MECHANISM OF WARMING AND INVIGORATING KIDNEY YANG IN PROTECTING PULMONARY FIBROSIS BY INHIBITING SMAD AND STAT3 SIGNALING PATHWAY

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ABSTRACT

Background and aim: *Pulmonary fibrosis is a kind of pulmonary disease, including heterogeneous groups of various interstitial lung diseases. We explore whether the warming and tonifying kidney yang can inhibit Smad and STAT3 signaling pathways, thus inhibiting the occurrence of pulmonary fibrosis.*

Methods: *To explore the mechanism of warming and tonifying kidney yang in protecting pulmonary fibrosis by inhibiting Smad and STAT3 signaling pathways, SD rats (6-8 weeks old, weight 200 ± 20g) were purchased from an experimental animal company. All feeding procedures were conducted according to the nursing guidelines of the national institutes of health. Further, the experiment and treatment were conducted by following the NIH experimental animal nursing and using guidelines. The pulmonary fibrosis model was induced by bleomycin. According to the experimental requirements, the rats were divided into control, induction, and treatment groups. The expressions of EMT, Smad, and STAT3 were analyzed by Western blot and immunohistochemistry.*

Result: *The pulmonary function of rats was detected by BUXCO pulmonary function test system. The lung sections of rats were observed by trichrome staining with hematoxylin-eosin and Masson. The expressions of the markers COL1A1, COL3A1, CTGF, and TGF-β in rats were analyzed using RT-qPCR. Moreover, TNF-α, IL-1 β, and IL-6 levels in bronchoalveolar lavage fluid were detected by enzyme-linked immunosorbent assay. The protein expression of P-Smad and P-STAT3 in the induction group was higher than those in the control group; also, it decreased in the treatment group compared with the induced group (P<0.05). Immunohistochemistry showed that warming and tonifying kidney yang inhibited the activation of P-Smad and P-STAT3. The lung resistance, dynamic pulmonary compliance, and vital capacity of the treatment group were lower than those in the control group (P<0.05). The HE staining and Ashcroft scores in the induction group were higher than those in the control group (P<0.05), and those in the treatment group were lower than those in the induction group (P<0.05). The expression of mRNA in the induction group was higher than that in the control group, and the expression of ctl1a1, ctl3a1, CTGF, and TGF-β in the treatment group were lower than those in the induction group (P<0.05). The levels of TNF- α, L-1 β, and IL-6 in the induction group were higher than those in the control group (P<0.05), and the levels of TNF-α, L-1 β, and IL-6 in the treatment group were lower than those in the induction group (P<0.05). Compared to the control group, the expression of E-cadherin decreased and α-SMA increased (P<0.05) in the induction group. Also, compared with the induction group, the expression of E-cadherin increased, and α-SMA decreased (P<0.05) in the treatment group.*

Conclusion: *Warming and tonifying kidney yang could improve the process of pulmonary fibrosis in rats by inhibiting the activation of Smad and STAT3. Thus, the method of warming and tonifying kidney yang may be a promising candidate strategy to treat pulmonary fibrosis.*

Keywords: *Pulmonary fibrosis, warming and tonifying kidney yang, smad, STAT3, EMT.*

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Introduction

Pulmonary fibrosis is a kind of pulmonary disease, including heterogeneous groups of various interstitial lung diseases. It involves progressive lung remodeling, alveolar destruction, excessive pathological extracellular matrix deposition, and lung scar formation(1). This destructive condition caused by the maladjustment of a wound healing process eventually leads to the interruption of basic gas exchange in the lungs, resulting in pulmonary dysfunction and ultimately respiratory failure. There are many triggers for the fibrosis process, the most important of which are β (Transforming

growth factor-beta (TGF-β) and its activation, the recruitment of fibroblasts and their epithelial-mesenchymal transition (EMT) to myofibroblasts, and the release of inflammation[2].

Finally, the disease spreads through continuous pathological fibroblast differentiation, fibrous hyperplasia, matrix deposition, lung remodeling, as well as scar and hard tissue formation[3]. Recently, sufficient evidence has proved that organ fibrosis involving the lung is highly malleable, and pulmonary fibrosis has the ability to subside under certain conditions[4].

