rats

As the primary inhibitor of tPA and uPA within an organism, PAI-1 controls plasminogen activation. PAI-1 inhibits uPA/tPA-mediated plasminogen activity in the lungs, liver, and kidneys, leading to matrix accumulation and fibrosis (Bienvenu et al., 2012; Makuszewska et al., 2023). We detected tPA, uPA and PAI-1 expression levels in rat lung tissue. The results revealed that the

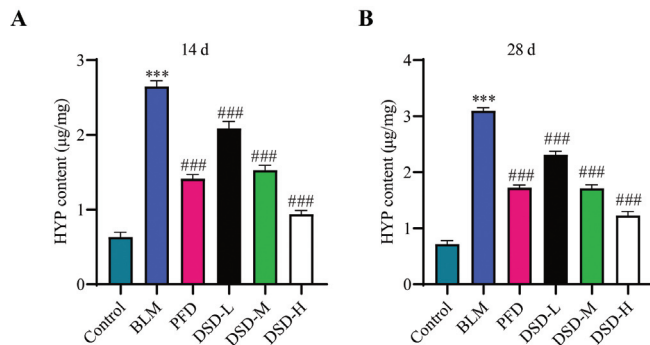


Fig. 3. DSD decreased Hyp content in the lung tissue of PF rats. **A, B.** Determination of hydroxyproline (Hyp) content, n=5. Compared with the control group, *** $p<0.001$; compared with the BLM group, ### $p<0.001$.

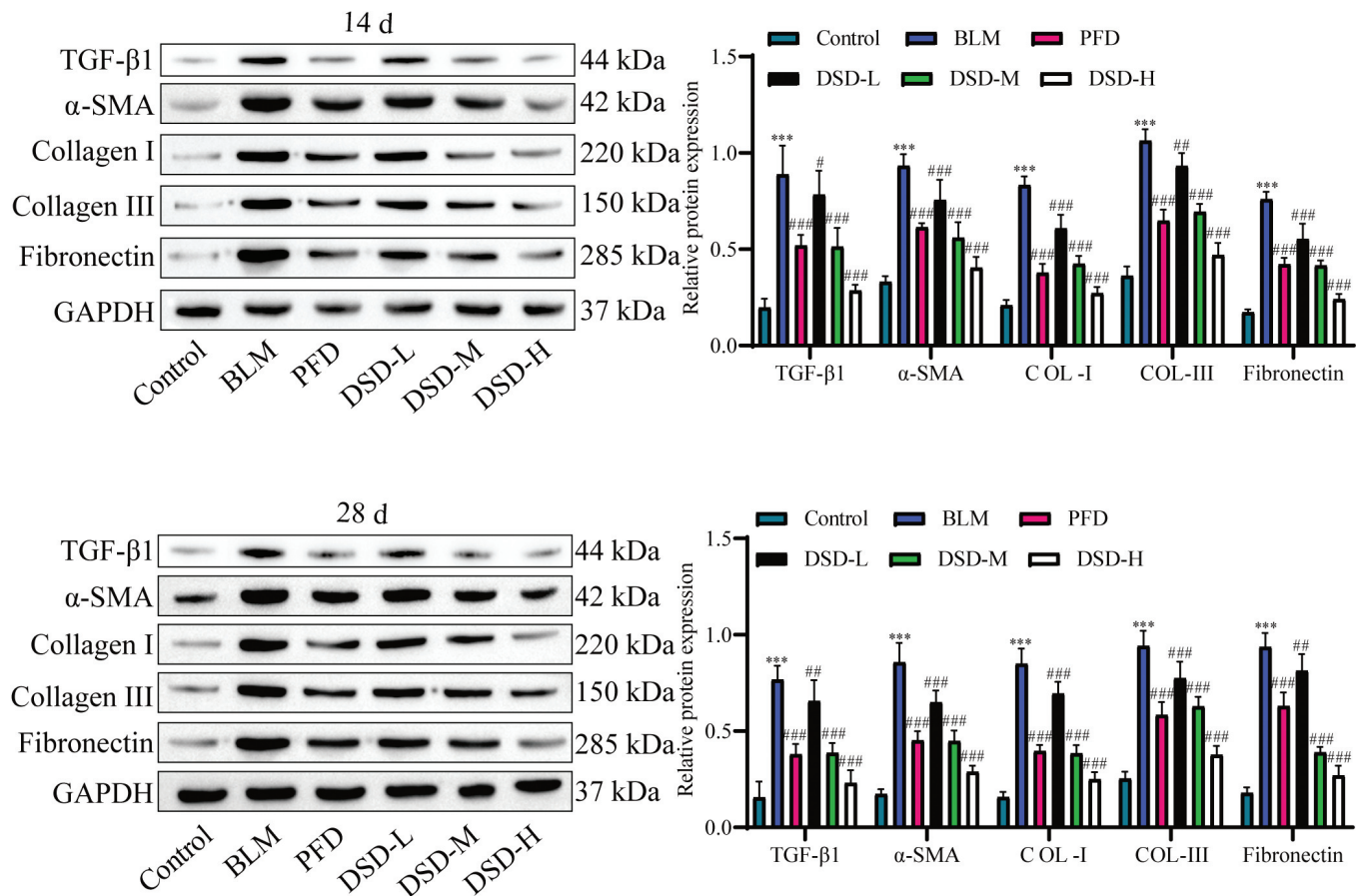


Fig. 4. DSD inhibits the expression of fibrosis-associated proteins in the lung tissue of PF rats. The expression levels of the fibrosis-associated proteins TGF- β 1, α -SMA, collagen I, collagen III, and fibronectin in lung tissue were detected by western blot. n=5; compared with the control group, *** $p<0.001$; compared with the BLM group, # $p<0.05$, ## $p<0.01$, and ### $p<0.001$.

