Prevention of Bleomycin-induced Pulmonary Fibrosis in Mice by the Combination of *Hirsutella sinensis* and *Panax notoginseng* Extracts

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Abstract: **Objective** *Hirsutella sinensis* (HS) is the anmorph of *Ophiocordyceps sinensis* (*Cordyceps sinensis*). *O. sinensis* and *Panax notoginseng* are two popular Chinese herbs, commonly used in traditional Chinese prescriptions for the treatment of various diseases. A combination of HS extract with *P. notoginseng* saponin (PNS) extract demonstrated more prominent lung-protective activity than the two herbs individually used in our preliminary studies. This study further investigated the action of their combination (HSPNS) on anti pulmonary fibrosis using a Bleomycin (BLM) induced mouse model. **Methods** BLM-treated Kunming mice was given HSPNS daily for 7, 14 or 28 d *via* ig administration. After treatment, following parameters were monitored using proper methods, respectively. Lung index, serum and lung malondialdehyde (MDA) and hydroxyproline (HYP) contents, lung superoxide dismutase (SOD) activity, transforming growth factor β1 (TGF-β1), and expression levels of collagen Ⅰ (Col-Ⅰ) and collagen Ⅲ (Col-Ⅲ). The lung biopsies were also dissected for semiquantitative histological analysis. **Results** The results indicated that HSPNS significantly reduced lung index, MDA and HYP contents, and expression levels of TGF-β1, Col-Ⅰ, and Col-Ⅲ. The combination also remarkably enhanced SOD activity compared with BLM-induced group. Moreover, the severe pulmonary fibrosis histopathological changes induced by BLM could be attenuated by HSPNS treatment. **Conclusion** These results suggest that HSPNS could significantly inhibit the progression of pulmonary fibrosis induced by BLM and its inhibitory effect might associate with its ability to scavenge free radicals, decrease TGF-β1 level, and inhibit collagen synthesis.

Key words: Bleomycin; *Hirsutella sinensis*; hydroxyproline; *Panax notoginseng*; pulmonary fibrosis; transforming growth factor-β1

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Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disease (ILD) with unknown etiopathogenesis. It is clinically characterized by lung volume reduction, relentless dyspnea, gas exchange impairment, and a histological pattern transformation of usual interstitial pneumonia on surgical lung biopsy (Verma and Slutsky, 2007; Sharma and Chan, 1999). The survival time varies considerably among different individuals, but the mean survival time is around 3–5 years (Khalil and O'Connor, 2004). A growing body of evidences demonstrates that IPF is a chronic inflamematory disease, occurring in response to an unknown stimulus, and if it keeps untreated, it will lead to progressive lung injury and ultimately fibrosis (Noble and Homer, 2005). Efforts have been made to search for effective anti-fibrotic agents but results have no avail. Therefore, the prevention of pulmonary fibrosis was a

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very significant both in theory and in practice.

