Effects of *Ginkgo biloba* leaf extract, shenmai and matrine on a human embryonic lung fibroblast fibrosis model

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Abstract. The aim of the present study was to investigate the effects of *Ginkgo biloba* leaf extract (GBLE), shenmai (S), and matrine (M) on human embryonic lung fibroblasts (HELFs). HELFs were allocated into the following groups: Group A (control group), group B [transforming growth factor β1 (TGF-β1) model group], groups C1-3 (TGF-β1 + low-, moderate- and high-dose GBLE), groups D1-3 (TGF-β1 + low-, moderate- and high-dose S) and groups E1-3 (TGF-β1 + low-, moderate- and high-dose oM). Cell proliferation was assessed with an MTT assay and apoptosis was measured by annexin V/propidium iodide double staining and flow cytometry analysis. Collagen type I (COL-I), collagen type III (COL-III), α-smooth muscle actin (α-SMA) and extracellular superoxide dismutase (ECSOD) mRNA expression levels were measured using semi-quantitative reverse transcription-polymerase chain reaction, and protein content was measured using ELISA. The cell growth inhibition rates of the S groups were significantly higher than those of the other treatment groups (P<0.05). The rate of apoptosis was significantly increased in the treatment groups compared with the model group (P<0.05), and S induced a significant increase in HELF apoptosis compared with the other treatment groups (P<0.05). The mRNA and protein expressions of COL-III, COL-I and α-SMA in the GBLE, S and M groups were significantly decreased, while the expression of ECSOD was significantly increased when compared with the model group (P<0.05). In conclusion, GBLE, S and M inhibited the pro-fibrotic role of TGF-β1 by targeting different steps in TGF-β1-mediated fibrosis.

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Introduction

The most prevalent type of idiopathic interstitial pneumonia is idiopathic pulmonary fibrosis (IPF) (1). Chronic progressive fibrosis in IPF is characterized by unexplained progressive dyspnea, decreased exercise tolerance and lung parenchyma interstitial infiltration or restricted pulmonary ventilation function (restrictive lung ventilation disorder) confirmed by histopathology and/or imaging (1). Following diagnosis, the average survival rate of patients ranges between 2.5-3.5 years, which is due to the difficulty of early diagnosis and lack of effective treatment methods (2,3). Over the past several decades, studies into the pathogenesis of IPF have made substantial progress, and an initial hypothesis suggested that chronic inflammation in early IPF may stimulate and damage the lung tissue, regulate fibroplasia and induce extracellular matrix (ECM)deposition, leading to late PF (4). However, mechanistic studies have indicated that chronic inflammation does not sufficiently explain the occurrence and development of IPF (5-8); therefore, a novel hypothesis that IPF is caused by abnormal damage and repair of alveolar epithelial cells (AECs) has been proposed (9). This hypothesis suggests that AECs, fibroblasts and their interaction, and not chronic inflammation, are the key factors in the pathogenesis of IPF. Recent studies have indicated that IPF results from abnormal cellular repair following sustained epithelial cell damage in the lung parenchyma, along with the proliferation and accumulation of fibroblasts/myofibroblasts and excessive ECM deposition (10-12). Injured AECs following repeated complex minor stimulations, including repeated infections and physical and chemical injuries, have been demonstrated to secrete pro-fibrotic factors, including transforming growth factor-β (TGF-β), tumor necrosis factor-α and platelet-derived growth factor (13-15), thereby initiating the proliferation, migration and differentiation of fibroblasts (9). Fibroblasts/myofibroblasts are able to stimulate the formation of fibroblast foci and secrete large quantities of ECM proteins, in addition to matrix metalloproteinases (MMPs) and MMP tissue inhibitors, thus resulting in the deposition of ECM (9). Furthermore, AECs have been demonstrated to induce the formation of a pro-coagulant alveolar environment, leading to increased fibrosis (9). In addition, myofibroblasts may secrete angiotensinogen to induce AEC apoptosis, thus resulting in continuous injury (9). Injured AECs have been reported to activate fibroblasts,

resulting in a vicious cycle that critically alters normal alveolar structure (9). A previous study indicated that IPF pathogenesis was a result of the combined effects of inflammatory reaction pathways, epithelial damage pathways and abnormal repair pathways (16).

