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Original Article

Protective Effect of Hydroxysafflor Yellow A on Inflammatory Injury in Chronic Obstructive Pulmonary Disease Rats*

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ABSTRACT **Objective**: To investigate the attenuating effect of Hydroxysafflor yellow A (HSYA) on inflammatory injury in chronic obstructive pulmonary disease (COPD). **Methods**: Rats were randomly assigned to 7 groups according to body weight including normal control group, HSYA blank group (76.8 mg/kg), COPD group, COPD+HSYA (30, 48, 76.8 mg/kg) groups and COPD+dexamethasone (2 mg/kg), 10 in each group. Passive cigarette smoke and intratracheal instillation of lipopolysaccharides were used to establish a COPD model in rats. Hematoxylin and eosin staining of lung tissue sections was used, real-time polymerase chain reaction (PCR) was used to assay mRNA levels of some cytokines in lung tissues, the cytokines in bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay (ELISA), Western blot analysis was used to determine phosphorylated p38 mitogen-activated protein kinase (MAPK) levels in lung tissues, and nuclear factor-κB (NF-κB) p65 protein levels in lung tissues were detected by immunohistochemistry. **Results**: Lung alveolar septa destruction, alveolus fusion, inflammatory cell infiltration, and bronchiole exudation were observed. These pathological changes were alleviated in the COPD+HSYA group. The mRNA expression of inflammatory factors were significantly increased in lung tissues from COPD rats (all $P<0.01$) and were inhibited by HSYA. Levels of inflammatory cytokines in BALF of COPD rats were significantly increased (all $P<0.01$) which were inhibited by HSYA (all $P<0.01$, 48, 76.8 mg/kg). The levels of p38 MAPK phosphorylation and p65 in lung tissues of COPD rats were significantly increased (all $P<0.01$) and were suppressed by HSYA (all $P<0.01$, 48, 76.8 mg/kg). **Conclusions**: HSYA could alleviate inflammatory cell infiltration and other pathological changes in the lungs of COPD rats. HSYA could inhibit inflammatory cytokine expression, and increase phosphorylation of p38 MAPK and NF-κB p65 in the lungs of COPD rats. The protective mechanism of HSYA to inhibit COPD inflammation might be by attenuating NF-κB and p38MAPK signal transduction.

KEYWORDS Hydroxysafflor yellow A, chronic obstructive pulmonary disease, inflammation, p38 mitogen-activated protein kinase, nuclear factor-κB

Chronic obstructive pulmonary disease (COPD), characterized by airflow limitation, is an irreversible disease. The air flow limitation of this disease is progressive and is associated with pulmonary inflammation injury.[1] COPD is a commonly and frequently encountered disease of the respiratory system and its prevalence in people over 40 years old is 8.2%.[2] COPD is one of the main diseases that increases mortality and economic burden. In recent years the prevalence of COPD has been increasing and it was predicted by the World Health Organization that it will become the third most common death-causing disease and a serious public health problem, causing a heavy financial burden by 2020.[3]

The pathology of COPD is unclear and currently it is widely accepted that the main pathogenetic mechanism is chronic inflammation in the airways, lung parenchyma and lung blood vessels.[2] In COPD, activated inflammatory cells secrete many inflammatory factors such as interleukin (IL)-6, IL-1β and tumor necrosis factor (TNF)-α,[4] which activate mitogen-activated protein kinase (MAPK) and nuclear factor-κB (NF-κB) signaling pathways to induce

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further inflammatory factor production. In COPD, increased growth factor expression initiates collagen and other extracellular matrix protein synthesis causing tissue remodeling and pulmonary fibrosis. This is an important feature of COPD.

The Chinese medicine *Carthamus tinctorius* L. has been used to activate blood circulation and dissipate blood stasis in China for over 2000 years. It is usually used as an aqueous extract in the clinic and Safflor Yellow (SY) is the effective component of *C. tinctorius* L.[5] and Hydroxysafflor yellow A (HSYA) is the main active ingredient of SY.[6] It was reported that HSYA inhibits pulmonary inflammation in rats or mice.[7] Wang, et al[8] reported that HSYA inhibited airway remodeling, but there has been no study of the protective effect of HSYA on chronic inflammation in COPD rats. Numerous studies have verified the effect of cigarette smoke exposure on responses to bacterial antigens, showing that smoke and lipopolysaccharide (LPS) can establish the COPD model to induce chronic inflammation and progressive airway obstruction and this model can be used to test therapeutic targets.[9] In this study, we investigated the inhibitory effect of HSYA on smoke and LPS induced chronic inflammatory injuries and related signal transduction in COPD rats to set the foundation for developing new treatments for COPD.

