Hydroxysafflor Yellow A Alleviates Early Inflammatory Response of Bleomycin-Induced Mice Lung Injury

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Hydroxysafflor yellow A (HSYA) is an effective ingredient of Chinese herb *Carthamus tinctorius* L. The aim of this study was to evaluate the protective effect of HSYA on inflammatory phase of bleomycin-induced pulmonary injury in mice. Three doses of HSYA (26.7, 40, 60 mg/kg/d) were intraperitoneally injected to mice consecutively for 1 week after bleomycin administration. It was found that HSYA attenuated the loss in body weight, the increase of myeloperoxidase activity and pathologic changes of pulmonary inflammation caused by bleomycin. Treatment with HSYA also alleviated bleomycin-induced increase of mRNA level of tumor necrosis factor (TNF)-α, interleukin (IL)-1β and transforming growth factor (TGF)-β1 in lung homogenates. Moreover HSYA inhibited the increased activation of nuclear factor (NF)-κB and phosphorylation of p38 mitogen-activated protein kinases (MAPK) in lung tissue. These findings demonstrated that HSYA had protective effect on bleomycin-induced lung inflammatory response.

Key words  hydroxysafflor yellow A; pulmonary inflammation; bleomycin

*Carthamus tinctorius* L. (safflower) has been used for treating blood stasis in traditional Chinese medicine for thousands of years. Phytochemical research shows that safflower yellow (SY) is the effective part of safflower and hydroxysafflor yellow A (HSYA) is the effective ingredient of SY. Many studies have demonstrated that HSYA possesses many pharmacological activities including anti-platelet aggregation, cardioprotective effects, neuroprotective effect against cerebral ischemia/reperfusion injury through its antioxidant action and anti-inflammatory action.

Bleomycin (BLM) is a chemotherapeutic drug used for a variety of human malignancies treatment. But its benefits are limited by severe side effect of inducing pneumonitis syndrome and progressing to fibrosis.

Therefore, bleomycin is frequently used in establishing acute lung injury model and pulmonary fibrosis model in vivo. In rodent model, lung injury induced by intratracheal delivery of bleomycin is characterized by two sequential pathological phases: acute lung injury in the early phase and pulmonary fibrosis in late phase. Previous study showed that the transition between inflammation and fibrosis remodeling approximately occurred on day 9 after bleomycin administration. Pro-inflammatory cytokines such as interleukin (IL)-1α, IL-1β, IL-6, and interferon (IFN)-γ were significantly elevated up to day 9, this sustained damage led to the initiation of the subsequent fibrotic processes which was observed from about day 9 up to a maximum on day 21. Inflammation is the initial response to various injuries. Activated inflammatory cells such as macrophages and neutrophils accumulate and release amounts of cytokines which are important regulators of the inflammatory and fibrotic process.

Our previous study has demonstrated that HSYA could alleviate mice acute lung injury (ALI) induced by intravenous administration of lipopolysaccharide (LPS). In that model the capillary endothelium was the initial site of injury. Given that the bleomycin model reproduces the pattern of ALI with early inflammation and intratracheal delivery of bleomycin results in direct damage initially to alveolar epithelium, our present study was conducted to investigate the potential effects of HSYA on the early inflammatory stage of bleomycin-induced pulmonary injury in mice model.