This delicate discovery has brought a glimmer of hope to this field's researchers, who hope to find drugs capable of reversing fibrosis as intervention measures to treat it[5]. TGF-β1 is considered as the key factor and "main switch" in the fibrosis process, which mainly regulates the cell growth and the production of extracellular matrix by activating the downstream receptor SMAD signal to form scar[6]. In addition to the TGF-β/Smad signaling pathway and EMT central mechanism, there are other signaling pathways in the pathogenesis of pulmonary fibrosis[7]. Activator of transcription 3 (STAT3) is a transcription factor that regulates the response of cells to growth factors and cytokines[8].

It is related to the activation, proliferation, and extracellular matrix deposition of fibroblasts, especially in the process of fibrogenesis[9]. In addition, STAT3 controls the crosstalk between epithelial cells and fibroblasts, and fibroblasts, in turn, promote disease progression[10]. IL-6/STAT3 axis is an important signal pathway in the pathogenesis of pulmonary fibrosis. Warming and tonifying kidney yang is a prescription developed and recorded in Synopsis of Golden Chamber (a classic traditional Chinese medicine).

The clinical application shows that warming and tonifying kidney yang can obviously improve the abnormal state of liver and kidney function. According to the traditional theory, lung-defensive qi protects the body from external pathogenic factors[11].

However, the lungs are closely related; they are connected with the kidneys through meridians. Lung-protecting qi originates from the lower energizer and nourishes the middle energizer, where the spleen and stomach are located.

This study aims to explore whether the warming and tonifying kidney yang can inhibit Smad and STAT3 signaling pathways, thus inhibiting the occurrence of pulmonary fibrosis.

Materials and methods

Experimental materials

Experimental rats

Forty-five male SD rats (6-8 weeks old, weight 200±20g) without specific pathogenic bacteria were purchased from an experimental animal company; they were given one week to adapt to the environment.

Animal model of bleomycin

All animal experiments and treatments were carried out according to NIH experimental animal care and use guidelines. The animals were anesthetized by IP injection of benzothiazine/ketamine and fixed on the tray in the supine position. They were intubated using a 20G vascular catheter. Then, tilting the plate at 45 degrees, the IA-1C microphotograph head (Pennscentury, Pennsylvania, Windmoor, Pennsylvania) was inserted into the vascular catheter lumen. After delivering Bleomycin by IT (50 μ L, 4u/kg), the tip was removed. The animals were returned to their cages after pulling out the pipes.

Chinese herbs applied in warming and invigorating kidney yang

The herbs applied in warming and invigorating kidney yang include cinnamon (1000g) and Morinda Officinalis (3000g) with a ratio of 1:3. All raw materials were purchased from the Chinese herbal medicine market (China). Quality inspection was conducted by the Department of Traditional Chinese Medicine Identification of the Medical University. The crude herbs were decocted twice after soaking for 60min, first with 24L water, then 32L water. The decocting time was strictly controlled. In the first decocting, a tube was used to maintain weak boiling. After heating in an electric furnace for 60min, the second boiling was conducted for 30min. The mixture was filtered with absorbent cotton and concentrated to 2000ml in a vacuum, with 2g of crude drug per ml. According to the formula in Pharmacopoeia of the People's Republic of China (PPRC-2012 edition), 6g of sodium benzoate was mixed in the prepared decoction; 1000 ml of liquid should contain 3 grams of sodium benzoate. Finally, the drug was stored in sterile glass in a refrigerator at 4°C.

Experimental grouping

According to the experimental requirements, the rats were divided into control group (injected

with normal saline as blank control, n=15), induction group (injected with 50μL in vivo; lung fibrosis induced by bleomycin at a dose of 4u/kg, n=15), and treatment group (based on induction group rats, warming and invigorating kidney yang was performed at a dose of 2 ml/day, n=15).

Medical ethics issues

All feeding procedures were carried out according to the nursing guidelines of the national health institutes (Registration number: 2020 NO.57).

Experimental methods

Western blot analysis

Using radioimmunoassay lysis buffer (R0010, Beijing Sun Biological Life Sciences Co., Ltd., Beijing, China), the whole lung tissue lysate was prepared with tissue protein extraction reagent (T-PER) to extract total protein from tissues, cells, or exosomes. The concentration of protein was evaluated by the Bicreatine Kit. Protein was separated by polyacrylamide gel electrophoresis; then, it was transferred to polyvinylidene fluoride membrane, sealed in 5%BSA for1h at room temperature, and incubated with primary rabbit antibodies against P-Smad (ab30871, 1:1000) and CD80 (ab109201, 1:1000), P-STAT3 (ab46154, 1:1000). Ab11939, 1:1000), α-SMA (ab13847, 1:1000). Later, the membrane was incubated with goat anti-rabbit (ab205718, 1:10000) or goat anti-mouse (ab6789, 1:5000) secondary antibody, labeled with horseradish peroxidase, at room temperature for1h.