Chinese materia medica (CMM), directed under the traditional Chinese medicines (TCM) theory, has been developed for thousands of years and many of them are proved to be effective for the treatment of lung diseases. In recent years, a number of studies have shown that some CMM have protective effects on experimental pulmonary fibrosis in animal models. These CMM include Feitai (Gong *et al*, 2004; Gong *et al*, 2005), DSQRL (Zhang *et al*, 2007; Zhang *et al*, 2008), *Fufang Biejia Fang* (Kong *et al*, 2005), *Huqiyin* (Zhou *et al*, 2007), curcumin (Xu *et al*, 2007), *Ginkgo biloba* L. (Iraz *et al*, 2006), total salvianolic acid (Lin *et al*, 2008), etc. The CMM used in our experiment consists of *Hirsutella sinensis* (HS) water extract and *Panax notoginseng* (Burk.) F. H. Chen saponin extract (PNS). HS is the anmorph of *Ophiocordyceps sinensis* (previous name as *Cordyceps sinensis*). *O. sinensis* as a traditional Chinese herb, is a time-honored herb in many East Asian cultures. Studies have shown that its various pharmacological activities, such as modulation of immune response, inhibition of tumor growth, decrease of blood pressure, increase of hepatic energy metabolism and blood flow, improvement of bioenergy in the liver, and secretion of adrenal hormone (Hsu *et al*, 2003). PNS, a principal ingredient extracted from the traditional Chinese herbal medicine *P. notoginseng*, has obvious effects on stopping haemorrhages, influencing blood circulation, and acting as a tonic agent (Wang *et al*, 2008; Wang *et al*, 2006). *In vivo* studies in our laboratory demonstrated that both HS and PNS had the anti-fibrotic effect (Wu *et al*, 2007; Cao *et al*, 2009). On TCM theory, it is a convention to combine different herbs in order to enhance a single effect without causing severe side effects (Nishiyama, Wang, and Saito, 1995). In this study, we tested a combination of HS and PNS (HSPNS) on anti-fibrotic activity and further explored its possible mechanisms using a Bleomycin (BLM)-induced pulmonary fibrosis of mice.

Materials and methods

Materials

HSPNS was provided by Shanghai Yang's Herb Institute (China). HPLC analysis revealed that HSPNS contain 0.64 mg/g of cordycepic adenosine, 23.9% cordycepic acid, 6.3% cordycepic polycose, 2.0% notoginsenoside R1, 7.2% ginsenoside Rg1, and 12.0% ginsenoside Rb1. BLM was purchased from Nippon Kayaku Co., Ltd. (Japan). Commercial kits used for determining superoxide dismutase (SOD), malondialdehyde (MDA), and hydroxyproline (HYP) were obtained from Nanjing Jiancheng Institute of Biotechnology (China). Immunohistochemistry kits for transforming growth factor-beta1 (TGF-β1), collagen I (Col-I) and collagen Ⅲ (Col-Ⅲ) detection were obtained from Wuhan Boster Biological Technology Ltd. (China). Prednisolone Acetate Tablets were purchased from Tianjin Pharmaceutical Jiaozuo Co., Ltd. Other chemicals used in the experiment were of analytical grade from commercial sources.

Animals and treatment

One hundred and forty-four male and female (1 : 1) Kunming mice weighing (18–22) g, were provided by Animal Breeding Center of Soochow University (Suzhou, China). All animals were housed in conventional cages under control conditions of temperature (23 ± 3) ℃, relative humidity 50% ± 20%, and illumination cycles (12 / 12 h light-dark cycle). All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Institute of Clinical Pharmacology, Soochow University. The animals were allowed free access to food and water throughout the acclimatization and experimental periods.

The procedures for preparing BLM-induced pulmonary fibrosis of mice were followed by the method described previously with some modifications (Gong *et al*, 2004; Gong *et al*, 2005). The mice were randomly divided into six groups: control group, BLM group, BLM and HSPNS (600, 300, and 150 mg/kg) groups, and BLM and Prednisolone (6.67 mg/kg) group. All groups except the control group were intratracheally injected with 5 mg/kg Bleomycin hydrochloride to induce pulmonary fibrosis. Control animals received the same volume of intratracheal saline instead. One day after BLM treatment, HSPNS (600, 300, and 150 mg/kg) or Prednisolone (6.67 mg/kg) were ig administrated for 7, 14 or 28 d. The control group was administered with the same volume of vehicle.

On the d 7, 14, and 28 after drug treatment, mice were anesthetized and sacrificed, samples of blood were drawn from the orbital vein and collected in

polyethylene tubes. The serum samples were obtained by centrifugation (3000 × *g*, 4 ℃) for 10 min and serum SOD and MDA were analyzed. After animals were killed, their lungs were immediately removed and weighed. The right lungs were kept in 4% formaldehyde solution for histological analyses including HE staining and immunohistochemical analysis. One portion of the left lung was used for biochemical analysis and the rest portion was kept for HYP analysis. Lung index was calculated as lung weight divided by body weight.