MATERIALS AND METHODS

Chemicals and Reagents  Safflower which was identified by Professor Jiashi Li (Beijing University of Chinese Medicine), was brought from Tacheng, the Xinjiang Uyghur Autonomous Region, China. HSYA was isolated and purified by macroporous resin-gel column chromatography from the aqueous extracts of *Carthamus tinctorius* L. as previously described. The molecular weight of HSYA is 612 with the molecular structure showed in Fig. 1. Its purity was determined using HPLC (Fig. 2) and HSYA with the purity of 98.8% was dissolved in sterile 0.9% NaCl for the subsequent use. BLM was purchased from Tianjin Taihe Pharmaceutical Co., Ltd. (Tianjin, China). The myeloperoxidase (MPO) activity assay kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The immunohistochemistry kit was purchased from Bio-High Technology Corporation (Hebei, China).
China); Antibodies to p38 mitogen-activated protein kinases (MAPK) and phospho-p38 MAPK were produced by Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Trizol reagent and M-MLV reverse transcriptase were purchased from Invitrogen Co. (Carlsbad, CA, U.S.A.); SYBR® Premix Ex Taq™ (Perfect Real Time) was purchased from Takara Bio Inc. (Otsu, Shiga, Japan).

**HPLC Analysis of HSYA**

HPLC analyses were performed using a Shimadzu Scientific Instruments (Kyoto, Japan) liquid chromatographic system with LC-10AT pump, SPD-6AV UV detector. Inertsil ODS-3 column (250 mm × 4.6 mm, 5 μm; GL Sciences, Japan) was used. The chromatographic mobile phase was methanol:acetonitrile:0.02% phosphoric acid solution (pH 2.6) at a flow-rate of 1.0 mL/min performed at 30°C. UV absorption was monitored at 405 nm. (Fig. 2)

**Animal Treatment and Experimental Groups**

Male C57BL/6 weighting 19–21 g were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH, 1996) and approved by the Institutional Animal Care and Use Committee of Capital Medical University. Seventy-two mice were randomly allocated into six groups (n=12 each group): sham group (saline+saline); BLM group (bleomycin+saline); HSYA groups (bleomycin+HSYA 26.7, 40, 60 mg/kg) and dexamethasone (DXM) group (bleomycin+dexamethasone 3 mg/kg).

After weighting, mice were anesthetized with isoflurane, then bleomycin in sterile 0.9% NaCl (4.5 mg/kg body weight) were introduced into the lungs via oropharyngeal aspiration with the mouse nose kept pinched, and the mice of sham group received the same volume of sterile saline instead. The date of bleomycin administration was defined as day 1. From day 1 mice in sham group and BLM group were intraperitoneally injected saline daily and mice of DXM group were intraperitoneally injected dexamethasone daily. Mice body weight was recorded before drug injection. On day 7, mice were anesthetized with pentobarbital sodium, blood samples were obtained from abdominals aorta with a heparinized 24-gauge needle for arterial blood gas analysis, then mice were sacrificed by over bleeding. Lung lobes were dissected and washed in ice-cold saline, and then right lung lobes were snap-frozen in liquid nitrogen for RNA isolation, MPO activity determination and p38 MAPK detection. Left lung was weighted and then fixed in 10% neutral formalin for histopathological and immunohistochemical examination.

**Arterial Blood Gas Analysis**

Blood samples were obtained from abdominals aorta and were measured using Radiometer ABL 700 series blood gas analyzer (Copenhagen, Denmark) within 1h at room temperature. Oxygen partial pressure (PaO2), carbon dioxide partial pressure (PaCO2), oxygen saturation (SO2), pH and HCO3− concentration were recorded.

**Left Lung Index**

Left lungs were taken out, rinsed and weighed. Left lung index was calculated as follow:

\[
\text{left lung index} = \frac{\text{left lung weight}}{\text{body weight (g)}}
\]

**Histopathology Studies**

Left lung specimens were fixed in 10% neutral formalin, embedded in paraffin. Sequential 5μm sections were stained with hematoxylin–eosin. Slides were scanned and images were taken under a light microscope (Nikon Eclipse 90i).