METHODS

Chemicals and Reagents

Safflower was identified by Prof. LI Jia-shi (Beijing University of Chinese Medicine) and was provided by Huahuikaide Pharmaceutical Co., Ltd. (Shanxi, China), and planted at Tacheng (Xinjiang Uygur Autonomous Region, China). HSYA was isolated and purified by macroporous resin-gel column chromatography from the aqueous extract of *Carthamus tinctorius* L. as previously described.[10] The molecular weight of HSYA is 612 (molecular structure is shown in Figure 1). HSYA was dissolved in sterile 0.9% NaCl for subsequent use. Zhongnanhai brand cigarettes, each containing 0.8 mg nicotine and 10 mg tar were obtained from the Beijing Tobacco Corporation in China. LPS (*Escherichia coli* O55:B5), was obtained from Sigma Chemicals (St. Louis, MO, USA) and it was dissolved in sterile 0.9% NaCl to make a stock solution. The LPS working solution was freshly prepared by dilution with normal saline before use. Dexamethasone (DXM) was obtained from the Tianjin Pharmaceutical Co., Ltd., China. Protein extraction kits and bicinchoninic acid (BCA) protein assay kits were obtained from the Beyotime Institute of Biotechnology (Jiangsu, China); TRIZOL reagent and M-MLV reverse transcriptase were obtained from Invitrogen Co. (Carlsbad, CA, USA); the SYBR® Premix Ex Taq (Perfect Real Time) kit was obtained from Takara Bio Inc. (Shiga, Japan). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from Shanghai ExCell Biology, Inc. (Shanghai, China); fluorescent-labeled secondary antibodies were obtained from LI-COR Biosciences company (Lincoln, NE, USA); rabbit anti rat NF-κB p65 multi-clone antibodies and p38 MAPK and phospho-p38 (Thr180/Tyr182) MAPK primary antibodies were obtained from Cell Signaling Technology (MA, USA). All other chemicals were analytical grade and were made in China.

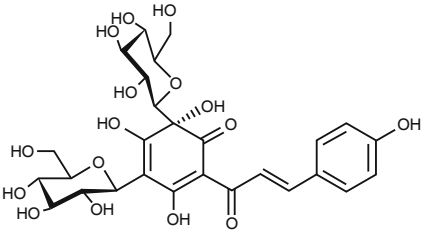

Figure 1. Molecular Structure of Hydroxysafflor Yellow A

High Performance Liquid Chromatographyanalysis of HSYA

High performance liquid chromatography (HPLC) analyses were performed with an Apollo C18 column (250 × 4.6 mm, 5 μm; Grace Davison) on a LC-10AT HPLC chromatographic system with an SPD-6AV UV detector (Shimadzu, Kyoto, Japan). The mobile phase consisted of acetonitrile (A) and 0.1% trifluoroacetic acid (B) at a flow rate of 1.0 mL/min. The gradient elution program was as follows: an initial 0 min at 1% solvent A, 99% solvent B; then from 0 to 50 min, solvent A was linearly increased from 1% to 35%, and solvent B was linearly decreased from 99% to 65%; from 50 to 60 min, solvent A was linearly increased from 35% to 45%, and solvent B was linearly decreased from 65% to 55%. The optical absorbance was monitored at 405 nm and the column temperature was 30 ℃. The purity of HSYA was quantitatively determined by the area normalization method. The purity of HSYA for this study was 95.9% (Figure 2).