Biochemical analysis

Serum SOD, MDA and lung HYP, SOD, MDA were analyzed using biochemical kits and with a 722 spectrophotometer (Shanghai Precision Scientific Instrument Co., Ltd., China).

Histological score

After being fixed in 4% formaldehyde solution for 48 h, the blocks of mice lung tissues were dehydrated and embedded in paraffin, cut into 5 μm slices, incubated at 60 ℃ overnight, dewaxed and finally stained with hematoxylineosin. The degree of lung alveolitis and lung fibrosis was categorized into four groups: Grade 0: no lung alveolitis or fibrosis; Grade 1: lung alveolitis area or fibrosis area < 20%; Grade 2: lung alveolitis area or fibrosis area > 20% and < 50%; Grade 3: lung alveolitis area or fibrosis area > 50% (Szapiel *et al*, 1979).

Immunohistochemical analysis of TGF-β1, Col-Ⅰ, and Col-Ⅲ

Slices for immunohistochemical analysis were treated at 58 ℃, dewaxed, rehydrated, and equilibrated in Tris buffered saline (TBS) containing 0.3% Triton X-100. Endogenous peroxide activity was blocked using 0.3% H_2O_2 in methanol for 30 min. Then slices were treated with 0.1% trypsin in 0.05 mol/L Tris, 0.02 mol/L $CaCl_2$ (pH 8.0). Non-specific binding was eliminated by blocking with 1.5% normal goat serum in TBS with 0.5% bovine serum albumin (BSA). Subsequently, slices were incubated overnight at 4 ℃ with primary antibodies (rabbit polyclonal IgG to mouse TGF-β1, Col-Ⅰ, and Col-Ⅲ) in TBS with 0.5% BSA, followed by incubation with secondary antibodies (goat anti-rabbit IgG-HRP for TGF-β1, Col-Ⅰ, and Col-Ⅲ) in TBS with 0.5% BSA, and then incubated with streptavidin-biotin complex (SABC) at 25 ℃ for 20 min. Immunoreactivity was detected by addition of diaminobenzidine (DAB). The sections were counterstained with Gill's haematoxylin, dehydrated and mounted with Permont. In this study, a negative control in which primary antibody was not added in the above incubation was used to verify the results of immunohistochemical analyses. The expression of TGF-β1, Col-Ⅰ, and Col-Ⅲ was assessed by quantitative image analysis system. Briefly, ten images were obtained from each lung sample slice using the 100 × microscope objective. For each image, ImageProPlus (IPP, Version 6.0) was used to measure the integrated optical density (IOD) of the image.

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and expressed as mean ± SD. Significant difference between groups was detected by Duncan's multiple range test using SPSS 12.0 software. Student's *t*-test was used for comparison between two groups. $P < 0.05$ indicated a statistically significant difference.

Results

Effect of HSPNS on pulmonary index

A significant increase of pulmonary index was recorded in BLM-treated animals after the d 7, 14, and 28. HSPNS (600, 300, and 150 mg/kg) treatment attenuated the increase of pulmonary index after BLM administration, the effect was the same as Prednisolone did (Table 1).

Table 1 Effect of HSPNS on pulmonary indexes ($\bar{X} \pm s$, $n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	7.330±1.171	6.862±0.628	6.382±0.831
Model	—	10.50±2.258$^{\#\#}$	10.450±2.399$^{\#\#}$	10.388±2.247$^{\#\#}$
HSPNS	600	7.993±1.656**	8.088±1.003*	7.005±0.803**
	300	8.378±1.021**	8.049±0.970*	6.727±1.569**
	150	8.456±1.279**	7.927±1.419 **	7.225±1.528**
Predni-solone	6.67	8.210±0.464**	7.770±1.057**	6.640±0.687**

$^{\#\#}P < 0.01$ *vs* control; $*P < 0.05$ $**P < 0.01$ *vs* model

Effect of HSPNS on lung HYP content

Compared with normal control group, HYP level in BLM-treated group was elevated after 14 d and reached the highest on the d 28 (Table 2). After treated

with HSPNS (600, 300, and 150 mg/kg), HYP contents were all significantly reduced on the day 28, and were 25%, 25%, and 27.3% less, respectively, compared with those in BLM group. However, Prednisolone at a dosage of 6.67 mg/kg failed to show such effect.

