

<sup>1</sup>State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, Guangdong, P. R. China; <sup>2</sup>School of Acupuncture & Moxibustion, Chengdu University of TCM, Chengdu 610075, Sichuan, P.R. of China; <sup>3</sup>National Diabetes Mellitus Clinical Research Base of TCM, the First Teaching Hospital of Chengdu University of TCM, Chengdu 610075, Sichuan, P.R. of China.

\*Corresponding author E-mail: [lr2269@163.com](mailto:lr2269@163.com)

## Abstract

**Background:** An increasing number of people suffered idiopathic fibrosis (IPF) and the current treatment was far from clinical satisfaction. Moxibustion, another effective and safe unconventional therapy, had been introduced to treat this refractory disease. The study aimed to investigate the effect of moxibustion on a bleomycin A5-induced pulmonary fibrosis model.

**Materials and Methods:** Sprague-dawley (SD) rats were randomly allocated to the blank group, model group, moxibustion group, and prednisone group, for which they received no treatment, modeling, moxibustion treatment and prednisone treatment. After four-week treatment, the rats were euthanized for Hematoxylin and Eosin (H.E.) staining, and TGF- $\beta$ 1 and IFN- $\gamma$  protein and mRNA detection in lungs.

**Results:** In the model group, TGF- $\beta$ 1 was significantly increased and IFN- $\gamma$  was significantly decreased at both protein and mRNA levels in comparison to the blank group. In the moxibustion and prednisone group, however, TGF- $\beta$ 1 was decreased and IFN- $\gamma$  was increased at both protein and mRNA levels in comparison to the model groups. Compared with prednisone, moxibustion showed comparable effect in lowering TGF- $\beta$ 1 ( $P>0.05$ ) and better effect in up-regulating IFN- $\gamma$  ( $P>0.05$ ).

**Conclusion:** The study concludes moxibustion protected pulmonary fibrosis by downregulating TGF- $\beta$ 1 and upregulating IFN- $\gamma$  cytokines at both mRNA and protein levels, and the effect was comparable to prednisone. Moxibustion could be used as a therapeutic alternative treatment for pulmonary fibrosis.

**Key words:** moxibustion; pulmonary fibrosis; TGF- $\beta$ 1; IFN- $\gamma$ ; rats

## Introduction

A large number of people have been suffered by idiopathic pulmonary fibrosis (IPF) in recent years (Ley and Collard 2013). In the United States, it was estimated that IPF affected 16.3 to 42.7 per 100,000 persons (Raghu et al. 2006). In Europe, IPF prevalence ranged from 1.25 to 23.4 cases per 100,000 population, and the annual incidence ranged between 0.22 and 7.4 per 100,000 population (Nalysnyk et al. 2012). The disease, to some extent, shares similar pathogenesis to chronic obstructive pulmonary disease (COPD). COPD is common disease characterized by airway obstruction and progressive lung inflammation. Both of them are chronic progressive disorders in which excessive deposition of extracellular matrix (ECM) leads to irreversible scarring of interstitial lung tissue (American Thoracic Society and European Respiratory society 2000, Gal and Staton 2005). Pulmonary fibrosis is generally believed to be the result of an aberrant wound-healing response to sequential lung injury with poor prognosis (Gross and Hunninghake 2001, Verma and Slutsky 2007). Therefore, it has been regarded as an intractable disease around the world. As stated in the article by Li et al, long time acupuncture treatment had a positive effect on airway smooth muscle of chronic obstructive lungs (Li et al. 2016). In analog to acupuncture, moxibustion is a commonly used therapy in Traditional Chinese Medicine. Moxibustion uses the heat generated by burning herbal preparation moxa to stimulate acupoints. Several observations had reported that moxibustion was effective in anti-fibrosis, and animal studies revealed of benefit (Li et al. 2005, Paterno et al. 2008, Wang et al. 2013). However, the precise molecular mechanism of moxibustion to treat pulmonary disease was not fully understood. Accumulating evidences were showing transforming growth factor- $\beta$  (TGF- $\beta$ ) and interferon- $\gamma$  (IFN- $\gamma$ ) are crucial to the pathological process of IPF (Ji et al. 2013, Russo et al. 2011, Verma and Slutsky 2007). Among the injury-responsive pulmonary cytokines, the transcription factor TGF- $\beta$ 1 acts as a critical regulator for tissue regeneration and immune responses by controlling pulmonary fibrosis. TGF- $\beta$  promotes fibroblasts to differentiate into myofibroblasts, stimulates ECM synthesis and inhibits ECM degradation (Ahn et al. 2011). On the other hand, IFN- $\gamma$  is the anti-fibrosis cytokine. Aberrant IFN- $\gamma$  expression is associated with a number of autoinflammatory and autoimmune diseases (Schoenborn and Wilson 2007). It could reduce collagen synthesis in fibroblasts and delay wound healing. Therefore, in an effort to better understand the mechanism of IPF, we conducted an experiment to investigate the effect of moxibustion on pulmonary fibrosis.