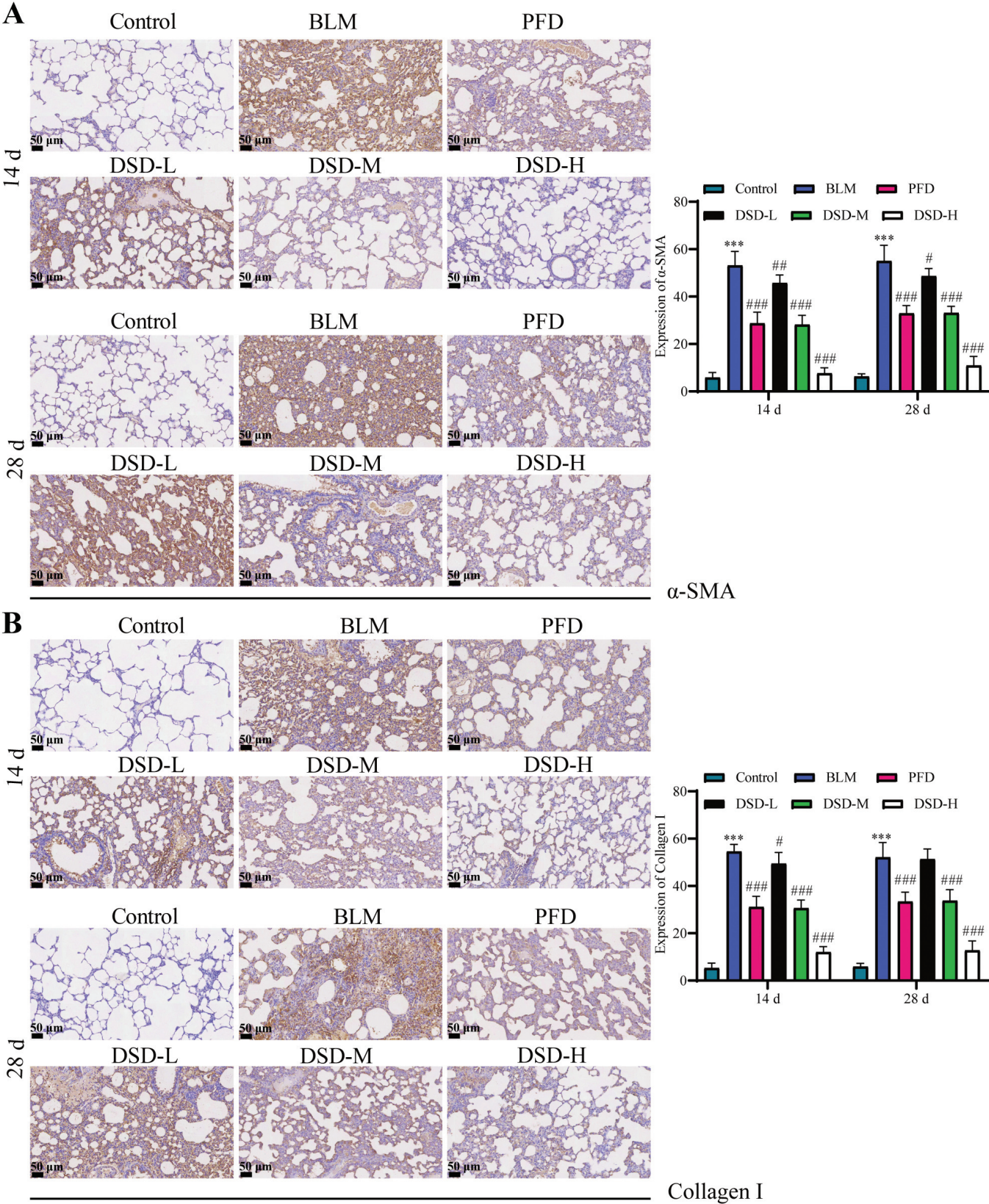


Fig. 5. A, B. The expression of α -SMA and collagen I was detected by immunohistochemistry. $n=5$; compared with the control group, *** $p<0.001$; compared with the BLM group, # $p<0.05$, ## $p<0.01$, and ### $p<0.001$.

levels of tPA and uPA decreased and the level of PAI-1 increased in BLM-induced rat lung tissue. The effect of BLM was partially reversed post-DSD treatment (Fig. 6A). The expression levels of tPA, uPA, and PAI-1 mRNAs were also assessed using RT-qPCR, and similar results were obtained (Fig. 6B).

DSD increases the expression of key proteins involved in autophagy in PF rats

Autophagy plays a crucial role in the development of lung fibrosis (Lv et al., 2020). To explore whether the ability of DSD to alleviate PF is related to autophagy, the levels of the autophagy-related proteins LC3II/I, Beclin1, and p62 in lung tissue were analyzed. Compared with those in normal rats, LC3II/I and Beclin1 levels were lower, whereas the protein expression of p62 was elevated in the BLM group. However, these levels were partially reversed after treatment with DSD in a dose-dependent manner (Fig. 7). Immunohistochemistry was used to assess LC3B

expression. These findings indicated that BLM induction suppressed LC3B expression, whereas DSD treatment led to increased LC3B expression (Fig. 8). These results suggest that DSD alleviates PF progression by activating autophagy.

Autophagy suppresses TGF- β -induced cell fibrosis by inhibiting PAI-1 expression

Our study demonstrated that DSD increases the expression of autophagy-associated proteins in PF model rats, and one study has shown that autophagy can inhibit the expression of PAI-1 (Kobayashi et al., 2020). Thus, we hypothesized that DSD could impact the PA/PAI-1 balance through the regulation of autophagy. To further verify the effect of autophagy on the PA/PAI-1 balance, TGF- β 1-stimulated cells were utilized to create an *in vitro* PF model. Detection of fibrosis-related proteins in cells revealed that TGF-1 stimulation increased the intracellular expression of α -SMA, collagen I, collagen III, and fibronectin (Fig. 9A). Subsequent immuno-