Table 2 Effect of HSPNS on lung HYP content ($\bar{X} \pm s$, $n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	0.55±0.06	0.48±0.23	0.63±0.09
Model	—	0.73±0.11	0.66±0.10$^{\#\#}$	0.88±0.03$^{\#\#}$
HSPNS	600	0.62±0.09	0.58±0.07	0.64±0.08**
	300	0.66±0.14	0.56±0.10*	0.66±0.14**
	150	0.59±0.15	0.57±0.08*	0.66±0.06**
Prednisolone	6.67	0.80±0.06	0.56±0.06*	0.86±0.06

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

Effect of HSPNS on serum and lung SOD and MDA

The depletion of SOD activity in the tissue or serum indirectly reflects generation of free radicals produced by BLM administration. Therefore, the serum and lung tissue SOD and MDA activities were measured in order to investigate whether HSPNS treatment could have effect on the redox state. As shown in Table 3, SOD activities in the lung tissue on the d 7, 14, and 28 were significantly decreased from the control value of (175.01 ± 18.22), (164.37 ± 20.75), and (163.61 ± 8.14) U/mg protein to (110.62 ± 11.10), (111.71 ± 15.24), and (112.11 ± 10.14) U/mg, respectively. The SOD activities in serum exhibited a similar tendency as that in lung tissue (Table 4). These data demonstrated that HSPNS could significantly prevent the depletion of SOD activity as Prednisolone did.

The MDA is an end-product and its level is an index of lipid peroxidation for many organs. As shown

Table 3 Effects of HSPNS on lung SOD activity ($\bar{X} \pm s$, $n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	175.01±18.22	164.37±20.75	163.61±8.14
Model	—	110.62±11.10$^{\#\#}$	111.71±15.24$^{\#\#}$	112.11±10.14$^{\#\#}$
HSPNS	600	132.28±19.52*	129.26±13.34*	126.66±16.66
	300	132.37±19.61**	134.20±13.36**	139.85±20.30**
	150	129.93±9.09*	129.58±16.79*	142.36±22.61**
Predni-solone	6.67	127.28±18.06*	112.85±15.50	121.03±17.85

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

Table 4 Effects of HSPNS on SOD activity ($\bar{X} \pm s$, $n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	230.92±27.91	245.58±18.61	251.10±33.04
Model	—	163.38±14.46$^{\#\#}$	193.61±18.79$^{\#\#}$	223.27±19.33
HSPNS	600	204.44±11.08	197.86±11.91	239.47±35.68
	300	222.41±17.38**	231.18±19.87**	259.40±26.80*
	150	215.18±18.58*	209.49±23.20	251.28±32.70
Predni-solone	6.67	190.96±31.29	200. 81±20.72	223.60±50.92

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

in Table 5, the lung MDA levels of the BLM model group were significantly higher than those in the control on the d 7 and 14. HSPNS at two lower dosages showed a similar effect as Prednisolone did, which significantly reduced the BLM-induced lipid peroxidation on the d 7 or 14. However, such effect was not observed on the d 28 and 14 in the highest dose group. Similar results were observed in serum samples (Table 6).