In the present study, human embryonic lung fibroblasts (HELFs) were cultured *in vitro* and used to construct a TGF-β1-induced HELF PF model, in order to characterize the effect of *Ginkgo biloba* leaf extract (GBLE), shenmai (S) and matrine (M) on PF. It has been previously reported that GBLE has a positive effect on the treatment of pulmonary interstitial fibrosis and tis mechanism of action may be via inhibiting the activity of nuclear factor-κB and decreasing TGF-β, which ameliorates inflammation and fibrosis (17). Shenmai may inhibit collagen synthesis and TGF-β and regulate the biological redox equilibrium (18). While matrine exerts an anti-PF effect by inhibiting the JAK-STAT signaling transduction pathways (19). This was hoped to elucidate an experimental basis for the treatment of PF with GBLE, S and M.

Materials and methods

HELF culture and grouping. HELFs (cat. no. KG062; Nanjing Keygen BioTech Co., Ltd., Nanjing, China) were cultured in α-Minimum Essential Medium (α-MEM; Nanjing Keygen BioTech Co., Ltd.) supplemented with 5% penicillin, 5% streptomycin and 10% fetal bovine serum (Nanjing Keygen BioTech Co., Ltd.) at 37°C in an atmosphere containing 5% CO_2. When the cells had reached ~80% confluence, trypsin (Sigma-Aldrich; Merck KGaA; Darmstadt, Germany) was utilized for passage. Cells generated from passage 3-4 were used for the following experiments.

Following trypsinization, the cells were centrifuged at 37°C at 256 x g for 10 min and resuspended in 4 ml fresh supplemented α-MEM (as described above) and counted using a DVM6 optical microscope (Leica Microsystems GmbH, Wetzlar, Germany). Cells were allocated into the following groups: Group A, control group; group B, TGF-β1 (model) group (1 ng/ml; Nanjing Keygen BioTech Co., Ltd.); group C1, TGF-β1 (1 ng/ml) + GBLE (approval number: X20010117; Dr. Willmar Schwabe GmbH & Co. KG, Karlsruhe, Germany; 5 µl/ml); group C2: TGF-β1 (1 ng/ml) + GBLE (10 µl/ml); group C3: TGF-β1 (1 ng/ml) + GBLE (20 µl/ml); group D1: TGF-β1 (1 ng/ml) + S (approval number: Z51022290; Sichuan Chuanda West China Pharmaceutical Co., Ltd., Chengdu, China; 10 µl/ml); group D2: TGF-β1 (1 ng/ml) + S (20 µl/ml); group D3: TGF-β1 (1 ng/ml) + S (40 µl/ml); group E1: TGF-β1 (1 ng/ml) + M (approval number: H22024149; Changchun Tiancheng Pharmaceutical Co., Ltd., Changchun, China; 10 µl/ml); group E2: TGF-β1 (1 ng/ml) + M (20 µl/ml); and group E3: TGF-β1 (1 ng/ml) + M (40 µl/ml). After treatments were administered, cells were incubated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. The treatment groups were incubated in 12-well plates (6 wells per group) and 96-well plates (5 wells per group).

MTT cell proliferation assay. An MTT assay was performed using 96-well plates with 5 repeat wells per group. Cells ($1x10^5$ cells/ml) were cultured in 200 µl α-MEM in the 96-well plates with 10 µl MTT solution (Amresco, Solon, OH, USA) at 37°C in an atmosphere containing 5% CO_2 for 4 h. The edges of wells were filled with sterile phosphate-buffered saline (PBS) to prevent evaporation. If the drug reacted with MTT, MTT was added after the culture was centrifuged at 256 x g at room temperature and washed two to three times with PBS. After 4 h, the supernatant was removed from the plates by gently tapping on a pre-prepared absorbent paper one to two times. To dissolve the crystals, 50 µl dimethylsulfoxide (Sigma-Aldrich; Merck KGaA) was added to the cells, and the plates were placed on a low-speed oscillator for 10 min at room temperature. The optical density (OD) of each well was measured at 490 nm and the growth inhibition rate of each group was calculated, as follows: Cell growth inhibition rate = (OD of the control group-OD of treatment group)/OD of the control group x100% (20).