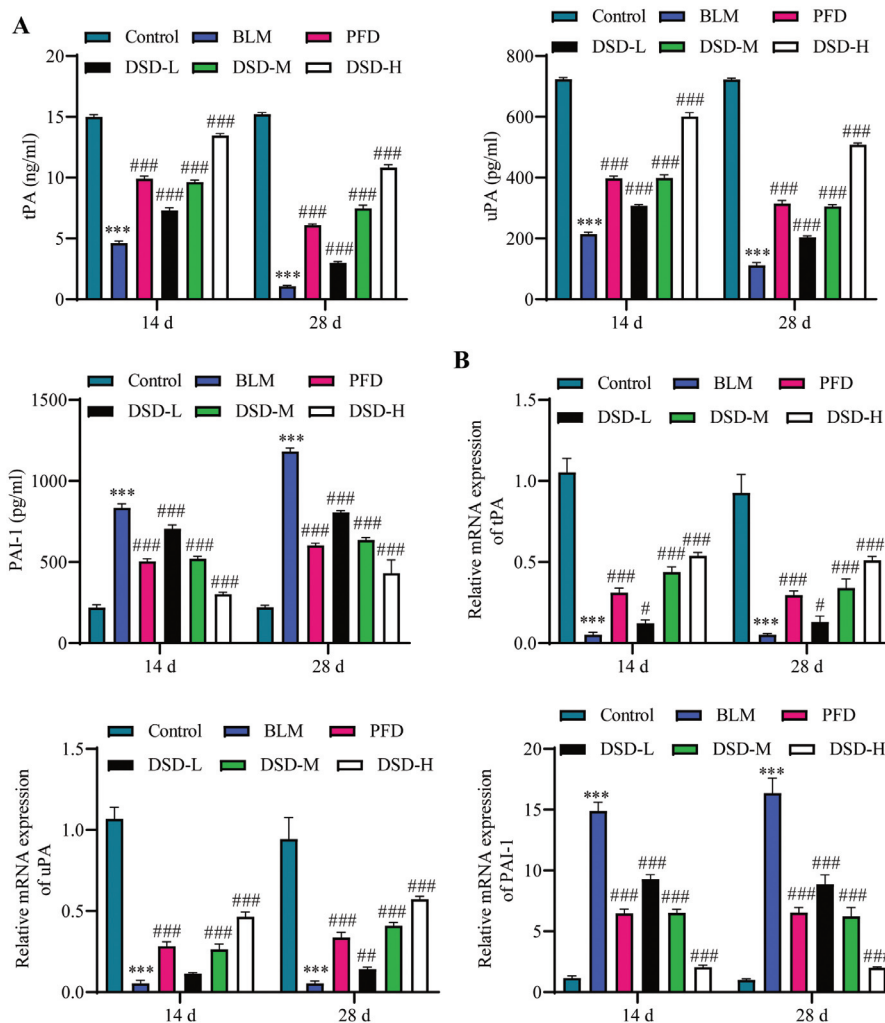


Fig. 6. DSD improved the PA/PAI-1 balance in the lung tissue of PF rats. **A.** ELISA detection of tPA, uPA, and PAI-1 expression in rat lung tissue. **B.** RT-qPCR detection of the expression of tPA, uPA, and PAI-1 mRNAs. $n=5$; compared with the control group, *** $p<0.001$; compared with the BLM group, # $p<0.05$, ## $p<0.01$, and ### $p<0.001$.

fluorescence analysis of α -SMA and collagen I expression demonstrated an increase in the TGF- β 1-treated cells compared with the normal cells (Fig. 9B).

These outcomes confirmed the successful construction of the PF cell model. Assessment of tPA, uPA, and PAI-1 protein and mRNA levels in the cells revealed an

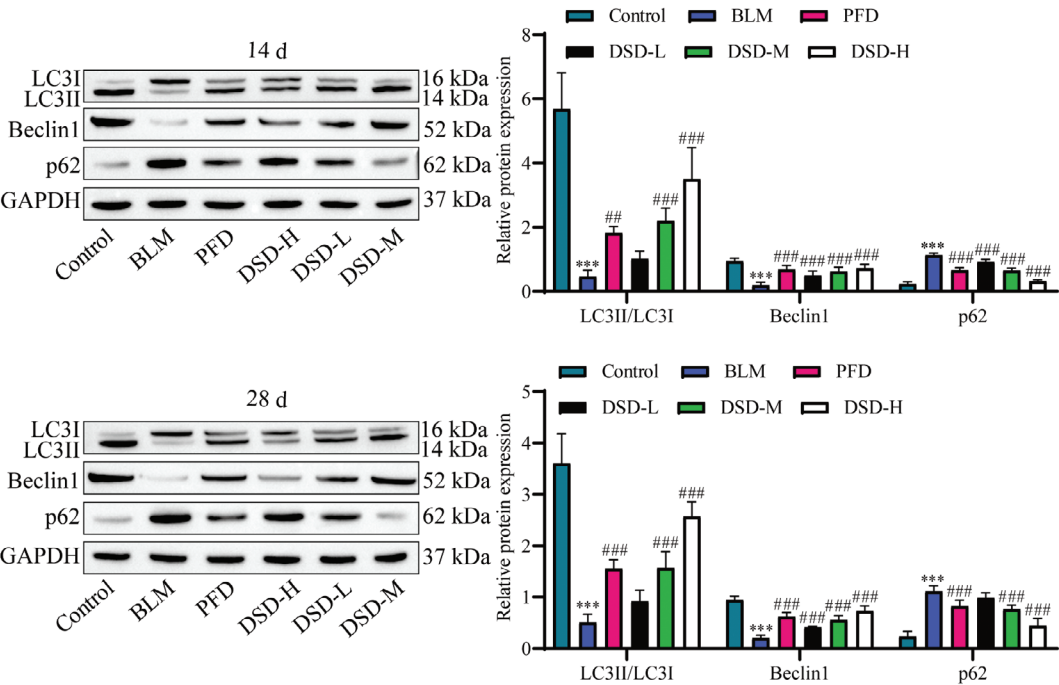


Fig. 7. DSD increases the expression of key proteins involved in autophagy in PF rats. The expression of the autophagy-related proteins LC3, Beclin1, and p62 was detected using western blot. n=5; Compared with the control group, *** $p<0.001$. Compared with the BLM group, ## $p<0.01$, and ### $p<0.001$.