Animals and Experimental Procedure

Specific pathogen free mature male Wistar

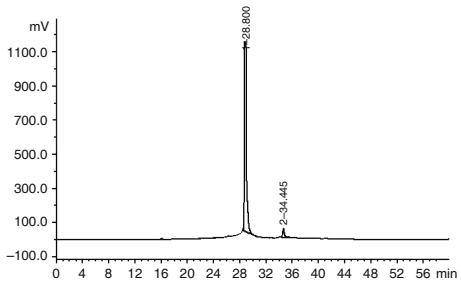

Figure 2. HPLC Analysis Result of HSYA

Note: The absorbance was measured at 405 nm and the 28 min peak is HSYA.

rats weighing from 160–180 g were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China; certificate No. 11400700013001). Animals were maintained in the animal department of Anzhen Hospital with controlled temperature (23 ± 2 ℃) and humidity (60% ± 10%), under a 12-h light/dark cycle. This study was approved by the Committee on the Ethics of Animal Experiments of Capital Medical University. All experimental procedures conformed to the Beijing Laboratory Animal Management Ordinance.

Animal Treatment and Experimental Groups

Passive cigarette smoke and intratracheal instillation of LPS was used to establish the COPD rat model in this study. Before the experiment, the rats were adaptively raised for 1 week. The rats were randomly assigned to 7 groups (*n*=10) according to body weight as follows: normal control group; HSYA blank group (76.8 mg/kg); COPD group; COPD+HSYA groups (30, 48, 76.8 mg/kg) and COPD+DXM (2 mg/kg) group. After being weighed, the rats were anesthetized with sodium pentobarbital (20 mg/kg). Rats in the COPD, COPD+HSYA and COPD+DXM groups received intratracheal instillation of LPS 200 μL (1,000 mg/L) twice, on the 1st and 14th day. Rats in the normal control group and the HSYA blank group received the same volume of sterile saline instead of LPS. Everyday except for the 1st and 14th day, rats in the COPD, COPD+HSYA and COPD+DXM groups were exposed to cigarette smoke in a smoking box (80 cm × 60 cm × 50 cm) connected to a vacuum pump. The smoke from 6 cigarettes was pumped into the box and was maintained for 60 min every day for 4 weeks, except for the 1st and 14th day. The carbon monoxide concentration in the box was measured with a CO detector (WT40, Weitai Technology Co., Ltd., Shanghai, China) and was maintained at 1000–1200 ppm. Rats in the COPD+HSYA and COPD+DXM groups were injected with HSYA or DXM (2 mg/kg) intraperitoneally daily except for the 1st and 14th day. Rats in the normal control group and the HSYA blank group were placed into the same box as for the other groups and exposed to fresh air instead of smoke for 60 min everyday for 4 weeks except the 1st and 14th day. Rats in the normal control group received an equal volume of sterilized saline and rats in the HSYA blank group received HSYA intraperitoneally (76.8 mg/kg) daily except for the 1st and 14th day. At the end of the experiment blood plasma was obtained from the abdominal aorta for transforming growth factor (TGF)-β1 assay and the left lobe of the lung was removed and fixed in 4% paraformaldehyde for histological and immunohistochemical observation. The right lung was snap-frozen in liquid nitrogen for RNA and protein sample preparation.

Histological Examination

The Lung samples were then sectioned at 5 μm and stained with hematoxylin and eosin (HE) or Masson's trichrome according to conventional methods. The slides were evaluated under a light microscope (Nikon Eclipse 90i) and histological analyses were performed blindly. Digital images at 100× magnification were captured from 5 randomly selected fields for each section, and positive areas were integrated by NIS-ELEMENTS quantitative automatic program (Nikon, Japan).

Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from frozen lung tissues using Trizol according to the manufacturer's instructions. RNA purity and concentration were assayed with a NanoDrop 2000 device (Thermo Scientific, Wilmington, DE, USA). The mRNAs were then reverse transcribed to cDNA using a reverse transcription-polymerase chain reaction (RT-PCR) kit with 2 μg RNA. The IL-6, IL-1β, TNF-α, intercellular cell adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 mRNA level of the target gene was quantified by real-time PCR using a SYBR® Premix Ex Taq kit on a Bio-Rad iCycler iQ5 Detection System. The sequences of the primer pairs and expected lengths (in bp) can be seen in Table 1. PCR amplification conditions were as follows: initial denaturation at 95 ℃ for 15 s followed by 40 cycles of denaturation at 95 ℃ for 5 s and annealing at 61 ℃ for 15 s. Relative mRNA level was calculated by the $2^{-\triangle\triangle Ct}$ method. All the results were normalized to the