Detection of cell apoptosis by flow cytometry. The rate of apoptosis was detected by flow cytometry, using 12-well plates with 6 repeat wells per group. Cells were seeded at a density of $1x10^5$ cells/ml in 2 ml/wells with α-MEM and FBS. Following incubation for 24 h at 37°C with the drug treatments, cells in the 12-well plates were removed from the incubator, imaged under a DVM6 optical microscope and 1 ml of the supernatant from each well was stored at -80°C for measurement of protein content by ELISA. The remaining supernatant was discarded and cells were washed twice with PBS. Trypsinized cells were collected, centrifuged at 650 x g for 7 min at 4°C and washed twice with PBS. Cells were subsequently resuspended in 500 µl of 1X binding buffer, filtered through a 80 µm filter and transferred to a tube for antigen labeling. Cells were then incubated with 4 µl fluorescein isothiocyanate-annexin V and 8 µl propidium iodide (PI) for 5 min in the dark at room temperature. A FACSCalibur flow cytometer was used for analysis using Winmidi 2.9 analysis software (both BD Biosciences, San Jose, CA, USA) to detect cells undergoing apoptosis. Normal living cells and early apoptotic cells were defined as those cells resistant to PI staining, whereas necrotic cells were identified by PI-positive staining.

ELISA. The levels of collagen type I (COL-1; cat. no. ab6308), collagen type III (COL-III; cat. no. ab7778), α-smooth muscle actin (α-SMA; ab5694) and extracellular superoxide dismutase (ECSOD; ab13534) in the ECM of HELFs were measured by ELISA (Abcam, Cambridge, UK), according to manufacturer's protocol.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). The levels of COL-I, COL-III, α-SMA and ECSOD mRNA expression were assessed by semi-quantitative RT-PCR. The primer sequences used are presented in Table I.

At 24 h after treatment, the 12-well plates were removed from the incubator and the cells were washed twice with PBS. Cells were resuspended in 1 ml TRIzol (Tianjin Kailiqi Biopharma Technology Co., Ltd., Tianjin, China) and placed into diethyl pyrocarbonate (DEPC; Fuzhou Maixin Biotech Co., Ltd., Fuzhou, China) water-treated Eppendorf tubes for total RNA extraction on ice. RT-PCR primers were designed using Primer 5.0 software (Premier, Inc., Charlotte, NC, USA).

Table I. Primer sequences used in the present study.

Gene	Direction	Sequence, 5'-3'	Size, bp
β-actin	Forward	GTGGGGCGCCCCAGGCACC	500
	Reverse	CTCCTTAATGTCACGCACGATTT	
COL-III	Forward	ACGGAAACACTGGTGGACAG	386
	Reverse	GTAGTCTCACAGCCTTGCGT	
COL-I	Forward	GCTCGTGGAAATGATGGTGC	449
	Reverse	CCTCGCTTTCCTTCCTCTCC	
α-SMA	Forward	GACCTTTGGCTTGGCTTGTC	418
	Reverse	AGCTGCTTCACAGGATTCCC	
ECSOD	Forward	GTGAAGGTGTGGGGAAGCAT	339
	Reverse	TCCAGCGTTTCCCGTCTTTG	

COL-III, collagen type III; COL-I, collagen type I; α-SMA, α-smooth muscle actin; ECSOD, extracellular superoxide dismutase.

RNA was extracted using TRIzol, and RNA concentration and purity were measured using a UV spectrophotometer at an absorbance (A) ratio of 260/280 nm. cDNA was synthesized from 2.5 μg total RNA according to RT kit instructions (MBI Fermentas; Thermo Fisher Scientific, Inc., Pittsburgh, PA, USA) using oligo dTs (Bio Basic, Inc., Markham, ON, Canada). PCR was performed with 5 μl cDNA, 2.5 μl 10 X PCR buffer, 2 μl 25 mM MgCl$_2$, 0.25 μl primers (25 pmol each), 0.5 μl 10 mM dNTPs, 0.5 μl Taq DNA polymerase (MBI Fermentas; Thermo Fisher Scientific, Inc.) and 14 μl DEPC water. The PCR reaction underwent the following conditions: 95°C for 5 min, 94°C for 1 min, 58-60°C for 1 min and 72°C for 1 min for 26 cycles, followed by an extension step at 70°C for 10 min. PCR products were electrophoresed and analyzed on a 1.5% gel stained with ethidium bromide and an Alpha Innotech Digital Imaging System (Bosch Institute, Sydney Medical School, University of Sydney, NSW, Australia) was used with Essential version 6 software (UVItec Ltd., Cambridge, UK) to determine the relative levels of target gene expression by calculating the gray value ratio between the target gene bands and β-actin control bands.