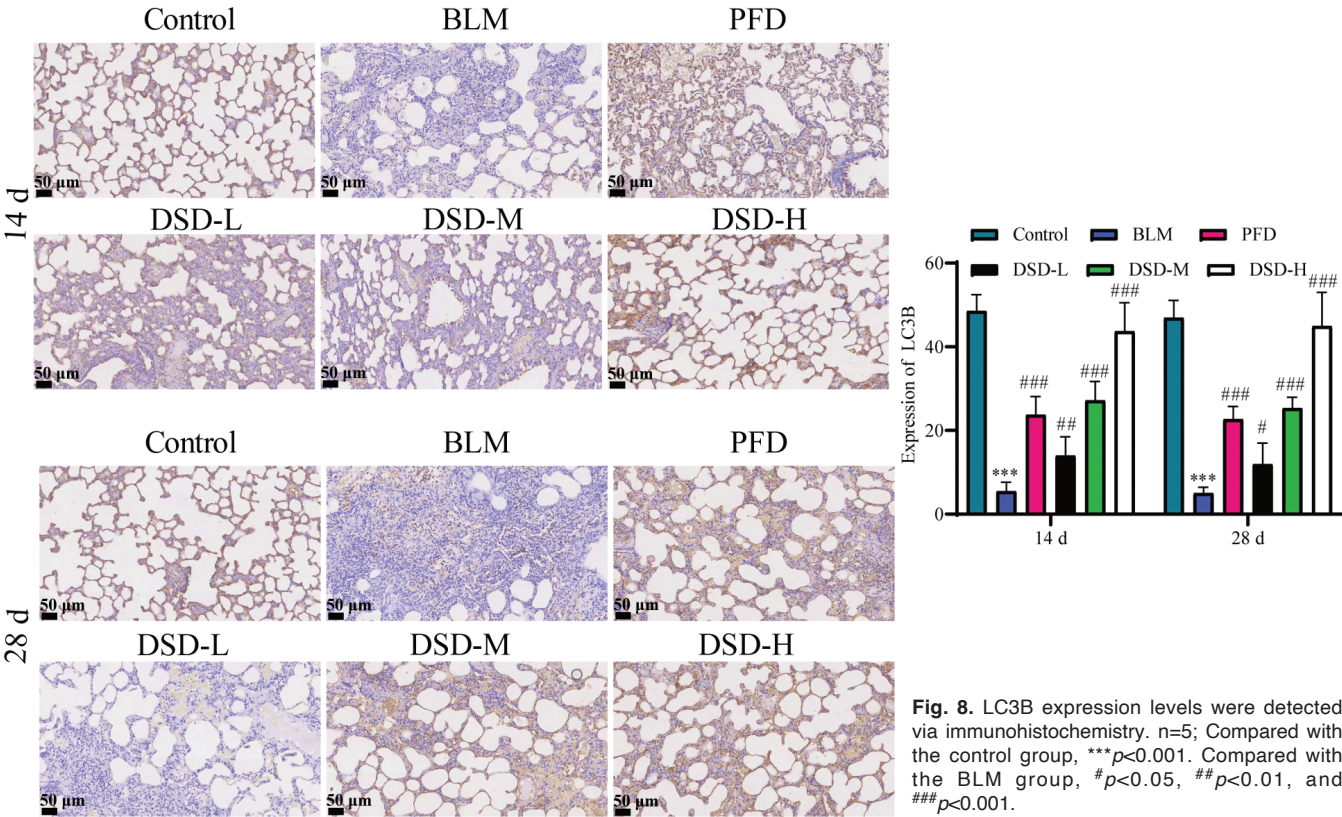


Fig. 8. LC3B expression levels were detected via immunohistochemistry. n=5; Compared with the control group, *** $p<0.001$. Compared with the BLM group, # $p<0.05$, ## $p<0.01$, and ### $p<0.001$.

increase in PAI-1 expression in the TGF-1-treated group, whereas tPA and uPA levels decreased (Fig. 9C,D). Compared with normal cells, TGF-1-treated cells

presented decreased levels of the autophagy-associated proteins LC3II/I and Beclin1 and increased expression of p62 (Fig. 9E). Immunofluorescence results also

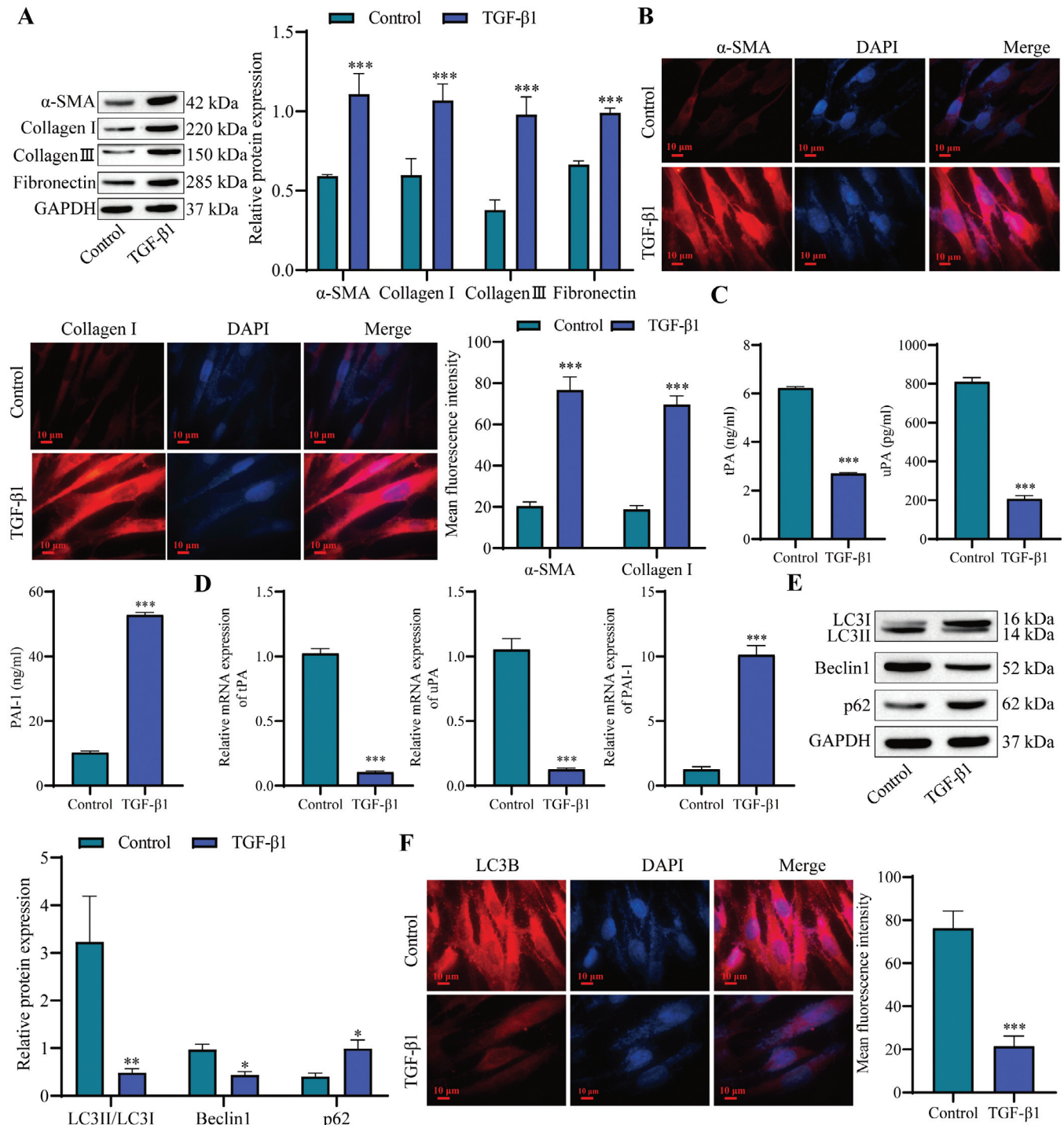


Fig. 9. TGF-β1 induced cell fibrosis, inhibited autophagy, and disrupted the balance of PA/tPA in cells. **A.** Expression levels of the fibrosis-associated proteins α-SMA, collagen I, collagen III, and fibronectin were detected via western blot. **B.** Expression of α-SMA and collagen I was detected via immunofluorescence. **C.** Detection of tPA, uPA, and PAI-1 via ELISA. **D.** Gene expression of tPA, uPA, and PAI-1 mRNAs was detected via RT-qPCR. **E.** Expression of the autophagy-related proteins LC3, Beclin1, and p62 was detected via western blot. **F.** LC3 expression was detected via immunofluorescence. Compared with the control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Dian sanguis draconis improves pulmonary fibrosis by activating autophagy