Table 1. The Sequences of the Primers

mRNA	Sequence	Product/bp
β-actin	F: TGG CAT CCA CGA AAC TAC CT	186
	R: TCA GGA GGA GCA ATG ATC TTG	
IL-6	F: GCT CTG GTC TTC TGG AGT TCC	236
	R: GAG TTG GAT GGT CTT GGT CCT	
IL-1β	F: ACA AGG AGA GAC AAG CAA CGA	243
	R: TCT GCT TGA GAG GTG CTG ATG	
TNF-α	F: GCC AAT GGC ATG GAT CTC AA	307
	R: ACT TGG GCA GGT TGA CCT CA	
ICAM-1	F: TCC GGT AGA CAC AAG CAA GAG	239
	R: AGA AGC CCA AAC CCG TAT GA	
VCAM-1	F: GGA TGC CGG AGT ATA CGA GTG	226
	R: CTT CTG TGC CTC CAC CAG ACT	

level of β-actin mRNA.

ELISA Analysis

The concentration of IL-6, IL-1 and TNF-α in bronchoalveolar lavage fluid (BALF) were measured by ELISA according to the instruction manual.

Western Blot Analysis

Frozen lung tissue was homogenized in lysis buffer in an ice bath and homogenates were centrifuged at 16,000×*g* for 15 min at 4 ℃ to remove cellular debris. Protein concentration was determined using the BCA method. Protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane using the wet transferring method. The membrane was first blocked with 5% nonfat dry milk in Tris-buffered saline-Tween (TBST) at room temperature for 1 h, and then hybridized with p38 MAPK and phospho-p38 MAPK primary antibodies followed by IR Dye-conjugated secondary antibody (1:5000) for 1 h. The membranes were scanned and analyzed using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Immunohistochemical Analysis

After deparaffinage with xylene and hydration with graded alcohol, the samples were incubated in citrate buffer (pH 6.0) at 96 ℃ for 20 min to retrieve the antigen. After being washed with phosphate buffered saline (PBS) 3 times, the samples were blocked with rabbit serum for 30 min. Then they were incubated with primary antibody against p65 (1:200) at 4 ℃ overnight. After washing with PBS, the samples were incubated in biotinylated rabbit anti-goat antibody for 60 min at 37 ℃. To verify the binding specificity, some sections were also incubated exclusively with a primary antibody or exclusively with a secondary antibody. There was no positive staining in these sections. Digital images at 100× magnification were captured from 5 randomly selected fields for each section, and positive areas were integrated by the NIS-ELEMENTS quantitative automatic program (Nikon).

Statistical Analysis

Data were presented as the mean±standard deviation ($\bar{x} \pm s$) and statistical analyses were performed using SPSS19.0 software. The results were analyzed by one-way analysis of variance (ANOVA), and LSD post hoc Multiple Comparisons. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

HSYA Protects against Pathological Changes of Lungs in COPD Rat

In the normal group, the left lungs was pinkish-red and soft. There was no apparent bleeding or edema. There was no significant difference in the left lungs between the HSYA blank group and the normal control group. In the COPD group, the color of the lungs was dark red with severe bleeding and the texture was flexile. These changes in the lungs of COPD rats were ameliorated in the COPD+HSYA and COPD+DXM groups but the protective effect was not significantly different from controls in the low dose HSYA group (Figure 3).