**Lung MPO Activity**

Lung tissue was homogenized and MPO activity was measured according to the manufacturer’s instructions. The absorbance at 460 nm of each sample was recorded using a UV-visible spectrophotometer. MPO activity was expressed in units per gram of wet tissue. A unit of MPO activity was defined as that converting 1 μmol of hydrogen peroxide to water in 1 min at 37°C.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis**

Total RNA was isolated from each specimen...
of frozen lung tissue using Trizol reagent according to the manufacturer’s instructions. RNA concentration and purity were determined by the Thermo Scientific NanoDrop 2000 (Wilmington, U.S.A.). First-strand cDNA synthesis was performed with 2 μg of total RNA in a reaction volume of 20 μL using M-MLV reverse transcriptase. One microliter of cDNA was amplified in 20 μL reactions using SYBR® Premix Ex Taq™ on an iCycler iQ Real-time Detection System. PCR amplification was carried out as follows: initial denaturation at 95°C for 15 s, 35 cycles with denaturation at 95°C for 5 s, annealing at 61°C for 15 s. The following gene-specific primers were used. Tumor necrosis factor (TNF)-α: sense 5’-CCC TCA CAC TCA GAT CAT CTT CT-3’ and antisense 5’-GCT ACG ACG TGG GCT ACA G-3’; IL-1β: sense 5’-CCA TGG CAC ATT CTG TTC AAA-3’ and antisense 5’-GCC CAT CAG AGG CAA GGA-3’; transforming growth factor (TGF)-β1: sense 5’-GAC TCT CCA CCT GCA AGA CCA T-3’ and antisense 5’-GGG ACT GGC GAG CCT TAG TT-3’; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense 5’-TGC GAT CAT CTT CT-3’ and antisense 5’-ATG TAG GCC ATG AGG TCC AC-3’. Relative quantification was determined using the 2⁻ΔΔCt method with data normalized to GAPDH housekeeping gene.

**Western Blot Analysis** Frozen lung tissue was homogenized in lysis buffer on ice and homogenates were centrifuged at 12000rpm for 15 min at 4°C to remove cellular debris. Protein concentration was determined using Bicinchoninic Acid (BCA) method. Equal amounts of total proteins were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the separated proteins were transferred onto nitrocellulose membrane using semi-dry transfer method. After blocked with 5% nonfat dry milk in PBS, sections were incubated for 10 min with streptavidin–biotin–peroxidase complex and were visualized using diaminobenzidine substrate. Cell nucleus was counterstained with hematoxylin. Positive staining for p65 was brown. For each sample, ten randomly selected fields were used for counting p65 positive cells.

**Statistical Analysis** Data were presented as mean±S.E. and statistical analyses were performed by SPSS 11.5 software. The results were analyzed by One-way analysis of variance (ANOVA), the post hoc Multiple Comparisons of ANOVA tests named LSD type was then used. A p value of less than 0.05 was considered statistically significant.

**RESULTS**

**Effects of HSYA on BLM-Induced Changes of Body Weight** To evaluate the protective effect of HSYA following bleomycin injury, mice body weight was recorded everyday. As shown in Fig. 3A, The body weight of mice treated by bleomycin decreased markedly to 19.1±1.8 g by day 7 compared with sham group (23.0±1.3 g, p<0.01). HSYA treatment attenuated the loss in body weight especially at a dose of 60 mg/kg, the protective effect is significant with a mean value of 20.8±1.4 g compared with the bleomycin group, p<0.05 (Fig. 3A).

**Effect of HSYA on Left Lung Index** Left lung index was calculated to exam the severity of pulmonary edema. Lung injury caused by bleomycin was demonstrated by a significant increase of left lung index due to lung edema, inflammatory cells infiltration and body weight loss. Treatment with HSYA and dexamethasone significantly lowered the left lung index compared with BLM group (Fig. 3B).

**Effects of HSYA on Arterial Blood Gas** In order to evaluate the effect of HSYA on relieving lung gas exchange dysfunction caused by pulmonary inflammation, arterial blood gas analysis was performed. As shown in Fig. 4, treatment with HSYA ameliorated the decrease of PaO2, SO2, pH and the increase of PaCO2 induced by bleomycin.