The above antibodies were purchased from Abcam, Cambridge, England. ImageJ 1.48u software (National Institute of Health Research, Bethesda, Maryland, USA) was used to develop the band and quantify its intensity; also, GAPDH was used as the internal reference standard.

Study of cardiac hemodynamics

After anesthetizing with isoflurane (2%-4%), a tracheotomy was performed for intubation, and mechanical ventilation with 1%-2% isoflurane and oxygen (tidal volume 6 mL/kg, respiratory rate 100 times /min) was done. The chest was open to enter the organ through sternotomy. Once the pericardium was opened got completely close to the heart, the ultrasonic flow probe was inserted into RV to collect right ventricular systolic pressure (RVSP). Hemodynamic data were recorded using Scisense PV control unit (Science, Ontario, Canada).

Assay of lung function

At the end of the experiment, 6 rats in the group were anesthetized with 1% normal saline pentobarbital sodium (45mg/kg, i.p.), and a tracheotomy was performed under the larynx and endotracheal intubation. After the operation, the rats were put in the operating room, and the cannula was connected to the machine. Lung function was measured by the BUXCO lung function test system in the USA, including functional residual capacity, quasi-static, fast flow capacity, and resistance compliance tests. Finally, the system software recorded and displayed pulmonary function parameters automatically.

Trichrome staining with hematoxylin-eosin and Masson

Lung tissues were collected, inflated with PBS/OCT (50:50), and fixed in OCT (frozen at –80°C). Slices were cut to 8μm and adhered to color frost slides (Thermo Fisher). Lung tissue slices were stained with hematoxylin-eosin (H&E) and Masson and observed under a light microscope. ImageJ software was used to quantify the thickness of the middle layer and collagen deposition. Ashcroft score is a semi-quantitative method to obtain a pulmonary fibrosis score. The score of the images ranged from 0 (normal lung) to 8 points (complete fibrous occlusion of the visual field). The average of all the scores of 5 parts was calculated.

Quantitative real-time PCR(RT-qPCR)

Total RNA was extracted with Trizol reagent. PrimeScript-RT-enzymix-mixi technology was applied to synthesize total RNA from RNA samples (cDNA (Dalian) Co., Ltd., Dalian, cat. No.BK501). SYBR-Premix-Ex-TaqII (Dalian Takara Co., Ltd, cat. No.BK402) was adopted for RT-qPCR. The gel image analysis system and gray analysis software were used to detect the average gray value. β actin was used for internal control. Further, primer premier software was applied to design primers.

Enzyme-linked immunosorbent assay (ELISA)

In all experimental groups, the same amount of lung tissue was weighed, and the buffer solution containing protease and phosphatase inhibitor was used to extract homogenate. The whole-cell lysate obtained after thorough homogenization was centrifuged at 10000 rpm for 10 minutes. The supernatant was collected after 10 minutes, divided into small portions to avoid freezing and thawing, and stored at -80°C. The expression of proinflammatory

cytokines, including IL-6, IL-1β, and TNF-α, was studied using commercially available ELISA kits. Enzyme-linked immunosorbent assay procedures were carried out according to the instructions. In brief, the 96-well plates were treated with the respective coated antibodies, fully sealed, and cultured at 4°C overnight.

On the next day, all wells of the culture plate were washed with washing buffer containing PBS and Tween 20 and cultured for 1h at room temperature with the diluent provided by the kit. The wells were washed once with washing buffer, treated with samples and standards, and incubated at 4°C overnight. On the next day, the samples and standards were replaced with washing buffer and treated with their respective detection antibodies for 1h at room temperature. Then, the wells were treated with avidin HRP antibody for 30 minutes and washed; next, it was added with substrate solution and incubated for 15 minutes. A stop solution (1M H3PO4) was added to the substrate solution, and absorbance was recorded at 450 nm and 570 nm.