Statistical analysis. SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for analysis of the data. The data were expressed as the mean ± standard deviation. One-way analysis of variance and paired t-tests were used for intergroup comparisons, and the Fisher's least significant difference test was used for pairwise comparisons among multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of cell proliferation. Compared with the control group, the OD value in the model group significantly increased (P<0.05). Compared with the model group, the OD value in each treatment group were significantly decreased (P<0.05). The OD values and cell growth inhibitory rates exhibited significant differences among Group C1, C2, and C3 (P<0.05), as well as among Group D1, D2, and D3 (P<0.05), and among

Table II. Results of human embryonic lung fibroblast MTT assay.

Group	OD value of cell proliferation	Growth inhibition rate (%)
Control	0.177±0.0071	-
Model	0.214±0.0045[a]	6.5±1.45
C1	0.166±0.0076[b]	0.0651±0.014[c,d]
C2	0.153±0.0056[b,c]	0.1342±0.0078[c,e]
C3	0.146±0.0043[b,d,e]	0.1762±0.0085[d,e]
D1	0.146±0.0060[b]	0.1783±0.0090[c,d]
D2	0.132±0.0076[b,c]	0.2551±0.0191[c,e]
D3	0.117±0.0046[b,d-f]	0.3384±0. 0125[d-f]
E1	0.153±0.0061[b]	0.1351±0.0083[c,d]
E2	0.143±0.0050[b,c]	0.1973±0.0107[c,e]
E3	0.128±0.0060[b,d,e]	0.2782±0.0094[d,e]

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. control group; [b]P<0.05 vs. model group; [c]P<0.05 different concentration of the same drug treatment, [d]P<0.05 for group 3 vs. group 1 of each drug treatment. [e]P<0.05 for group 3 vs. group 2 of each drug treatment; [f]P<0.05 vs. the C3 group. C1-3, transforming growth factor β1 + low-, moderate- and high-dose Ginkgo leaf extract, respectively; D1-3, transforming growth factor β1 + low-, moderate- and high-dose shenmai, respectively; E1-3, transforming growth factor β1 + low-, moderate- and high-dose matrine, respectively; OD, optical density.

Group E1, E2, and E3 (P<0.05). The OD value in Group D3 was significantly lower (P<0.05) and the cell growth inhibition rate was significantly higher, compared with other treatment groups with the same concentrations (P<0.05). The results are reported in Table II.