Table 5 Effects of HSPNS on tissue MDA level ($\bar{X} \pm s$, $n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	6.84±0.82	6.60±1.38	6.91±1.03
Model	—	9.76±0.82$^{\#\#}$	10.27±2.79$^{\#\#}$	8.27±1.44
HSPNS	600	7.58±0.45**	8.64±1.86	8.51±1.73
	300	7.09±0.64**	7.41±1.84**	7.57±1.38
	150	7.71±0.89**	7.74±2.60*	8.31±1.57
Prednisolone	6.67	6.98±0.75**	7.84±1.31*	8.14±1.27

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

Table 6 Effects of HSPNS on serum MDA level ($\bar{X} \pm s$, $n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	2.41±0.85	2.82±1.46	2.93±0.70
Model	—	5.48±0.75$^{\#\#}$	6.88±2.25$^{\#\#}$	3.79±0.74
HSPNS	600	3.01±0.46**	4.07±2.52**	3.28±0.64
	300	3.80±1.28*	4.02±0.88**	2.94±1.08
	150	3.97±1.82	3.43±0.29**	3.43±0.85
Prednisolone	6.67	4.03±0.48	4.20±1.14*	3.81±1.12

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

Effect of HSPNS on lung histological examination

Tables 7 and 8 and Fig. 1 show the HE stained lung histopathologic sections of BLM treatment, control, and HSPNS treatment groups. Under the photomicroscope, no pathological structural variation in the control group was observed, while BLM treatment

Table 7 Effects of HSPNS on the degree of alveolitis of lung induced by BLM ($\bar{X} \pm s, n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	0.375±0.518	0.25±0.463	0.125±0.354
Model	—	2.375±0.518$^{\#\#}$	2.25±0.707$^{\#\#}$	1.25±0.463$^{\#\#}$
Feiteling I	600	0.250±0.463**	0.625±0.518**	0.625±0.518
	300	1.375±0.518**	1.125±0.354**	0.375±0.518
	150	1.25±0.463**	1.375±0.518**	0.250±0.46*
Prednisolone	6.67	1.25±0. 463**	1. 5±0.535**	0.50±0.535

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

Table 8 Effects of HSPNS on lung fibrosis degree induced by BLM ($\bar{X} \pm s, n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	0.125±0.354	0.25±0.463	0.125±0.354
Model	—	1.625±0.518$^{\#\#}$	1.75±0.707$^{\#\#}$	1.75±0.707$^{\#\#}$
HSPNS	600	1.00±0.00	0.375±0.518**	1.00±0.756*
	300	1.00±0.00	0.500±0.756**	0.75±0.886**
	150	1.25±0.463	0. 500±0.756**	0.875±0.641*
Predni-solone	6.67	1.25±0. 641	0. 5±0.535**	1.00±1.069*

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

group exhibited severe histopathological changes, such as collapsed and thickened alveolar spaces and lung inflammatory infiltration. Prednisolone group reduced histological degeneration and HSPNS also prevented such histopathological changes induced by BLM.

Effect of HSPNS on TGF-β1, Col-Ⅰ, and Col-Ⅲ production

Since the pulmonary fibrosis induced by BLM is predominantly driven by TGF-β1, the protein levels of TGF-β1 in lung were analyzed and showed in Table 9. As expected, treatment with BLM significantly elevated the expressions of TGF-β1 protein compared with the control group, and could be reduced by Prednisolone treatment. Such attenuative effects were also observed in HSPNS group at all three dosages on the d 7 and 14, but not on the d 28 (Table 9). Similarly, the expression of lung Col-Ⅰ and Col-Ⅲ were significantly decreased by HSPNS and Prednisolone treatment compared to the model group (as shown in Tables 10 and 11). These results demonstrated HSPNS at all three doses protected mice against BLM-induced overexpression of the two proteins in lungs as Prednisolone did.