**Lung Histopathology** To elucidate the effect of HSYA on lung histopathological changes and cellular infiltration associated with bleomycin-induced lung injury, left lung slices were stained and observed. Normal alveolar structure was shown from lung tissue sections of mice in sham group. Sever lung

**Fig. 3.** Effect of HSYA on Changes of Mice Body Weight (A) and Left Lung Index (B) Following Bleomycin Injury

Data are presented as mean±S.E., n=12 mice per group, *p<0.01 versus sham group, *p<0.05, **p<0.01 versus BLM group.
inflammatory response in bleomycin group was observed with obvious infiltration of inflammatory cells and marked thickening of the interalveolar septa. The alveolitis was attenuated dose-dependently in HSYA groups and also in DXM group as shown in Fig. 5.

MPO Activity in Lung Tissue MPO activity has been used as a biochemical indicator for neutrophil granulocytes infiltration. To assess the effect of HSYA on inhibition of inflammatory cellular infiltration, lung MPO activity was measured. As shown in Fig. 6 the lung tissue MPO activity was significantly higher in bleomycin group (0.707±0.175 U/g, p<0.01) compared with that in sham group (0.471±0.107 U/g). Bleomycin induced elevation of MPO activity was attenuated by dexamethasone (0.551±0.155 U/g) and dose-dependently by HSYA (0.582±0.141 U/g at 60 mg/kg).

Effects of HSYA on TNF-α, IL-1β and TGF-β1 mRNA Expression in Lung Homogenates RT-qPCR was performed to evaluate the expression of major inflammatory
factors after bleomycin administration and HSYA treatment. The mRNA level of TNF-α, IL-1β and TGF-β1 in lung tissue was significantly elevated in BLM group compared with sham group. The augmented expression was attenuated by HSYA with the maximum effect at 60mg/kg. The effect was also observed in DXM group (Fig. 7).

**Immunohistochemistry Detection of Nuclear Factor (NF)-κB p65** In order to examine whether the observed anti-inflammatory effect could be through the inhibition of NF-κB activity, the activated subunit p65 was detected and assessed by immunohistochemistry study. As shown in Fig. 8 the p65 positive cells rate were significantly increased after beomycin administration. Treatment with HSYA and dexamethasone significantly attenuated the increased p65-positive cell rate induced by bleomycin.

**Effects of HSYA on p38 MAPK Activation** The p38 MAPK is known to be one of the intracellular signal transducers of inflammatory reaction which can be triggered by various extracellular stimuli. Western blot analysis was carried out to assess the p38 activation in the lung tissue after bleomycin administration and HSYA treatment. The level of phosphorylated p38 MAPK in the BLM group was markedly augmented and the augmentation was attenuated by HSYA and dexamethasone (Fig. 9).

**DISCUSSION**

In this study, we demonstrated the beneficial effects of HSYA on early inflammatory stage in bleomycin model. Bleomycin exposure resulted in pulmonary edema and significant leukocyte infiltration in the lung tissue on day 7. Arterial blood gas analysis showed hypoxemia and acidosis which indicated lung respiratory dysfunction. As a result of the injury a marked body weight loss and lung index increase were observed. Intraperitoneal injection of HSYA alleviated the destruction by reducing lung index and inflammatory cells infiltration which was also supported by MPO activity measurement. MPO is a peroxidase enzyme released as a response to various stimulatory substances and it most abundantly exists in neutrophil granulocytes.