Comparison of apoptosis. Compared with the control group, the apoptosis rate in the model group was significantly decreased (P<0.05; Fig. 1). Compared with the model group, the apoptosis rate in each treatment group was significantly
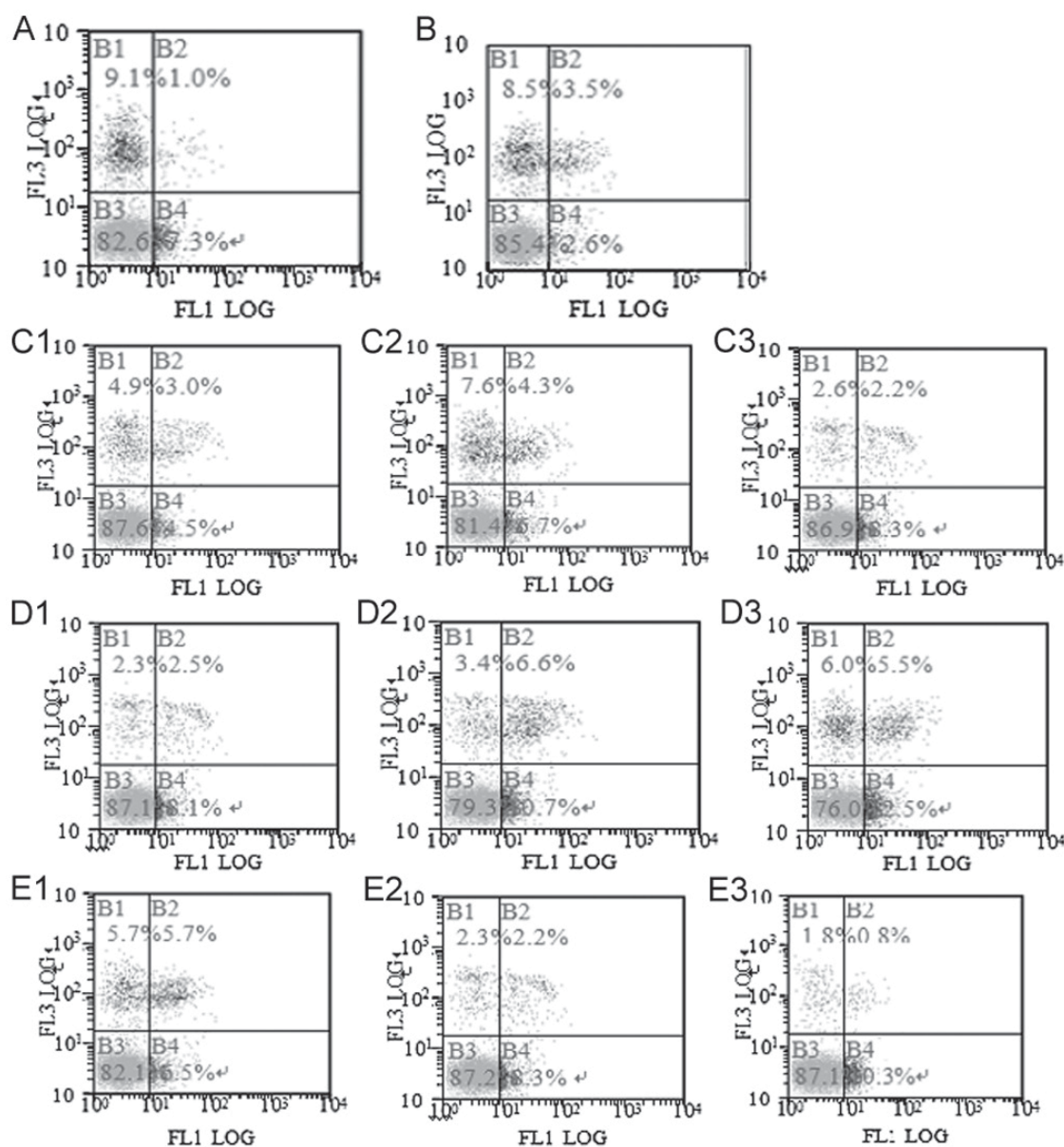
Figure 1. Flow cytometry scatter plots of cell apoptotic rate in each group. (A) Control group; (B) model group; (C1) low-, (C2) moderate- and (C3) high-dose Ginkgo leaf extract groups; (D1) low-, (D2) moderate- and (D3) high-dose shenmai groups; and (E1) low-, (E2) moderate- and (E3) high-dose matrine groups. B1, necrotic cells; B2, necrotic cells + late apoptotic cells; B3, living cells, B4, early apoptotic cells.

increased (P<0.05). The apoptosis rate had significant difference among the C1, C2 and C3 groups (P<0.05), among the D1, D2 and D3 groups (P<0.05), and among the E1, E2 and E3 groups (P<0.05). The apoptosis rate in the D3 group was significantly higher than in the other treatment groups at the same dose (P<0.05). The results are presented in Table III and Fig. 1.

Comparison of extracellular matrix protein expression levels. Compared with the control group, the protein and mRNA expression levels of COL-III, COL-I and α-SMA in the model group were significantly increased (P<0.05; Fig. 2 and Table IV). Compared with the model group, the above indexes in each treatment group were significantly decreased (P<0.05). There was a significant difference in each index among the C1, C2 and C3 groups (P<0.05), among the D1, D2 and D3 groups (P<0.05), and among the E1, E2 and E3

groups (P<0.05). The protein and mRNA expression levels of COL-III, COL-I and α-SMA in the E3 group were significantly lower than in the other treatment groups (P<0.05). The results are presented in Compared with the control group, the expressions of COL-III, COL-I, α-SMA protein and mRNA in the model group increased significantly (P<0.05). Compared with the model group, the expressions of COL-III, COL-I, α-SMA protein and mRNA, exhibiting significant differences among Group C1, C2, and C3 (P<0.05), as well as among Group D1, D2, and D3 (P<0.05), and among Group E1, E2, and E3 (P<0.05). The expressions of COL-III, COL-I, α-SMA protein and mRNA in Group Matrine Injection was significantly lower (P<0.05). The results are shown in Tables IV, V and Fig. 2.