## Materials and Methods

Pulmonary fibrosis was induced by bleomycin A5 in Sprague-Dawley (SD) rats. Bleomycin-induced pulmonary fibrosis is a globally accepted animal model due to its high successful rate and close to the pathophysiology of human IPF (Fireman et al. 2001, Giri et al. 1980, Veninga et al. 1988). Rats in blank group (n=17), model group (n=15), moxibustion group (n=16), and prednisone group (n=15) were analyzed. The study was approved by the animal facility of Chengdu University of TCM (ethical approval number: 0411). The rats were half male and half female, three-month old, and body weight was  $180\pm 20$ g. Except the blank group, the other three groups received intratracheal injection of bleomycin A5 (5mg/kg), resulting in direct damage to the alveolar epithelial cells (Moore and Hogaboam 2008). Blank group injected saline and air. After six days' modeling, the moxibustion group received moxibustion at "Feishu (BL13)" as well as "Gaohuangshu (BL43)", once per day for continuous six days then followed by one day rest. Four consecutive weeks in terms of this routine have been executed. The acupoints were identified bilateral in rats, based on the instructions in *Experimental Acupuncture Science* (Li 2007) and *Traditional Chinese Veterinary Acupuncture and Moxibustion* (Chuan 1995). Vaseline was smeared to the acupoints and a slide of ginger was placed on the top. 5mg pyramid-shaped moxa wool were then applied to the acupoints. Treatment was terminated till the moxa went to the bottom. Repeat the above procedure for three times as one moxibustion treatment. The prednisone group received intragastric infusion of prednisone (5mg/kg) in the same way while the blank group and model group didn't receive any treatment. After the treatment, the rats were euthanized and the lung samples were dissected for Hematoxylin and Eosin (H.E.) staining as well as TGF- $\beta$ 1 and IFN- $\gamma$  cytokines detection.

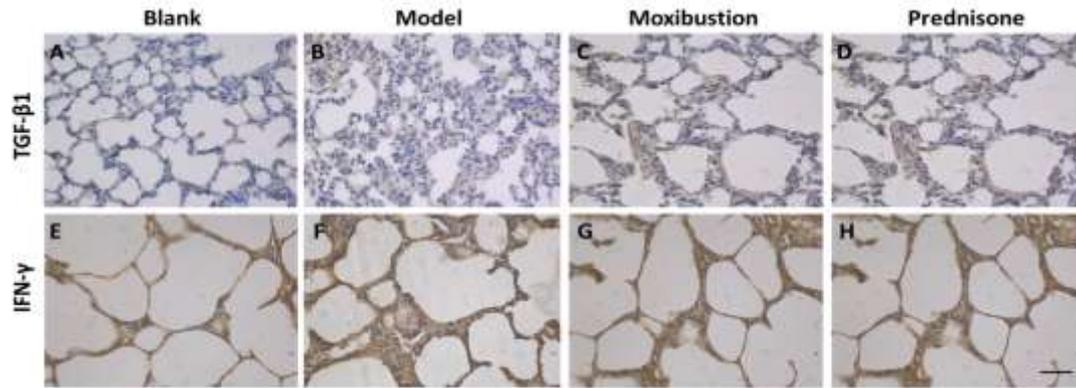
The protein and mRNA levels of TGF- $\beta$ 1 and IFN- $\gamma$  in lungs were detected by the StreptAvidin-Biotin Complex (SABC) staining and RNA in-situ hybridization (hybrid with known probes), respectively. Eight lung specimens were randomly selected for each rat. The expression location and levels were analyzed. The grade of alveolitis and fibrosis was determined by the Szapiel et al. scoring method (Szapiel et al. 1979). Alveolitis was divided into four degrees in H.E. staining: no alveolitis (-); mild alveolitis (+): alveolar walls got wider due to cell infiltration, the lesion range was limited to 20% of the whole lung; moderate alveolitis (++) : the lesion range was 20%~50% of the whole lung; severe alveolitis (+++): diffused lesions in the whole lung, and the range was more than 50%. The pulmonary interstitial fibrosis was categorized into four degrees: no fibrosis (-); mild fibrosis (+): the lesion range was limited to 20%; moderate fibrosis (++) : 20%~50% whole lung involvement with alveolar structure disorder; severe fibrosis (+++): more than 50% involvement of alveolar fusion and lung parenchyma structure disorder. The protein and mRNA expression images were quantified by Leica image analysis software. The average gray value was inversely linked to the stain intensity. The average gray can be divided into 256 grades. The blackest is 0 grade and the brightest is 256 grade.