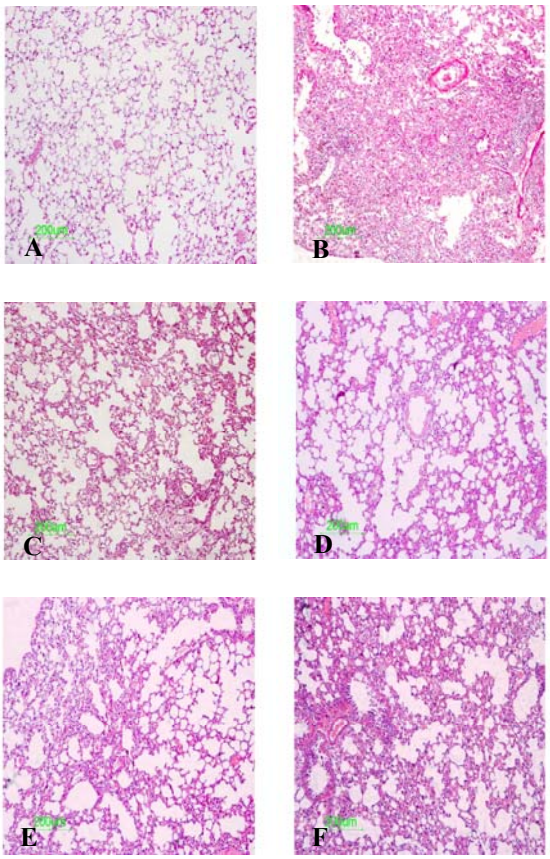

Fig. 1 Effect of HSPNS on lung histopathologic changes induced by BLM

A: normal group, saline + water; B: BLM + water; C–E: BLM + HSPNS at doses of 600, 300, and 150 mg·kg^{-1} per day, respectively; F: BLM + prednisolone at a dose of 6.67 mg·kg^{-1} per day; Tissues were sampled on the d 28 after BLM treatment. All panels were stained with HE and were shown at the same magnification

Table 9 Effects of HSPNS on the expression of TGF-β1 IOD in lung tissue ($\bar{X} \pm s, n = 8$)

Group	Dose / (mg·kg^{-1})	d 7	d 14	d 28
Control	—	265.63±59.61	252.25±56.65	272.00±51.29
Model	—	423.25±52.70$^{\#\#}$	402.13±50.02$^{\#\#}$	331.13±56.82$^{\#}$
HSPNS	600	306.00±41.39**	291.00±39.40**	284.63±38.43
	300	304.63±68.47**	289.38±64.95**	283.13±63.75
	150	320.38±76.55**	304.38±72.91**	298.00±71.03
Predni-solone	6.67	327.63±66.73*	311.25±63.28**	304.75±61.79

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

Discussion

IPF is a chronic inflammatory interstitial lung disease with unknown etiopathogenesis and pathogenesis (Dempsey, 2006). Several inflammatory mediators have been implicated in the pathogenesis. These mediators

Table 10 Effect of HSPNS on the expression of Col-Ⅰ IOD in lung tissue (*n* = 8)

Group	Dose/ (mg·kg^{-1})	d 7	d 14	d 28
Control	—	199.38±44.73	200.63±30.78	190.63±29.20
Model	—	305.00±45.92$^{\#\#}$	320.13±48.30$^{\#\#}$	339.50±51.29$^{\#\#}$
HSPNS	600	229.63±31.35**	240.88±32.34**	255.50±34.65**
	300	228.63±51.17**	239.88±54.05**	254.25±57.28**
	150	240.63±57.60**	252.13±60.26**	267.38±64.09 **
Predni-solone	6.67	245.75±50.03**	258.00±52.59**	273.50±55.79**

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

Table 11 Effects of HSPNS on the expression of Col-Ⅲ IOD in lung tissue (*n* = 8)

Group	Dose/ (mg·kg^{-1})	d 7	d 14	d 28
Control	—	290.13±45.79	296.00±46.55	293.13±46.29
Model	—	419.88±65.11$^{\#\#}$	432.63±66.93$^{\#\#}$	458.50±71.13$^{\#\#}$
HSPNS	600	357.50±48.12*	368.13±49.71*	390.25±52.86*
	300	355.88±80.20*	366.63±82.52*	388.75±87.45*
	150	360.50±80.97*	371.38±83.53*	393.88±88.69*
Predni-solone	6.67	361.75±68.17*	372.63±70.34*	394.88±74.59*