Impact of antioxidant factors on ECSOD. Compared with the control group, the expression of ECSOD mRNA in the

Table III. Results of human embryonic lung fibroblast apoptosis assay.

Group	Apoptotic rate, %
Control	0.0731±0.0060
Model	0.0262±0.0036[a]
C1	0.0451±0.0049[b-d]
C2	0.0673±0.0045[b,c,e]
C3	0.0831±0.0046[b,d,e]
D1	0.0812±0.0021[b-d]
D2	0.1071±0.0030[b,c,e]
D3	0.1252±0.0051[b,d-f]
E1	0.0651±0.0038[b-d]
E2	0.0833±0.0040[b,c,e]
E3	0.1034±0.0050[b,d,e]

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. control group; [b]P<0.05 vs. model group; [c]P<0.05 for group 2 vs. group 1 of each drug treatment, [d]P<0.05 for group 3 vs. group 1 of each drug treatment. [e]P<0.05 for group 3 vs. group 2 of each drug treatment; [f]P<0.05 vs. the C3 group. C1-3, transforming growth factor β1 + low-, moderate- and high-dose Ginkgo leaf extract, respectively; D1-3, transforming growth factor β1 + low-, moderate- and high-dose shenmai, respectively; E1-3, transforming growth factor β1 + low-, moderate- and high-dose matrine, respectively.

Table IV. Extracellular matrix protein content.

Group	COL-III	COL-I	α-SMA
Control	8.44±0.28	17.43±0.33	180.93±2.24
Model	55.28±0.17[a]	25.85±0.47[a]	252.20±4.87[a]
C1	51.74±0.37[b-d]	24.51±0.37[b-d]	246.49±0.79[b-d]
C2	48.37±0.41[b,c,e]	22.55±0.22[b,c,e]	234.07±4.75[b,c,e]
C3	43.30±0.42[b,d,e]	19.71±0.31[b,d,e]	225.20±2.76[b,d,e]
D1	49.15±1.60[b-d]	23.48±0.33[b-d]	234.94±3.92[b-d]
D2	45.43±0.24[b,c,e]	21.59±0.27[b,c,e]	217.39±5.41[b,c,e]
D3	40.14±0.83[b,d,e]	18.99±0.30[b,d,e]	207.90±3.13[b,d,e]
E1	44.26±0.27[b-d]	22.89±0.25[b-d]	219.34±3.85[b-d]
E2	40.46±0.22[b,c,e]	20.50±0.25[b,c,e]	205.78±2.47[b,c,e]
E3	37.76±0.20[b,d-f]	17.89±0.20[b,d-f]	193.41±1.76[b,d-f]

Data are represented as the mean ± standard deviation. [a]P<0.05 vs. control group; [b]P<0.05 vs. model group; [c]P<0.05 for group 2 vs. group 1 of each drug treatment, [d]P<0.05 for group 3 vs. group 1 of each drug treatment. [e]P<0.05 for group 3 vs. group 2 of each drug treatment; [f]P<0.05 vs. the C3 group. C1-3, transforming growth factor β1 + low-, moderate- and high-dose Ginkgo leaf extract, respectively; D1-3, transforming growth factor β1 + low-, moderate- and high-dose shenmai, respectively; E1-3, transforming growth factor β1 + low-, moderate- and high-dose matrine, respectively; COL-III, collagen type III; COL-I, collagen type I; α-SMA, α-smooth muscle actin.
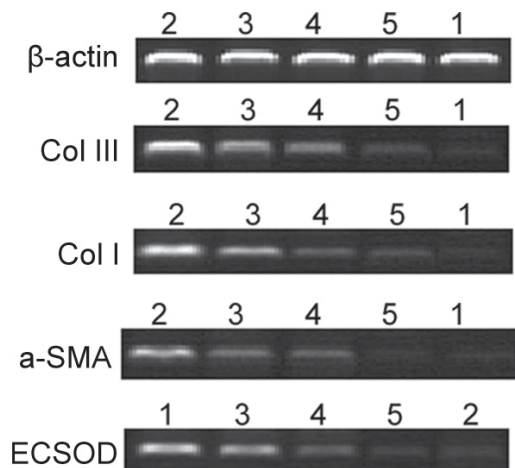