Independent-sample t-test was used on numerical variables. ANOVA was applied for comparisons of data among each group. Chi-square test was used on categorical variables. Two-sided test was applied on all available data.

## Results

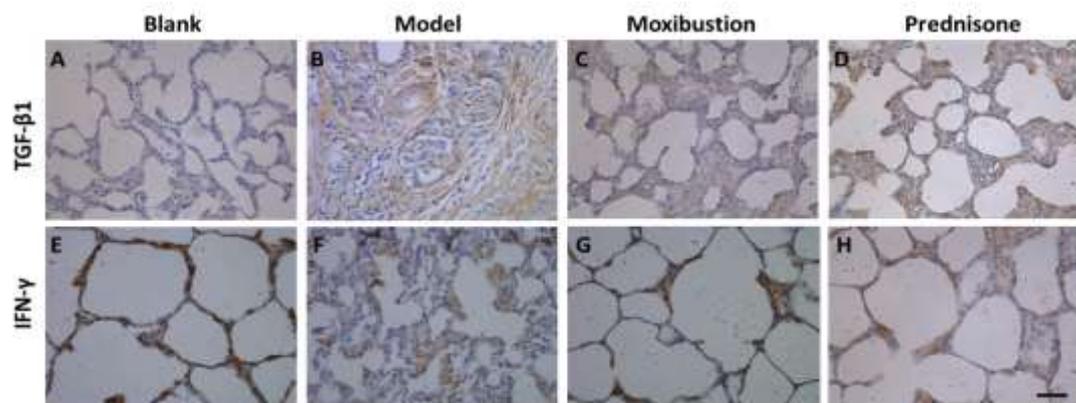
The severity of the inflammatory responses was evaluated according to the H.E. staining. The blank control group showed no significant inflammatory changes. Whereas, the model group had mild or moderate pulmonary alveolitis as well as moderate to severe interstitial fibrosis. The moxibustion group showed moderate pulmonary fibrosis and some lymphocytic infiltration, and there were limited pathological changes compared to the model group. The prednisone group showed comparable pulmonary response to that of moxibustion group (Figure S1). It seems moxibustion can relieve traumatic pulmonary alveolitis and inhibit pulmonary interstitial fibrosis, and the effect was as good as prednisone. The comparison of alveolitis and fibrosis grades in each group was shown in Table S1. The moxibustion and prednisone groups had less severe alveolitis comparing to the model group, and the differences were statistically significant ( $P<0.05$ ). For fibrosis, the moxibustion and prednisone groups showed alleviated physical signs compared to the model group ( $P<0.01$ ). The moxibustion group showed comparable alveolitis and fibrosis grades to that of prednisone group ( $P>0.05$ ).

In terms of TGF- $\beta$ 1 and IFN- $\gamma$  detection, the positive protein and mRNA staining exhibited as fine brown particles or brown mesh networking in the lungs. The blank control rats had clear alveolar macrophages, interstitial connective tissues, vascular endothelium and smooth muscle cells. And only some positive cells could be found (Figure 1 A, E; Figure 2 A, E). In the model group, TGF- $\beta$ 1 was significantly increased and IFN- $\gamma$  was significantly decreased at both protein and mRNA levels in comparison to the blank group (Figure 1 B, F; Figure 2 B, F). TGF- $\beta$ 1 was decreased and IFN- $\gamma$  was increased in moxibustion and prednisone groups at both protein (Figure 1 C,D,G,H) and mRNA (Figure 2 C,D,G,H) levels compared with the model group.



**Figure 1:** SABC immunohistochemistry staining of TGF- $\beta$ 1 and IFN- $\gamma$  protein ( $\times 400$ ). (A) and (E) illustrated the rats in blank group; (B) and (F) indicated the model group; (C) and (G) showed the moxibustion group; (D) and (H) represented the prednisone group. Scar bar: 50 $\mu$ m.