$^{\#\#}P < 0.01$ *vs* control; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* model

include cytokines, chemokines, growth factors, and reactive oxygen species (ROS) (Noble and Homer, 2004; Bhatt *et al*, 2006). An oxidant/antioxidant imbalance in the lower respiratory tract has been proposed as one of the mechanisms of the lung injury in IPF patients (MacNee and Rahman, 1995; Rahman and MacNee, 2000). The accumulation of inflammatory cells in lower respiratory tract, such as activated alveolar macrophages and neutrophils, will cause an increase of oxidant stress in lung (Crystal *et al*, 1984) and consequently result in a destruction of the protective antioxidant shield of epithelial lining fluid (ELF) (Carr and Winterbourn, 1997). The destruction may lead to the damages of alveolar epithelial cells and lung parenchyma which are hallmarks of interstitial fibrosis (Crystal *et al*, 1976). It is known that BLM is a potent oxygen free radical inducer which generates severe oxidant stress. Local administration of BLM produces fibrotic changes in lung parenchyma that are similar to those in IPF (Moeller *et al*, 2006). To diminish oxidants by antioxidants could block BLM-induced pulmonary fibrosis.

Under normal conditions, various antioxidant components in the lower respiratory tract are adequately to protect it from oxidative stress (MacNee and Rahman, 1995). However, evidences suggest that IPF patients lose the balance between oxidants and antioxidants. Their antioxidant levels as well as glutathione levels in ELF are decreased whereas they have elevated the levels of catalase, myeloperoxidase, inducible nitric oxide synthase (iNOS), and plasma superoxide dismutase, compared with that in normal subjects (Rahman and MacNee, 2000). Our study showed that activity of serum and lung SOD decreased in the BLM group on the d 7, 14, or 28. HSPNS upregulated the lowered SOD activity to different extents following 7, 14, or 28 days' treatment. In addition to SOD and level of MDA, one of the main lipid peroxidation products, is also an important index that could reflect the degrees of lipid peroxidation injury in pneumonocytes. The increased MDA in BLM group could be reversed by HSPNS treatment. These results imply that the primary mechanism of HSPNS on protecting lung fibrosis is associated with neutralization of lipid peroxidation.

TGF-β1 has multiple pathologic and physiologic functions in inflammation, immune reactions, tissue destruction, tissue remodelling, proliferation, differentiation, and healing/repairing (Evans *et al*, 2003). Many evidences implied TGF-β1 might take a role in the development and progression of pulmonary fibrosis. In lung, extracellular matrix deposition and induction of myofibroblasts are all the outcome of overexpression of TGF-β1, and consequently they promote transcription of Col-Ⅰ, fibronectin, and Col-Ⅲ in fibroblasts through an intricate signaling cascade (Foronjy *et al*, 2003; Sheppard, 2006). On the basis of these, we predicted that HSPNS would have downregulation effects on these molecules in BLM-induced pulmonary fibrosis mice. This prediction seems to be verified in our experiments. The abnormal elevated levels of TGF-β1, Col-Ⅰ, and Col-Ⅲ expression induced by BLM could be markedly downregulated by HSPNS. It implies that HSPNS also slows down fibrosis progression *via* inhibition of TGF-β1, Col-Ⅰ, and Col-Ⅲ expression. However, some results of our study did not show a good dose-dependent effect, which may be related to the complexity of Chinese herbal medicine, a limited number of experimental animals, and animal individual differences.

In summary, HSPNS has shown to possess significant beneficial effects in experimental studies of pulmonary fibrosis in mice and its mechanism may be related to its anti-oxidative and free radical-scavenging activity, inhibition of TGF-β1 signal transduction, and diminishment of collagen accumulation.

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