Figure 2. mRNA expression of β-actin (control) and the extracellular matrix components COL-III, COL-I, α-SMA and ECSOD. Lanes 1-5 represent the control, model, C3, D3 and E3 groups, respectively. COL-I collagen type I; COL-III, collagen type III; α-SMA, α-smooth muscle actin; ECSOD, extracellular superoxide dismutase.

model group was significantly decreased (P<0.05; Table V). Compared with the model group, the expression of ECSOD mRNA in each treatment group was significantly increased (P<0.05). There was a significant difference in each index among the C1, C2 and C3 groups (P<0.05), among the D1, D2 and D3 groups (P<0.05) and among the E1, E2 and E3 groups (P<0.05). The expression of ECSOD mRNA in the C3 group was significantly lower than in the other treatment groups at the same dose (P<0.05). The results are presented in Tables V, VI and Fig. 3).

Discussion

Under homeostatic conditions, fibroblasts in the lung tissues assume a resting state; however, during fibrosis, various internal and external factors stimulate and activate fibroblasts to transition from a resting state to a hyperproliferative state, leading to the formation of a lung fibroblastic focus (10). Inducing fibroblast apoptosis may alleviate PF, thereby promoting recovery (21). In the lung tissue, increased expression of ECM proteins, particularly of collagens, is an important indicator of the degree of fibrosis (22,23), as demonstrated by previous studies that identified large quantities of collagen deposition within fibrotic alveoli, in which COL-I and COL-III were the primary constituents (24,25). The detection of ECM protein expression at the mRNA and protein level may be of significant value in the assessment of anti-fibrotic drug efficacy. Activated fibroblasts transform into myofibroblasts following functional and phenotypic changes, leading to the expression of α-SMA (26). Myofibroblasts are the primary cell responsible for the abnormal synthesis and deposition of ECM proteins, and α-SMA may be used as a marker of myofibroblasts (27). A previous study demonstrated that the degree of PF in patients was positively correlated with the level of oxidative stress in vivo (28). ECSOD as the primary antioxidant within lung tissue, exhibits the highest enzyme activity in the lungs (29). ECSOD removes superoxide from cells and inhibits the reaction between nitric oxide and superoxide, which protects lung tissue and collagen from free radical-induced injury during the inflammatory process (30). Therefore, ECSOD exerts critical protective effects against oxidative stress-induced PF (30).

Table V. COL-III, COL-I, α-SMA and ECSOD mRNA (n=6).

Group	COL-III/β-actin	COL-I/β-actin	α-SMA/β-actin	ECSOD/β-actin
Control	0.1988±0.0078	0.1929±0.0109	0.2002±0.0007	0.8987±0.0027
Model	0.9822±0.0224[a]	0.9762±0.0193[a]	0.9912±0.0014[a]	0.0977±0.0012[a]
C3	0.6152±0.0113[b]	0.6098±0.0125[b]	0.6023±0.0445[b]	0.7024±0.0106[b]
D3	0.4351±0.0223[b]	0.4289±0.0089[b]	0.4278±0.0169[b]	0.3532±0.0072[b,c]
E3	0.2221±0.0121[b,c]	0.2091±0.0435[b,c]	0.2332±0.0651[b,c]	0.1977±0.0124[b,c]

Data are presented as the mean ± standard deviation. [a]$P<0.05$ vs. the control group; [b]$P<0.05$ vs. the model group; [c]$P<0.05$ vs. the C3 group. C3, transforming growth factor β1 + high-dose Ginkgo leaf extract; D3, transforming growth factor β1 + high-dose shenmai; E3, transforming growth factor β1 + high-dose matrine.