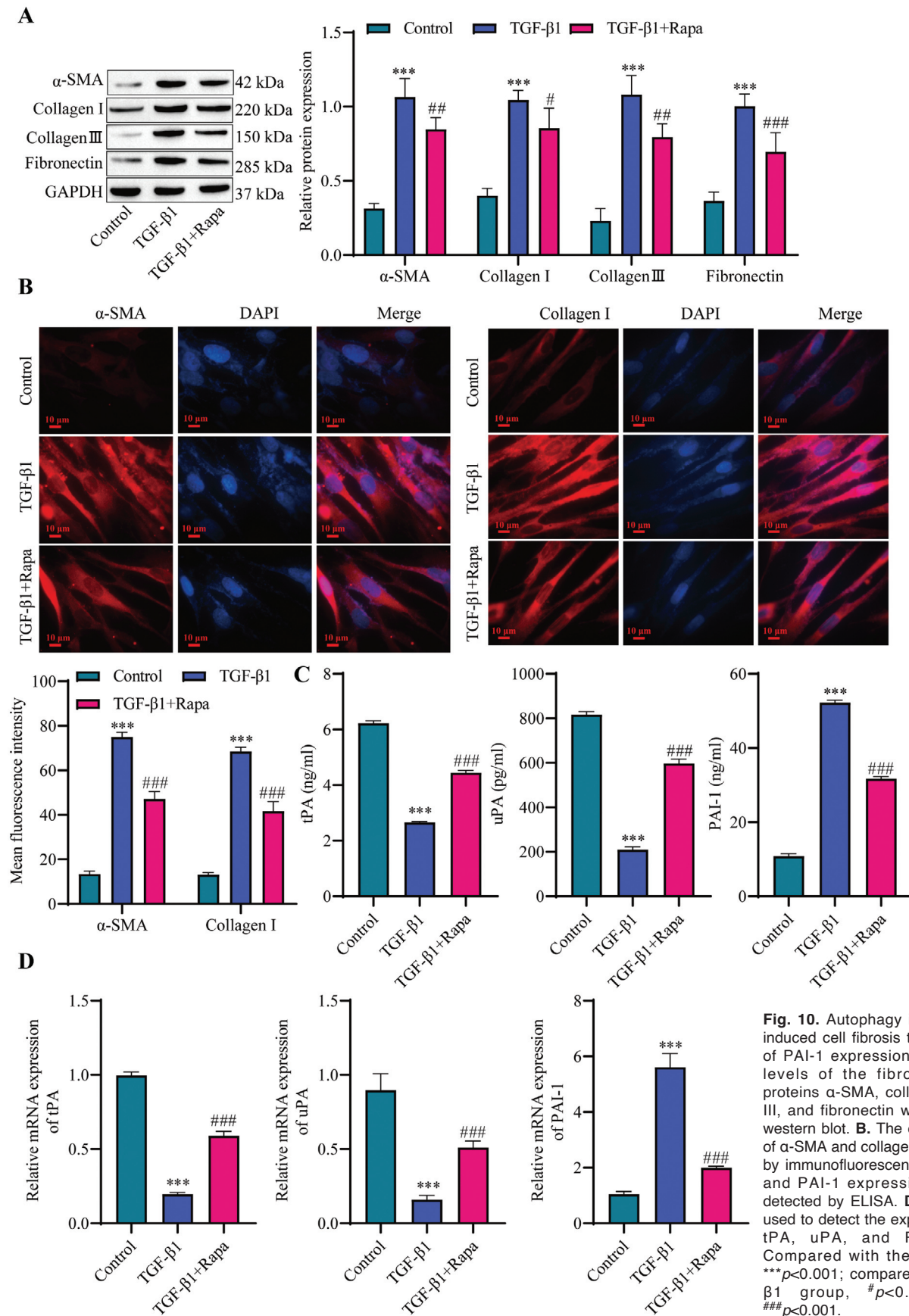


Fig. 10. Autophagy inhibits TGF-β1-induced cell fibrosis through inhibition of PAI-1 expression. **A.** Expression levels of the fibrosis-associated proteins α-SMA, collagen I, collagen III, and fibronectin were detected by western blot. **B.** The expression levels of α-SMA and collagen I were detected by immunofluorescence. **C.** tPA, uPA, and PAI-1 expression levels were detected by ELISA. **D.** RT-qPCR was used to detect the expression levels of tPA, uPA, and PAI-1 mRNAs. Compared with the control group, *** $p < 0.001$; compared with the TGF-β1 group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

revealed that TGF- β 1 reduces LC3B expression (Fig. 9F). In summary, TGF- β 1 induces cell fibrosis, inhibits autophagy, and disrupts the PA/PAI-1 balance.

To investigate the impact of autophagy on the PA/PAI-1 balance, an autophagy activator (rapamycin [Rapa]) was introduced into the media during TGF- β 1 treatment. The results showed that rapamycin weakened the effect of TGF- β 1 and inhibited the expression of α -SMA, collagen I, collagen III and fibronectin (Fig. 10A). The immunofluorescence results suggested that rapamycin alleviated the increase in α -SMA and collagen I expression induced by TGF- β 1 (Fig. 10B). These observations imply that activating autophagy may hinder the progression of lung fibrosis. Additionally, ELISA and RT-qPCR analyses were used to measure tPA, uPA, and PAI-1 protein and mRNA levels. The results revealed that rapamycin treatment restored the PA/PAI-1 balance, increasing tPA and uPA expression while reducing PAI-1 expression (Fig. 10C,D). These results suggest that autophagy may play an important role in the process of PF by regulating the PA/PAI-1 balance. In addition, we treated TGF- β 1-induced MRC-5 cells with DSD. A CCK-8 assay was used to detect cell viability. The results revealed that 50 μ g/mL DSD had a significant effect on cell viability (Fig. 11A). Therefore, 50 μ g/mL DSD was subsequently selected for experimental analysis. The expression levels of the autophagy-related proteins LC3II/I, Beclin1 and p62 were further detected. Compared with those in the TGF- β 1 group, LC3II/I and Beclin1 expression was increased after DSD treatment, whereas p62 levels were decreased (Fig. 11B). tPA, uPA and PAI-1 mRNA expression levels were detected via RT-qPCR. The results revealed that

tPA and uPA expression increased after DSD treatment, whereas PAI-1 expression decreased (Fig. 11C). These results suggest that DSD may affect the PA/PAI-1 balance by regulating autophagy.

Discussion

In recent years, the unique advantages of traditional Chinese medicine in the treatment of lung diseases have gradually attracted increasing attention. Among the various herbs, Rhodiola has been used in China as part of traditional Chinese medicine to protect lung tissue against inflammation and fibrosis (Zhang et al., 2016). Additionally, DSD, a traditional medicine in Yunnan, has many pharmacological activities, including anti-inflammatory, analgesic, antithrombotic, and antioxidative effects (Jing et al., 2019). Given the challenging nature of PF research, researchers are diligently exploring relevant mechanisms through isolated drug compounds or complex prescriptions to identify effective drugs against this condition. In our research, we successfully developed both a PF cell model and a rat PF model, and our findings revealed that DSD can markedly promote tPA and uPA expression and inhibit PAI-1 expression in both cells and rat lung tissues. Moreover, DSD inhibited the levels of fibrosis-related proteins such as α -SMA, collagen I, collagen III, and fibronectin. Our results demonstrate that DSD can effectively mitigate the progression of lung fibrosis in rats, offering new insights and potential strategies for the treatment of PF.