The protein content of cytokine expression was standardized by the Bradford reagent, and the data were expressed in pg/mg protein.

Immunohistochemistry

The slide glass containing 5μ-thick lung tissue slices was dewaxed at 65°C and incubated for 20 min. Xylene was changed twice every 5 minutes. The slices were rehydrated with gradient alcohol (100%, 90%, 80%, and 70%) and put in distilled water. After rehydration, the antigen was recovered with 1X citrate buffer, and the endogenous peroxidase was inactivated with 3%H_2O_2. Lung slices were incubated with 3%BSA blocking solution at room temperature for 1h to avoid nonspecific binding. Then, they were washed twice with washing buffer (PBST phosphate-buffered saline mixed with Tween-20) and were incubated with primary antibodies (pSTAT3 and P-Smad in 3%BSA blocking solution with a dilution of 1:100) at 4°C overnight. On the second day, PBST was used to fully wash the slices, and the poly-Excel HRP/DAB detection system was applied for the color reaction. The slides were counterstained with hematoxylin and mounted with DPX mounting medium. Images of stained sections were captured by Olympus microscope to show immunoreactivity.

Statistical analysis

All experiments were conducted at least three times. The unpaired t-test was used to compare the two conditions, and GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis. $P<0.05$ indicated a statistically significant difference.

Results

Inhibition of the protein expressions of Smad and STAT3 by warming and invigorating kidney yang

The protein expression of the Smad/STAT3 signaling pathway was analyzed by western blot. Compared with the control group, the protein expressions of P-Smad and P-STAT3 in the induction group increased, while those in the treatment group decreased ($P<0.05$).

The Smad/STAT3 signaling pathway was inhibited after treatment. Immunohistochemistry showed that warming kidney yang inhibited the activation of P-Smad and P-STAT3, as provided in Table 1.

Group	P-Smad	P-STAT3
Control group	1.08±0.08	1.14±0.10
Induction group	2.15±0.23	1.96±0.21
Treatment group	1.15±0.12	1.24±0.15
F-value	10.263	13.026
P-value	0.014	0.035

Table 1: Western blot analysis of Smad/STAT3 protein ($\bar{x}\pm s$).

Comparison of pulmonary function parameters in rats

In the treatment group, the lung resistance increased, while the lung's dynamic compliance and vital capacity decreased ($P<0.05$) compared to the control group.

Warming and invigorating kidney yang can reverse bleomycin-induced pulmonary fibrosis, as presented in Table 2.

Group	Lung resistance (cmH20*secsml)	Dynamic compliance (ml/cmH20)	Vital capacity (ml)
Control group	0.26±0.03	0.36±0.03	14.73±2.49
Induction group	0.53±0.06	0.14±0.01	6.32±0.68
Treatment group	0.34±0.05	0.32±0.02	12.55±1.42
F-value	11.527	13.429	10.836
P-value	0.014	0.025	0.015

Table 2: Comparison of pulmonary function parameters in rats ($\bar{x}\pm s$).

Reduction effect of warming and tonifying kidney yang on pulmonary fibrosis injury

The staining and Ashcroft scores were higher in the induction group (P<0.05), while they were lower in the treatment group (P<0.05) compared to the control group . Fig. 1 shows the pulmonary fibrosis scores of representative H&E and Ashcroft stained rat lung slices. After treatment, the bleomycin-induced pulmonary fibrosis decreases, as presented in Table 3.

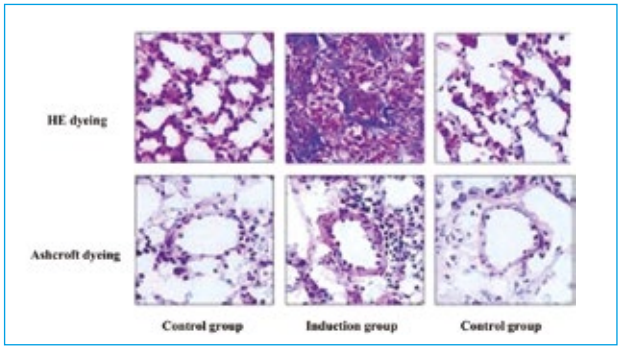

Fig. 1: Pulmonary fibrosis score of rat lung slices stained by H&E and Ashcroft.