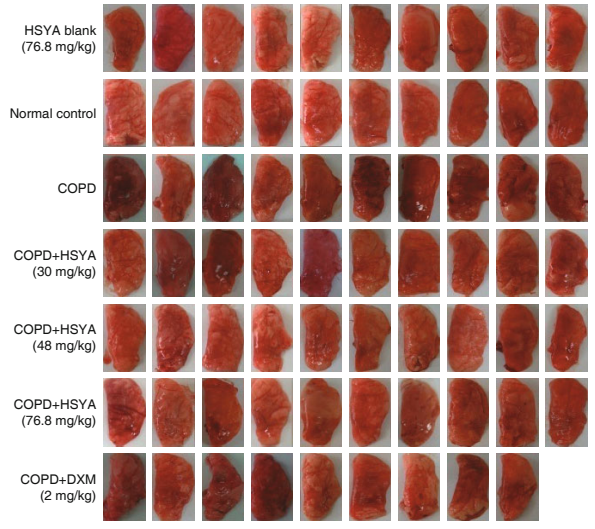

Figure 3. Protective Effect of HSYA on the Lungs of Every Rat in Each Group

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HSYA Reduces the Morphological Changes of Lungs in COPD Rats

Lung tissue from the HSYA blank group and normal control group rats displayed normal histological features (Figure 4). In the normal control group, pulmonary alveoli were clear and intact, with no inflammatory cell infiltration, and no secretion in the bronchioles. The COPD group exhibited severe inflammation with alveolar septa destruction, alveolus fusion, inflammatory cell infiltration, and bronchiole exudation. These injuries were attenuated in animals treated with DXM and HSYA, especially the medium and high dose HSYA treatment groups.

Inhibitory Effect of HSYA on Elevated IL-6, IL-1β, TNF-α, ICAM-1 and VCAM-1 mRNA Levels in COPD Rat Lungs

Figure 5 shows that IL-6, IL-1β, TNF-α, ICAM-1 and VCAM-1 mRNA levels in the lung increased in the COPD group compared with the normal control group (all $P<0.01$). The HSYA+COPD groups (especially the medium and high dose groups) and DXM group showed inhibition of the elevated mRNA levels ($P<0.05$ or $P<0.01$).

Inhibitory Effect of HSYA on Elevated IL-6, IL-1β and TNF-α Levels in the BALF of COPD Rats

IL-6, IL-1β and TNF-α levels in the BALF increased in the COPD group (all $P<0.01$) compared with normal control group and HSYA, especially the medium and high dose or DXM, inhibited this elevation ($P<0.01$, Figure 5).

Attenuating Effect of HSYA on p38 MAPK Phosphorylation in the Lungs of COPD Rats

The p38 MAPK phosphorylation levels in the lungs of COPD rats was increased compared with the normal control group ($P<0.01$). The medium and high dose of HSYA or DXM inhibited the increased phosphorylation ($P<0.01$, Figure 6).

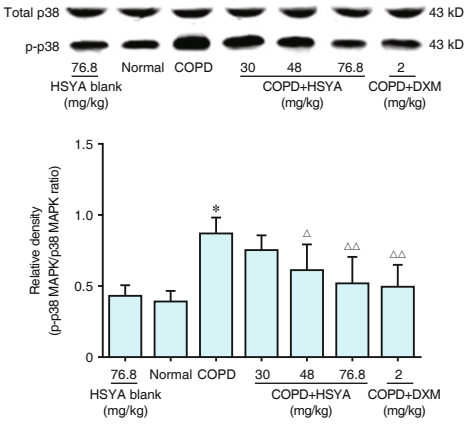

Figure 6. Effect of HSYA on p38 MAPK Phosphorylation in COPD Rats (n=10, $\bar{x}\pm s$)

Notes: $^{*}P<0.01$ *vs*. normal control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. COPD group

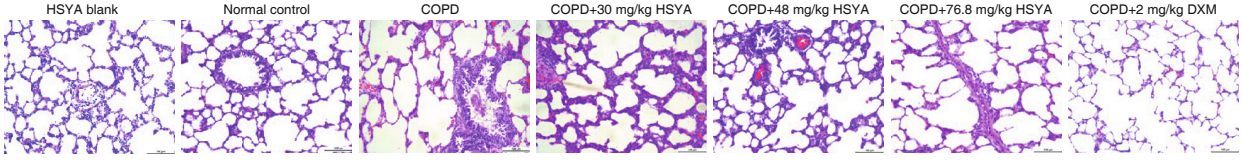

Figure 4. HSYA Protects against COPD Lung Pathology (HE staining, ×200)