Autophagy is a core mechanism for maintaining cell homeostasis and responding to intracellular stress by

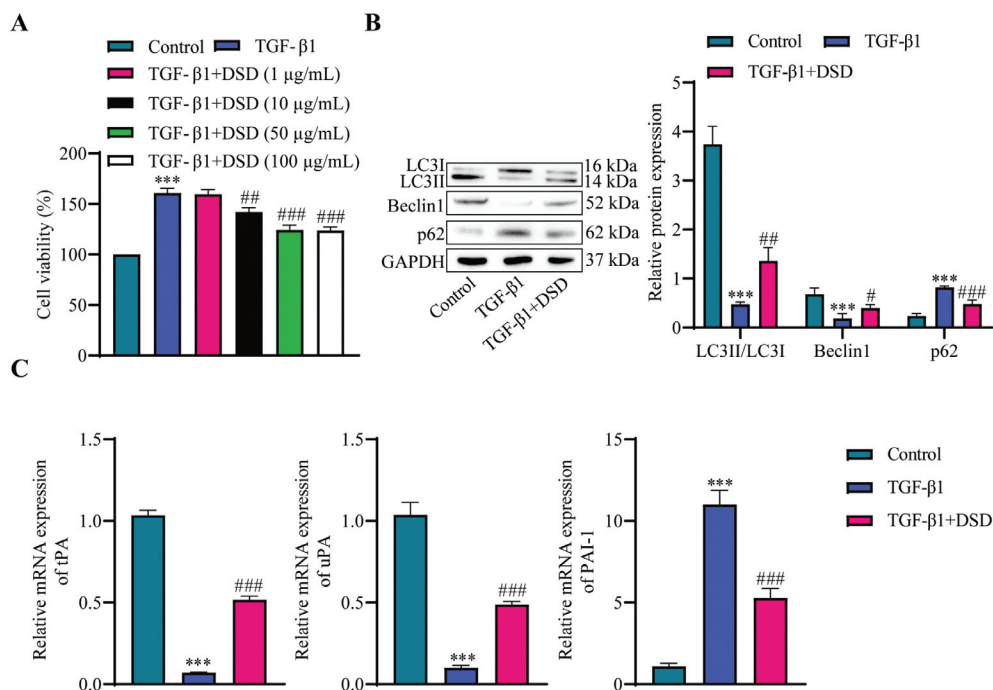


Fig. 11. DSD affects TGF- β 1-induced autophagy and PA/PAI-1 balance. **A.** Cell viability was measured by CCK-8 assay. **B.** The expression of the autophagy-related proteins LC3, Beclin1, and p62 was detected via western blot. **C.** The expression of tPA, uPA, and PAI-1 mRNAs was detected via RT-qPCR. Compared with the control group, *** p <0.001; compared with the TGF- β 1 group, # p <0.05, ## p <0.01, ### p <0.001.

degrading and recycling damaged cytoplasmic components (Filomeni et al., 2015). Recent studies have demonstrated the pathogenic role of impaired autophagy in a variety of diseases and revealed the potential ameliorative effect of autophagy induction on disease progression (Liu et al., 2019; Baek et al., 2020). In addition, the autophagy response is decreased in patients with idiopathic pulmonary fibrosis (IPF) (Araya et al., 2013; Romero et al., 2016; Mercader-Barceló et al., 2023). Specifically, PF progression is closely linked to the level of autophagic flux in the pathogenesis of the condition (Lv et al. 2020). This study investigated the potential role of autophagy in PF and detected the expression levels of the autophagy-related proteins LC3II/I, Beclin1, and p62. The results of these experiments demonstrated that DSD significantly promoted the expression of the autophagy proteins LC3II/I and Beclin1 in the lung tissue of PF rats and inhibited the expression of p62. Moreover, high-dose DSD significantly affected the expression of autophagic proteins. These results offer a novel perspective for enhancing our understanding of the pathological progression of PF. Moreover, PAI-1 expression is associated with autophagy (Kobayashi et al., 2020). In the present study, we evaluated the role of PAI-1 and autophagy in the pathological process of PF and revealed that autophagy has a negative regulatory effect on PAI-1, which disrupts the PA/PAI-1 balance and provides a new perspective on the pathogenesis of pulmonary fibrosis.

The fibrinolytic process requires tPA and uPA. These proteins work in tandem to facilitate the transformation of plasminogen into plasmin, an enzyme that subsequently breaks down cross-linked fibrin. Predominantly located in the circulatory system on endothelial cells and the fibrin surface, tPA is essential for the effective solubilization of fibrin through its efficient conversion mechanism (Rosenberg, 2003; Ruszkowska-Ciastek et al., 2020). In contrast, PAI-1, a major fibrinolytic inhibitor, inhibits both tPA and uPA activity (Nordeng et al., 2022). Studies have shown that uPA and tPA play an antifibrosis role by regulating collagen production and matrix clearance, whereas PAI-1 promotes fibrosis by affecting inflammatory cell migration, adhesion, and plasminase activity (Johnson et al., 2020). In a mouse model of PF, PA and PAI-1 levels play important roles in the development of fibrosis (Osterholzer et al., 2012; Schuliga et al., 2018). In this study, we observed that BLM reduced tPA and uPA expression levels and increased PAI-1 expression in rats. After treatment with DSD, the PA/PAI-1 balance improved. Our results provide new perspectives and strategies for fibrosis treatment.

PF, a prevalent lung illness, is frequently encountered in the medical field and can be fatal in extreme cases. Currently, the standard approaches for treatment include glucocorticoids, immunosuppressants, and pirfenidone, with hydrogen-oxygen inhalation therapy providing some relief for patients as well (Song et al., 2022; Ishida et al., 2023). However, these

therapies do not reverse the progression of PF. PF, a pathological manifestation of various pulmonary interstitial diseases, is a secondary lesion that often leads to respiratory failure (Yu and Tang, 2022; Ahangari et al., 2023). Histopathological analysis using HE and Masson staining in this study revealed that rats induced by BLM presented severe structural lung tissue abnormalities characterized by a thickened alveolar septum and excessive deposition of collagen fibers. Through western blot and immunohistochemistry assessments, a notable increase in the expression of α -SMA, collagen I, collagen III, and fibronectin was observed, confirming the successful establishment of the rat model. Following treatment with DSD, the pathological damage to the lung tissues improved, and the levels of proteins associated with lung fibrosis decreased. These findings indicate that DSD has an antifibrotic effect.

In conclusion, this study revealed that DSD improves PF progression by activating autophagy and subsequently regulating the PA/PAI-1 balance. Our study confirms the significant potential of DSDs in the treatment of PF. This discovery provides new drug candidates for the treatment of PF. However, there are limitations to this study. Future studies will further explore the key molecular targets to explain how DSD regulates the PA/PAI-1 balance by affecting the autophagy pathway and to reveal its exact mechanism of action. In addition, conducting rigorously designed clinical trials to evaluate the efficacy, safety, and long-term impact of DSD in patients with PF represents an important step in advancing its clinical application.