The qualification of TGF- $\beta$ 1 and IFN- $\gamma$  protein and mRNA expressions were shown in Table S2 and Table S3, respectively. The TGF- $\beta$ 1 protein average gray in the model group was significantly decreased compared to that in the blank rats ( $P < 0.01$ , Table S2 a). Moxibustion and prednisone groups exhibited significantly elevated average grays when compared with the model group, suggesting they could decrease the TGF- $\beta$ 1 protein expression ( $P < 0.05$ , Table S2 b). The TGF- $\beta$ 1 protein level in moxibustion group was as much as that in the prednisone group ( $P > 0.05$ , Table S2 c). IFN- $\gamma$  protein average gray was increased in the model group, but showed no statistical difference comparing to the blank group ( $P > 0.05$ , Table S2 d). Moxibustion and prednisone group had significantly low average gray of IFN- $\gamma$ , indicating they can increase IFN- $\gamma$  proteins ( $P < 0.01$ , Table S2 e). In further, moxibustion showed more decreased IFN- $\gamma$  protein average gray, suggesting moxibustion was more effective in increasing IFN- $\gamma$  protein than prednisone ( $P < 0.05$ , Table S2 f). In Table S3, TGF- $\beta$ 1 mRNA average gray was significantly decreased in the model group ( $P < 0.05$ , Table S3 a). Moxibustion and prednisone groups had elevated average grays when compared with the model group, suggesting their significance in reducing TGF- $\beta$ 1 mRNA ( $P < 0.05$ , Table S3 b). The effect of moxibustion in lowering TGF- $\beta$ 1 mRNA was comparable to that of prednisone ( $P > 0.05$ , Table S3 c). IFN- $\gamma$  mRNA average gray was increased in the model group with no statistical difference compared to the blank group ( $P > 0.05$ , Table S3 d). Moxibustion and prednisone treatment showed lower average gray of IFN- $\gamma$ , indicating their effect in increasing IFN- $\gamma$  mRNA expression ( $P < 0.01$ , Table S3 e). Moxibustion had a lower IFN- $\gamma$  mRNA average gray, suggesting a better effect than prednisone in upregulating IFN- $\gamma$  mRNA ( $P < 0.05$ , Table S3 f).



**Figure 2:** In situ hybridization of TGF- $\beta$ 1 and IFN- $\gamma$  mRNA ( $\times 400$ ). (A) and (E) illustrated the rats in blank group; (B) and (F) indicated the model group; (C) and (G) showed the moxibustion group; (D) and (H) represented the prednisone group. Scar bar: 50  $\mu$ m.

## Discussion

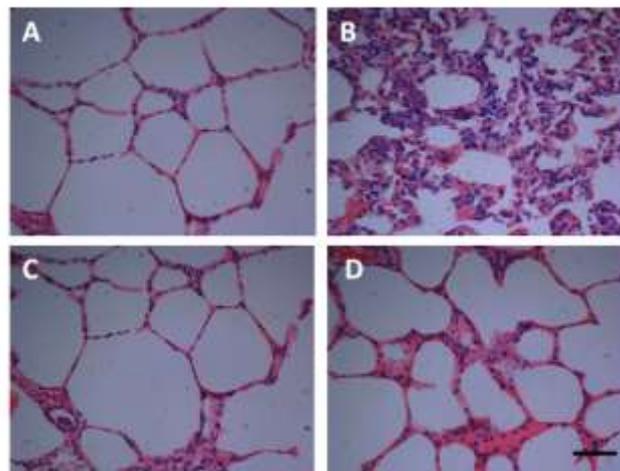
The results in the study was consistent with the previous studies, assuming that lowering TGF- $\beta$  and enhancing IFN- $\gamma$  could attenuate the disease of pulmonary fibrosis (Ji, Wang, Wei, Lu, Jiang, Xia and Dai 2013, Tulek et al. 2011). Although IFN- $\gamma$  was increased in the model group at both protein and mRNA levels, the difference was not statistically remarkable compared to that in the blank group. This may be explained by the short treating period. The treatment effect can be reflected by comparing moxibustion with the negative-control model group, and the positive-control prednisone group. Among the three groups, moxibustion could effectively reduce TGF- $\beta$ 1 and upregulate IFN- $\gamma$ , with comparable effect to prednisone in lowering TGF- $\beta$ 1 and better effect in increasing IFN- $\gamma$ . Therefore, it concludes that moxibustion

protects lung against fibrosis deterioration by downregulating TGF-β1 and upregulating IFN-γ. TGF-β1 and IFN-γ play some role in the pathogenesis of pulmonary fibrosis.