Group	HE staining score	Ashcroft score
Control group	0.43±0.02	1.35±0.24
Induction group	2.85±0.34	6.59±1.24
Treatment group	1.37±0.15	3.06±0.84
F-value	9.382	12.926
P-value	0.004	0.017

Table 3: Pulmonary fibrosis score ($\bar{x}\pm s$).

RT-qPCR quantitative fibrosis marker level

The mRNA expressions of COL1A1, COL3A1, CTGF, and TGF-β were analyzed by RT-qPCR. The mRNA expressions of col1a1, COL3A1, CTGF, and TGF-β in the induction group were higher than those in the control group, while those in the treatment group were lower than those in the induction group (P<0.05). Warming and tonifying kidney yang reduced the degree of pulmonary fibrosis in model rats, as presented in Table 4.

Group	COL1A1	COL3A1	CTGF	TGF-β
Control group	1.14±0.08	1.05±0.07	1.07±0.08	1.12±0.08
Induction group	2.37±0.25	2.15±0.23	2.46±0.35	2.33±0.24
Treatment group	1.25±0.11	1.36±0.14	1.28±0.15	1.33±0.16
F-value	10.446	12.375	9.152	13.815
P-value	0.024	0.031	0.004	0.012

Table 4: RT-qPCR analysis of fibrosis markers ($\bar{x}\pm s$).

Enzyme-linked immunosorbent assay

The levels of TNF-α, IL-1β, and IL-6 in the bronchoalveolar lavage fluid of rats were detected by enzyme-linked immunosorbent assay. Compared with the control group, the levels of TNF-α, L-1β, and IL-6 in the induction group were higher (P<0.05), while those in the treatment group were lower than those in the induction group (P<0.05), as presented in Table 5.

Group	TNF-α (ng/ml)	IL-6 (ng/ml)	IL-1β (ng/ml)
Control group	64.26±11.85	24.23±5.33	52.16±8.43
Induction group	263.18±37.29	87.24±11.24	96.28±13.24
Treatment group	124.58±19.26	38.37±7.23	64.43±12.08
F-value	11.725	13.407	13.54
P-value	0.003	0.013	0.024

Table 5: ELISA analysis ($\bar{x}\pm s$).

Inhibition of EMT by warming and invigorating kidney yang

Western blot was used to analyze the effect of warming and invigorating kidney yang on EMT protein. Compared with the control group, the expression of E-cadherin decreased and the expression of α-SMA increased in the induction group (P<0.05), while the expressions of E-cadherin and α-SMA decreased in the treatment group (P<0.05), indicating that warming and invigorating kidney yang could regulate EMT process; the results are provided in Table 6.

Group	E-cadherin	α-SMA
Control group	1.95±0.22	1.15±0.11
Induction group	1.06±0.09	2.25±0.27
Treatment group	1.86±0.20	1.23±0.13
F-value	13.994	10.527
P-value	0.013	0.027

Table 6: Inhibition of the occurrence of EMT (±s) by warming and invigorating kidney yang.

Discussion

Pulmonary fibrosis is a destructive and refractory disease, mainly affecting middle-aged and older people. It is a heterogeneous group of lung diseases involving the progressive accumulation of scar tissue and fibrous hyperplasia, leading to respiratory failure[12]. The respiratory function of pulmonary fibrosis decreases gradually, developing related pulmonary hypertension. Although the

etiology and mechanism of pulmonary fibrosis are complex and speculative, their pathogenesis can be divided into three stages: susceptibility, pathogenesis, and disease progression[13]. Various factors, including environmental exposure to certain drugs, chemotherapy, aging, and genetic factors, induce pulmonary fibrosis.

The TGF-β/Smad signaling pathway is very important among the signaling pathways leading to pulmonary fibrosis. TGF-β1 is a key subtype of the TGF-β superfamily and a fibrotic factor, which combines with receptors to transmit intracellular signals[14]. The number of TGF-β type I and type II receptors is associated with the synthesis of the extracellular matrix, which increases in many fibrosis cases. In addition, activation of Smad2/3 promotes fibroblast proliferation, differentiation, and ECM remodeling[15]. Considerable evidence emphasizes the role of STAT3 in pulmonary fibrosis. Further, the activation of STAT3 promotes fibroblast activation and pulmonary fibrosis induced by TGF-β1. Many small-molecule STAT3 inhibitors are being developed and evaluated in clinical trials, especially in cancer treatment, showing that this treatment is relatively safe and effective. Due to the importance of STAT3 in the development of pulmonary fibrosis, it may be a potential target for the treatment of pulmonary fibrosis. Warming and invigorating kidney yang is a natural strategy to inhibit Smad/STAT3. Studies have shown that warming and invigorating kidney yang can reduce inflammation and joint destruction of arthritis mice induced by type II collagen by inhibiting STAT3 acetylation mediated by p300.