Funding. The present study was supported by the Yunnan Province Science and Technology Department of Traditional Chinese Medicine application basic research joint special youth project (202101AZ070001-295); the National Natural Science Foundation of China Project (82274374); the Yunnan Province Science and Technology Department of Traditional Chinese Medicine application basic research joint special project (202101AZ070001-169); and the Kunming Health Science and Technology Personnel training project and "ten hundred thousand" project training plan (2023-SW(Reserve)-63).

Availability of data and materials. The datasets analyzed during this study can be obtained from the corresponding author upon request.

Consent for publication. All the authors agreed on the publication of this manuscript.

Conflicts of interest. The authors declare that they have no competing financial interests.

Ethics approval and consent to participate. All animal procedures were approved by the Animal Ethics Committee of Kunming Medical University (approval number: R-062021LH-074).

Authors' contributions. All the authors contributed substantially to this manuscript. Jiayi Song, Qian Li and Yi Fu designed the study. Jiayi Song, Chunyan Yang, Qing Liu, Jianmei Li and Yi Fu performed the research and analyzed the data. Jiayi Song contributed new methods and wrote the paper. Yi Fu revised the manuscript. All the authors have read and approved the final manuscript.

References

- Ahangari F., Price N., Malik S., Chioccioli M., Bärnthaler T., Adams T., Kim J., Pradeep S., Ding S., Cosmos C., Rose K., McDonough J., Aurelien N., Ibarra G., Omote N., Schupp J., Deluliis G., Villalba Nunez J., Sharma L., Ryu C., Dela Cruz C., Liu X., Prasse A., Rosas I., Bahal R., Fernández-Hernando C. and Kaminski N. (2023). microRNA-33 deficiency in macrophages enhances autophagy, improves mitochondrial homeostasis, and protects against lung fibrosis. *JCI Insight* 8, e158100.
- Araya J., Kojima J., Takasaka N., Ito S., Fujii S., Hara H., Yanagisawa H., Kobayashi K., Tsurushige C., Kawaishi M., Kamiya N., Hirano J., Odaka M., Morikawa T., Nishimura S.L., Kawabata Y., Hano H., Nakayama K. and Kuwano K. (2013). Insufficient autophagy in idiopathic pulmonary fibrosis. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 304, L56-69.
- Baek A., Hong J., Song K., Jang A., Kim D., Chin S. and Park S. (2020). Spermidine attenuates bleomycin-induced lung fibrosis by inducing autophagy and inhibiting endoplasmic reticulum stress (ERS)-induced cell death in mice. *Exp. Mol. Med.* 52, 2034-2045.
- Barham H., Osborn J., Snidvongs K., Mrad N., Sacks R. and Harvey R. (2015). Remodeling changes of the upper airway with chronic rhinosinusitis. *Int. Forum Allergy Rhinol.* 5, 565-572.
- Bienvenu L., Morgan J., Rickard A., Tesch G., Cranston G., Fletcher E., Delbridge L. and Young M. (2012). Macrophage mineralocorticoid receptor signaling plays a key role in aldosterone-independent cardiac fibrosis. *Endocrinology* 153, 3416-3425.
- Chen Q., Shou W., Wu W., Wang G. and Cui W. (2019). Performance evaluation of thrombomodulin, thrombin-antithrombin complex, plasmin- α 2-antiplasmin complex, and t-PA: PAI-1 complex. *J. Clin. Lab. Anal.* 33, e22913.
- Filomeni G., De Zio D. and Cecconi F. (2015). Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death Differ.* 22, 377-388.
- George P., Spagnolo P., Kreuter M., Altinisik G., Bonifazi M., Martinez F., Molyneaux P., Renzoni E., Richeldi L., Tomassetti S., Valenzuela C., Vancheri C., Varone F., Cottin V. and Costabel U. (2020). Progressive fibrosing interstitial lung disease: Clinical uncertainties, consensus recommendations, and research priorities. *Lancet. Respir. Med.* 8, 925-934.
- Godtfredsen A., Sidelmann J., Dolleris B., Jørgensen J., Johansen E., Pedersen M., Palarasah Y. and Gram J. (2022). Fibrinolytic changes in women with preeclampsia. *Clin. Appl. Thromb. Hemost.* 28, 10760296221126172.
- Huebner B., Moore E., Moore H., Stettler G., Nunns G., Lawson P., Sauaia A., Kelher M., Banerjee A. and Silliman C. (2018). Thrombin provokes degranulation of platelet α -granules leading to the release of active Plasminogen Activator Inhibitor-1 (PAI-1). *Shock* 50, 671-676.
- Hussein Z., Abu-Raghiif A., Tahseen N., Rashed K., Shaker N. and Fawzi H. (2024). Vinpocetine alleviated alveolar epithelial cells injury in experimental pulmonary fibrosis by targeting PPAR- γ /NLRP3/NF- κ B and TGF- β 1/Smad2/3 pathways. *Sci. Rep.* 14, 11131.
- Ishida Y., Kuninaka Y., Mukaida N. and Kondo T. (2023). Immune mechanisms of pulmonary fibrosis with bleomycin. *Int. J. Mol. Sci.* 24, 3149.
- Jing H., Fu Y., Yang C., Zhang A., Wang X., Wu H., Liu Q. and Li J. (2019). Effect of Dian Sanguis Draconis on IL-2 and IL-5 in rats with pulmonary fibrosis. *J. Chinese Med.* 37, 1068-1070. (in Chinese)
- Johnson S., Shaikh S., Muneesa F., Rashmi B. and Bhandary Y. (2020). Radiation induced apoptosis and pulmonary fibrosis: Curcumin an effective intervention? *Int. J. Radiat. Biol.* 96, 709-717.
- Kobayashi M., Yoshino O., Nakashima A., Ito M., Nishio K., Ono Y., Kusabiraki T., Kunitomi C., Takahashi N., Harada M., Hattori K., Orisaka M., Osuga Y. and Saito S. (2020). Inhibition of autophagy in theca cells induces CYP17A1 and PAI-1 expression via ROS/p38 and JNK signalling during the development of polycystic ovary syndrome. *Mol. Cell. Endocrinol.* 508, 110792.
- Liu M., Su M., Tang D., Hao L., Xun X. and Huang Y. (2019). Ligustrazin increases lung cell autophagy and ameliorates paraquat-induced pulmonary fibrosis by inhibiting PI3K/Akt/mTOR and hedgehog signalling via increasing miR-193a expression. *BMC Pulm. Med.* 19, 35.
- Liu Y., Shu L., Jiang X., Zhang Y., Chen Q., Shen Y. and Yang Y. (2023). The development of nasal polyps involves early middle meatus mucous remodeling via TGF- β 1 mediated PAI-1 reduction. *Braz. J. Otorhinolaryngol.* 89, 366-373.
- Long X. (2018). Randomized controlled study of the efficacy of Sanguis Draconis and Meibao in the treatment of pressure ulcers in patients with chronic kidney disease. *J. Clin. Nursing's Practicality.* 3, 25-26.
- Lv X., Li K. and Hu Z. (2020). Autophagy and pulmonary fibrosis. *Adv. Exp. Med. Biol.* 1207, 569-579.
- Makuszczyńska M., Cieślirńska M., Winnicka M., Skotnicka B., Niemczyk K. and Bonda T. (2023). Enhanced expression of plasminogen activators and inhibitor in the healing of tympanic membrane perforation in rats. *J. Assoc. Res. Otolaryngol.* 24, 159-170.
- Mercader-Barceló J., Martín-Medina A., Truyols-Vives J., Escarrer-Garau G., Elowsson L., Montes-Worboys A., Río-Bocos C., Muncunill-Farreny J., Velasco-Roca J., Cederberg A., Kadefors M., Molina-Molina M., Westergren-Thorsson G. and Sala-Llinàs E. (2023). Mitochondrial dysfunction in lung resident mesenchymal stem cells from idiopathic pulmonary fibrosis patients. *Cells* 12, 2084.
- Nordeng J., Solheim S., Åkra S., Schandiz H., Hoffmann P., Roald B., Bendz B., Arnesen H., Helseth R. and Seljeflot I. (2022). Gene expression of fibrinolytic markers in coronary thrombi. *Thromb. J.* 20, 23.
- Osterholzer J., Christensen P., Lama V., Horowitz J., Hattori N., Subbotina N., Cunningham A., Lin Y., Murdock B., Morey R., Olszewski M., Lawrence D., Simon R. and Sisson T. (2012). PAI-1 promotes the accumulation of exudate macrophages and worsens pulmonary fibrosis following type II alveolar epithelial cell injury. *J. Pathol.* 228, 170-180.
- Raghu G., Remy-Jardin M., Myers J., Richeldi L., Ryerson C., Lederer D., Behr J., Cottin V., Danoff S., Morell F., Flaherty K., Wells A., Martinez F., Azuma A., Bice T., Bouros D., Brown K., Collard H., Duggal A., Galvin L., Inoue Y., Jenkins R., Johkoh T., Kazerooni E., Kitaichi M., Knight S., Mansour G., Nicholson A., Pipavath S., Buendía-Roldán I., Selman M., Travis W., Walsh S. and Wilson K. (2018). Diagnosis of idiopathic pulmonary fibrosis. An official ATS/ERS/JRS/ALAT clinical practice guideline. *Am. J. Respir. Crit. Care Med.* 198, e44-e68.
- Rajan S., Cottin V., Dhar R., Danoff S., Flaherty K., Brown K., Mohan A., Renzoni E., Mohan M., Udawadia Z., Shenoy P., Currow D., Devraj A., Jankharia B., Kulshrestha R., Jones S., Ravaglia C., Quadrelli S., Iyer R., Dhooria S., Kolb M. and Wells A. (2023). Progressive pulmonary fibrosis: An expert group consensus statement. *Eur. Respir. J.* 61, 2103187.