**Conclusions**

The study provides evidence that moxibustion is beneficial for pulmonary fibrosis by downregulating TGF-β1 and upregulating IFN-γ. The study concludes that moxibustion had a protective effect on pulmonary fibrosis. Moxibustion, as a therapeutic alternative treatment, could be used for pulmonary fibrosis.

**Supplementary Materials:** Figure S1: Post-treatment histopathological manifestations of lung tissues in different groups, Table S1: The grading of alveolitis and fibrosis in each group, Table S2: The TGF-β1 and IFN-γ protein expressions in bleomycin A5-induced pulmonary fibrosis rats, Table S3: The TGF-β1 and IFN-γ mRNA expressions in bleomycin A5-induced pulmonary fibrosis rats.



**Figure S1:** Post-treatment histopathological manifestations of lung tissues in different groups (H.E. staining, ×400). (A) No significant changes were observed in the blank control group. (B) Disrupted alveolar walls, big lung bubbles, wide alveolar septa, alveolar space infiltrate and lymphocytic infiltration were observed in the model group. (C) Moderate pulmonary fibrosis, a few lymphocytic infiltrations and limited range of pathological changes were observed in the moxibustion group. (D) Wide alveolar septa, interstitial edema, some necrosis and focal abscess, and cell infiltration at pulmonary alveolis were observed in the prednisone group.

**Table S1:** The grading of alveolitis and fibrosis in each group

Group	Numbers	Pathological Grades							
		Alveolitis -			Fibrosis -				
		-	+	++	+++	-	+	++	+++
Blank	17	17	0	0	0	17	0	0	0
Model	15	0	5	8	2	0	2	6	7
Moxibustion	16	4 <sup>a</sup>	8	2	2	0 <sup>b</sup>	7	6	3
Prednisone	15	4 <sup>a</sup>	7	3	1	0 <sup>b</sup>	8	4	3

a:  $P < 0.05$  vs model group in alveolitis, b:  $P < 0.01$  vs model group in fibrosis.

**Table S2:** The TGF-β1 and IFN-γ protein expressions in bleomycin A5-induced pulmonary fibrosis rats ( $\bar{X} \pm S$ )

Group	TGF-β1 protein			IFN-γ protein	
	N	TGF-β1(average gray)	n	IFN-γ(average gray)	
Blank	9	147.47±8.13	10	136.09±2.13	
Model	7	120.07±5.01 <sup>a</sup>	10	139.34±2.30 <sup>d</sup>	
Moxibustion	8	137.39±5.67 <sup>b</sup>	10	127.24±2.25 <sup>e</sup>	
Prednisone	7	136.82±3.21 <sup>b,c</sup>	10	129.24±2.15 <sup>e,f</sup>	

a:  $P < 0.01$  vs blank group, b:  $P < 0.05$  vs model group, c:  $P > 0.05$  vs moxibustion group; d:  $P > 0.05$  vs blank group, e:  $P < 0.01$  vs model group, f:  $P < 0.05$  vs moxibustion group.

Table S3: The TGF- $\beta$ 1 and IFN- $\gamma$  mRNA expressions in bleomycin A5-induced pulmonary fibrosis rats ( $\bar{X} \pm S$ )

Group	TGF- $\beta$ 1 mRNA		IFN- $\gamma$ mRNA	
	n	TGF- $\beta$ 1 mRNA(average gray)	n	IFN- $\gamma$ mRNA (average gray)
Blank	9	126.31 $\pm$ 2.58	10	127.67 $\pm$ 2.42
Model	7	107.71 $\pm$ 19.23 <sup>a</sup>	10	129.45 $\pm$ 2.00 <sup>d</sup>
Moxibustion	8	120.89 $\pm$ 9.50 <sup>b</sup>	10	119.85 $\pm$ 2.63 <sup>e</sup>
Prednisone	7	122.02 $\pm$ 7.49 <sup>b,c</sup>	10	120.79 $\pm$ 1.97 <sup>e, f</sup>

a:  $P < 0.05$  vs blank group, b:  $P < 0.05$  vs model group, c:  $P > 0.05$  vs moxibustion group; d:  $P > 0.05$  vs blank group, e:  $P < 0.01$  vs model groups, f:  $P < 0.05$  vs moxibustion group.

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**Author Contributions:** L.C., R.L., and X.L. conceived and designed the experiments; M.Z., F.L., Q.C., and C.L. performed lab experiments, analyzed the data; L.C. wrote the paper. All the above authors contributed to the final paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

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