The lung function test in this study directly reflected that warming and invigorating kidney yang inhibited the development of pulmonary fibrosis in rats and improved the lung function of rats. Pharmacokinetic studies have shown that after oral or intravenous administration, the drug for warming and invigorating kidney yang is widely distributed, preferentially in the lungs. In a recent report, it has been pointed out that warming and invigorating kidney yang reduces the lung inflammatory infiltration in rats with radiation-induced lung injury in the early stage and collagen deposition in the late stage, suggesting its better therapeutic effect on interstitial pulmonary diseases[16]. These pieces of evidence indicate that warming and invigorating kidney yang may improve pulmonary fibrosis by inhibiting the Smad/STAT3 signaling pathway. In this study, it was observed that the drug had a significant inhibitory effect on P-STAT3 and P-Smad induced by bleomycin in rat lungs. Furthermore, according to the results of an immunohistochemical microscope, the method inhibited the translocation of STAT3 and Smad to the nucleus stimulated by T bleomycin. Static inhibition led to a decrease in ECM deposition. In addition, warming and invigorating kidney yang reduced the expression of bleomycin-induced fibrosis markers COL1A1, COL3A1, CTGF, and TGF-β, slowing down the progression of pulmonary fibrosis in rats.

Many studies have reported that bleomycin induces inflammation with pulmonary fibrosis as a side effect[17]. Indeed, there is a shred of important evidence that bleomycin induces the production of fibroblasts and proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6. In this research, the evidence indicated that warming and invigorating kidney yang had anti-inflammatory and anti-fibroblast effects. Further, the results showed that the method could effectively inhibit bleomycin-induced inflammation. Pulmonary fibrosis is a kind of pulmonic disease caused by persistent injury, leading to the disintegration of the alveolar epithelium and the accumulation of various ECM components. At present, the established concepts about the pathogenesis of pulmonary fibrosis mainly focus on TGF-β/Smad signal transduction, EMT communication disorder, and epithelial cell repair. Fibrogenesis is an irreversible dynamic process, and the traditional assumption that therapeutic intervention is ineffective is now being replaced by a new belief in the plasticity of fibrous tissue, indicating the reversibility of fibers and is used to develop restorative treatment[18]. One recent report elegantly describes the importance of natural products and how they can help fill the gap of treatment intervention for organ fibrosis.

Repeated injury to lung epithelium by various triggers initiates the process of tissue regeneration and remodeling, being gradually abnormal due to the persistence of pathogens and/or tissue injury[19]. The unsuccessful control of the chronic wound healing process leads to fibroblasts' activation, proliferation, and transdifferentiation to more active counter-myofibroblasts. This dynamic process called EMT involves the transcription regression of key epithelial markers such as E-cadherin and the increased expression of mesenchymal markers such as α-SMA. According to the results, the expression of E-cadherin and α-SMA increased in rats induced by bleomycin, promoting pulmonary fibrosis and

decreasing the expression of EMT markers after treatment.

Persistent inflammation caused by continuous stimulation of lung epithelium is the key factor leading to disease progression[20]. Therefore, the expression of inflammatory cytokines after treatment was studied by enzyme-linked immunosorbent assay. Simultaneously, in vivo study showed the bleomycin-induced inflammatory cells to be at a low level after treatment by warming and invigorating kidney yang. Besides, ELISA results showed that the expressions of IL-6, IL-1β, and TNF-α were inhibited.

Conclusion

This study proves that warming and invigorating kidney yang has a protective effect on bleomycin-induced pulmonary fibrosis in rats and inhibits the fibrosis reaction of lung fibroblasts.

Further, it is indicated that the anti-pulmonary fibrosis effect of this method is related to the inhibition of Smad and STAT3 activation. Although the specific molecular target of CTS needs further study, the present research provides important evidence, indicating warming and invigorating kidney yang might be a promising treatment for treating pulmonary fibrosis.

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