Dian sanguis draconis improves pulmonary fibrosis by activating autophagy

- Romero Y., Bueno M., Ramirez R., Álvarez D., Sembrat J.C., Goncharova E.A., Rojas M., Selman M., Mora A.L. and Pardo A. (2016). mTORC1 activation decreases autophagy in aging and idiopathic pulmonary fibrosis and contributes to apoptosis resistance in IPF fibroblasts. *Aging Cell* 15, 1103-1112.
- Rosenberg S. (2003). The urokinase-type plasminogen activator system in cancer and other pathological conditions: Introduction and perspective. *Curr. Pharm. Des.* 9, 4p.
- Ruszkowska-Ciastek B., Kwiatkowska K., Bielawska S., Robakowska M., Bielawski K. and Rhone P. (2020). Evaluation of the prognostic value of fibrinolytic elements in invasive breast carcinoma patients. *Neoplasma* 67, 1146-1156.
- Schuliga M., Grainge C., Westall G. and Knight D. (2018). The fibrogenic actions of the coagulant and plasminogen activation systems in pulmonary fibrosis. *Int. J. Biochem. Cell Biol.* 97, 108-117.
- Song S., Fu Z., Guan R., Zhao J., Yang P., Li Y., Yin H., Lai Y., Gong G., Zhao S., Yu J., Peng X., He Y., Luo Y., Zhong N. and Su J. (2022). Intracellular hydroxyproline imprinting following resolution of bleomycin-induced pulmonary fibrosis. *Eur. Respir. J.* 59, 2100864.
- Takabayashi T. and Schleimer R. (2020). Formation of nasal polyps: The roles of innate type 2 inflammation and deposition of fibrin. *J. Allergy Clin. Immunol.* 145, 740-750.
- Tanner L., Single A., Bhongir R., Heusel M., Mohanty T., Karlsson C., Pan L., Clausson C., Bergwik J., Wang K., Andersson C., Oommen R., Erjefält J., Malmström J., Wallner O., Boldogh I., Helleday T., Kalderén C. and Egesten A. (2023). Small-molecule-mediated OGG1 inhibition attenuates pulmonary inflammation and lung fibrosis in a murine lung fibrosis model. *Nat. Commun.* 14, 643.
- Wijssenbeek M., Suzuki A. and Maher T. (2022). Interstitial lung diseases. *Lancet* 400, 769-786.
- Xie M., Yang L., Cheng J., Qu H., Gu Y., Ding C., Xu X., Zhao C., Huang X. and Wang L. (2023). Gracillin relieves pulmonary fibrosis by suppressing the STAT3 axis. *J. Ethnopharmacol.* 316, 116704.
- Yanagihara T., Sato S., Upagupta C. and Kolb M. (2019). What have we learned from basic science studies on idiopathic pulmonary fibrosis? *Eur. Respir. Rev.* 28, 190029.
- Yu Q. and Tang X. (2022). Irreversibility of pulmonary fibrosis. *Aging Dis.* 13, 73-86.
- Zanetto A., Campello E., Bulato C., Gavasso S., Saggiorato G., Shalaby S., Burra P., Angeli P., Senzolo M. and Simioni P. (2022). Global hemostatic profiling in patients with decompensated cirrhosis and bacterial infections. *JHEP Rep.* 4, 100493.
- Zhang K., Si X.P., Huang J., Han J., Liang X., Xu X.B., Wang Y.T., Li G.Y., Wang H.Y. and Wang J.H. (2016). Preventive effects of rhodiola rosea L. on bleomycin-induced pulmonary fibrosis in rats. *Int. J. Mol. Sci.* 17, 879.
- Zhang X., Rao Y., Zhang Y., Yang K., Zhou H. and Ni W. (2018). Prevention and treatment of restenosis after coronary intervention by Dragon's Blood. *Cardiovasc. Dis. J. Integrated traditional Chinese and Western Medicine.* 6, 19-20 (in Chinese).

Accepted February 14, 2025