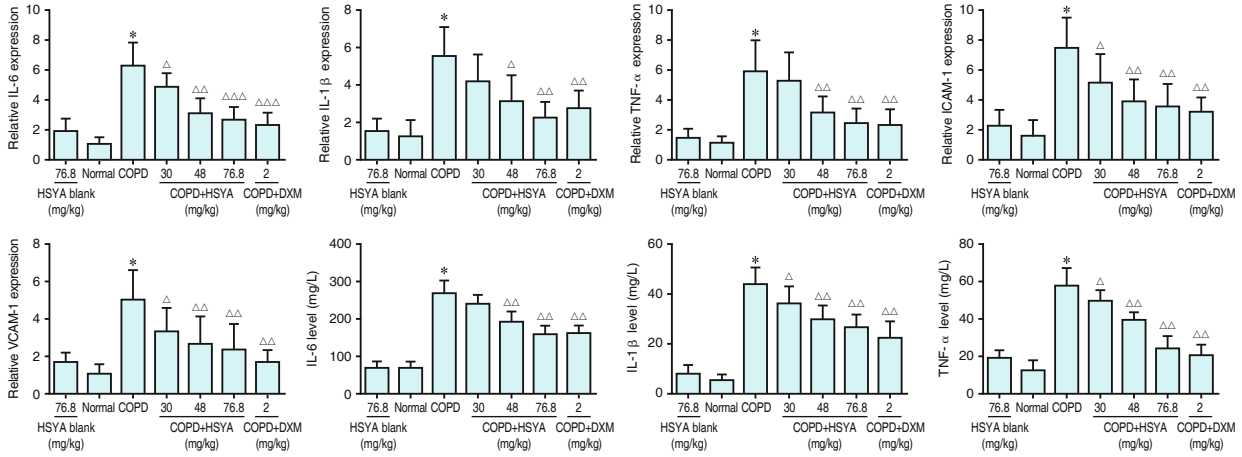

Figure 5. Effect of HSYA Treatment on Lung Tissue IL-6, IL-1β, TNF-α, ICAM-1, VCAM-1 mRNA (A-E) and Inflammatory Factor Protein in BALF (F-H) Expression in COPD Rats (n=10, $\bar{x}\pm s$)

Notes: $^{*}P<0.01$ *vs*. normal control group; $^{\triangle}P<0.05$, $^{\triangle\triangle}P<0.01$ *vs*. COPD group

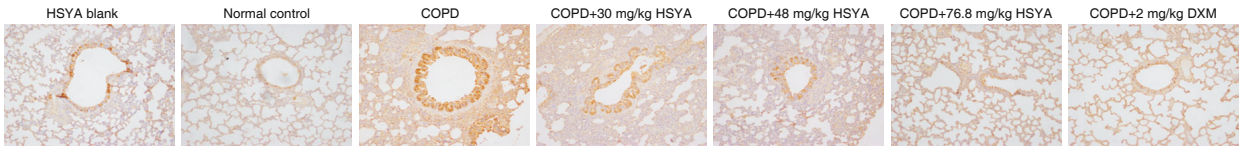

Figure 7. Effect of HSYA on the Level of NF-κB p65 (Immunohistochemical staining, ×100)

Attenuating Effect of HSYA on Elevated NF-κB p65 Levels in the Lungs of COPD Rats

P65 positive cells were rarely observed in the lungs of rats in the normal control group. In the COPD group, the rate of p65 positive cells was significantly increased. Compared with the normal control group, the NF-κB p65 level in the lungs of COPD rats increased (P<0.01). Treatment with HSYA reduced the rate of p65 positive cells, which was more apparent in the high dose HSYA group and the DXM group (P<0.05 or P<0.01, Figures 7 and 8).