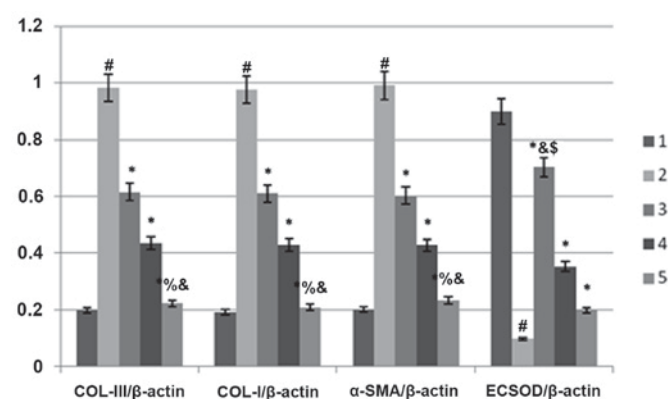Figure 3. mRNA expression of COL-III, COL-I, α-SMA and ECSOD. 1, control; 2, Model; 3, C3-transforming growth factor β1 + high-dose Ginkgo leaf extract; 4, D3-transforming growth factor β1 + high-dose shenmai; 5, E3-transforming growth factor β1 + high-dose matrine. COL-I collagen type I; COL-III, collagen type III; α-SMA, α-smooth muscle actin; ECSOD, extracellular superoxide dismutase. #$P<0.05$ vs. the control group; *$P<0.05$ vs. the model group; %$P<0.05$ vs. the C3 group; &$P<0.05$ vs. the D3 group; $$P<0.05$ vs. the E3 group.

Table VI. ECSOD protein content.

Group	ECSOD (mU/l)
Control	49.67±0.95
Model	34.82±0.43[a]
C1	39.98±0.26[b-d]
C2	44.40±0.84[b,c,e]
C3	48.46±0.65[b,d,e]
D1	38.53±0.44[b-d]
D2	42.74±0.77[b,c,e]
D3	46.24±0.43[b,d-f]
E1	37.30±0.37[b-d]
E2	40.81±0.36[b,d,e]
E3	43.89±0.67[b,d,e]

Data are represented as mean ± standard deviation. [a]$P<0.05$ vs. control group; [b]$P<0.05$ vs. model group; [c]$P<0.05$ for group 2 vs. group 1 of each drug treatment, [d]$P<0.05$ for group 3 vs. group 1 of each drug treatment. [e]$P<0.05$ for group 3 vs. group 2 of each drug treatment; [f]$P<0.05$ vs. the C3 group. C1-3, transforming growth factor β1 + low-, moderate- and high-dose Ginkgo leaf extract, respectively; D1-3, transforming growth factor β1 + low-, moderate- and high-dose shenmai, respectively; E1-3, transforming growth factor β1 + low-, moderate- and high-dose matrine, respectively; ECSOD, extracellular superoxide dismutase.

Previous studies have suggested that the predominant active ingredients in GBLE are flavonoids (31-33). These flavonoids have the ability to dilate blood vessels, improve microcirculation, resist oxidation, scavenge oxygen free radicals and prevent or reverse the fibrosis of multiple organs, thus alleviating PF and reducing the degree of alveolitis and fibrosis (33,34). Ginseng saponin and Ophiopogonin D are the primary active ingredients in S, which may inhibit the activation of myofibroblasts by scavenging free radicals and blocking the free-radical-generation enzyme system, thus acting as antioxidants and reducing PF (35-37). Tetracyclic quinodines are active ingredients in M that have been reported to directly scavenge cytotoxic free radicals, aid in the inhibition of fibroblast division and proliferation, promote the expression of TGF-β1 and CT-GF and prevent tissue injury and PF (38,39).

The present data demonstrated that GBLE, S and M inhibited the proliferation and induced the apoptosis of lung fibroblasts, inhibited the synthesis and secretion of COL-I, COL-III and α-SMA, and promoted the synthesis and secretion of ECSOD.S exhibited the greatest activity in suppressing the abnormal proliferation of lung fibroblasts and promoting their apoptosis, while M exhibited the greatest activity in reducing the synthesis of collagens, potentially by inhibiting expression of the collagen gene. In addition, GBLE exhibited the greatest activity in promoting the secretion of the antioxidant factor ECSOD. The above activities were all significantly associated with drug concentration.

The present findings suggest that different traditional Chinese medicines have various anti-fibrotic targets. Thus, drug treatment should be selected based on the particular stage of fibrosis and mechanism of drug action.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

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