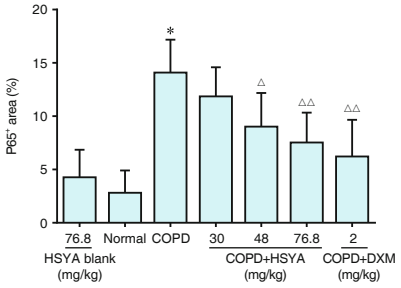

Figure 8. Analysis of p65 Immunohistochemical Staining

Notes: *P<0.01 *vs*. normal control group; △P<0.05, △△P<0.01 *vs*. COPD group

DISCUSSION

The pathogenesis of COPD is very complex and is still unclear, although it is associated with inflammation induced by deleterious gas and granules. Smoking-induced COPD is caused by pulmonary inflammation and lung destruction. This may damage normal defense and repair mechanisms leading to further damage. Lung injury includes increased mucus secretion, airway stenosis and fibrosis, emphysema and changes in the structure of blood vessels. These pathological changes can improve airflow limitations and other pathophysiological changes.[11] LPS is a component of the cell wall of Gram negative bacterium that exist in air pollutants.[12] When bacteria invades living organisms, LPS of their cell wall triggers monocytes, macrophages, epithelial cells and nuclear cells to release proinflammatory factors such as TNF-α and IL-1 that induce inflammatory reactions in the airways and lung tissues. In this study, we established a rat COPD model by smoke inhalation and LPS intratracheal instillation, similar to cases observed in the clinic.[13]

C. tinctorius L. is a Chinese medicine used for activating blood circulation to dissipate blood stasis. SY is the effective part of *C. tinctorius* L. and its inflammatory protective effect has been reported.[14] The current study showed that the IL-6, IL-1β, TNF-α, ICAM-1 and VCAM-1 mRNA levels of lungs in the COPD group were increased and that HSYA alleviated this dose dependently compared with the normal control group. BALF IL-6, IL-1β, and TNF-α levels were increased in the COPD group and were dose dependently attenuated by HSYA.

To explore the mechanism of increased inflammatory factor expression during COPD, we measured p38 MAPK phosphorylation and NF-κB p65 levels in the lungs. MAPK is an important transducer of inflammatory signals from the cell membrane to the nucleus and is activated by many inflammatory factors.[15,16] In this process, the tyrosine and threonine residues are phosphorylated to form activated p38 MAPK that phosphorylates serine/threonine residues of regulatory proteins to activate further inflammatory reactions. NF-κB is involved in the expression and regulation of many inflammatory factors. Promotors that encode inflammatory factors such as IL-6, IL-1β, TNF-α, ICAM-1 and VCAM-1 contain a NF-κB binding segment and the activation of NF-κB may induce their expression, which in turn trigger NF-κB activation further resulting in a vicious cycle of excessive inflammation that causes tissue damages. In this study, p38 MAPK phosphorylation and p65 levels were increased. IL-6, IL-1β, TNF-α, ICAM-1 and VCAM-1 mRNA levels in lung tissues and inflammatory factors in BALF were increased in the COPD group. These pathological changes in the COPD rats were dose-dependently alleviated by HSYA. Thus, the mechanism of how HSYA attenuates COPD may be by the inhibition of inflammatory signal transduction and inflammatory factor expression.

This study demonstrated that HSYA attenuated

COPD similar to DXM. DXM is currently used clinically to treat COPD but has many disadvantages. HSYA is the main active ingredient of SY, which has been used to treat coronary heart disease the last 10 years. SY (80–160 mg once a day) was reported to be effective with no obvious adverse reactions.[17] Therefore, SY might have advantages over the use of glucocorticoids clinically. Our findings may provide new drug targets for the treatment of COPD. We previously reported that the intraperitoneal injection of 16 mg/kg SY, which matches the dose used clinically, was effective for the treatment of acute lung injury(ALI) rats.[14] The content of HSYA in SY is >90% as measured by HPLC.[18] SY may be useful to treat COPD. In this study, rats were intraperitoneally injected to 60 mg/kg HSYA, which is beyond the routine clinical dose; therefore, its safety needs further investigation.

HSYA is effective at inhibiting increased p38 MAPK phosphorylation and NF-κB p65 levels, the high expression of inflammatory factors, and alleviating COPD symptoms in rats.

Conflict of Interest

The authors declare there is no conflict of interest.

Author Contributions

Jin M designed and supervised the experiments and revised the primary manuscript. Xue CJ completed the animal experimental work and wrote the paper. Wang Y, Dong F, Peng YY, and Zhang YD took part in the animal experiment. Zang BX was responsible for HPLC analysis of HSYA. Tan L applied a part of the financial support.

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