Increased lung MPO activity due to bleomycin was effectively decreased by HSYA at dose 40mg/kg and 60mg/kg. Moreover HSYA ameliorated the gas exchange disturbance dose-dependently by decreasing arterial blood PaCO2, and increasing arterial blood PaO2, SO2 and pH. Anti-inflammatory agent dexamethasone was also shown to attenuate the inflammatory response by reducing leukocyte infiltration and MPO activity and ameliorated gas exchange impairment in this bleomycin model, but the loss of mice body weight was more severe after dexamethasone treatment, this should be correlated with its side effects of inhibiting protein synthesis and promoting protein decomposition. HSYA significantly attenuated the body weight loss especially at dose 60mg/kg. These results demonstrated the protective effect of HSYA on inflammatory phase of mice lung injury induced by bleomycin with fewer side effects.
Bleomycin is a chemotherapeutic antibiotic produced by Streptomyces verticillus. Previous study showed that the recognition of bleomycin as a pathogen-associated molecular pattern by toll-like receptor (TLR) 2 triggered the activation of intracellular signaling pathways involving transcription factors such as NF-κB and MAPKs that led to the upregulation of genes encoding for chemokines and pro-inflammatory cytokines such as TNF-α and IL-1β.13,14 On the other hand, bleomycin has been known to bind to DNA/Fe²⁺ and form a DNA/Fe²⁺/BLM complex which undergone redox cycling and generated reactive oxygen species (ROS).15 Overproduction of ROS caused an inflammatory response with activation of macrophages and neutrophils and induction of pro-inflammatory cytokines. There was also study demonstrated TLR2 translated oxidative tissue damage into inflammatory responses by mediating the effects of oxidized phospholipids. It also showed a TLR2-dependent inflammatory gene expression and c-Jun

Fig. 8. Inhibitory Effect of HSYA on the Expression of NF-κB p65 in Mice Lung Tissue
Immunohistochemical staining for p65 in lung sections by day 7 (100×). Positive cells are stained brown. (A) sham group, (B) BLM group, (C) BLM+DXM group, (D, E and F) BLM+HSYA (26.7, 40, 60 mg/kg) group. (G) Quantification of NF-κB p65 staining. For each sample, the average positive stained cells number was calculated from 10 different 400× fields. Data are presented as means±S.E., n=10 mice per group. ##p<0.01 versus sham group, *p<0.05, **p<0.01 versus BLM group.

Fig. 9. Inhibitory Effect of HSYA on Activation of p38 MAPK in Mice Lung Tissue
Data are presented as means±S.E., n=7 mice per group. ***p<0.01 versus sham group, *p<0.05, **p<0.01 versus BLM group.
N-terminal kinase (JNK) and p38 signaling in macrophages.\(^5\)

In the initiating stage of bleomycin-induced lung injury increased expressions of many inflammatory cytokines in the lung are accompanied with recruitment of inflammatory cells and the network of cytokines regulates the inflammatory process and they are also related with accumulation of extracellular matrix components in fibrosis development. Previous studies showed that the expression of TNF-\(\alpha\) and IL-1\(\beta\) was up-regulated in both bleomycin-induced animal and IPF patients.\(^6\)\(^7\)\(^8\)\(^9\)\(^10\) These two cytokines were related to recruitment of inflammatory cells and induction of the pro-fibrotic cytokine TGF-\(\beta\).\(^9\)\(^10\)\(^11\) Therefore, in our study the mRNA level of TNF-\(\alpha\) and IL-1\(\beta\) in lung tissue was tested by RT-qPCR. Our data confirmed that bleomycin administration induced a significant increase of TNF-\(\alpha\) and IL-1\(\beta\) mRNA expression. The increased level of the pro-inflammatory cytokines was significantly attenuated by treatment with HSYA.

Under resting state, NF-\(\kappa B\) acts on regulating the expression of genes involved in normal immunologic responses. During lung injury, NF-\(\kappa B\) is activated persistently resulting in gene expression of many cytokines, chemokines and growth factors.\(^10\) We further assessed the activity of NF-\(\kappa B\) using immunohistochemical method to detect activated subunit p65. The activation of NF-\(\kappa B\) was enhanced after bleomycin administration. Treatment with HSYA significantly attenuated the increased p65 positive cell rate. The p38 MAPK is known to be one of the intracellular signal transducers, pharmacological inhibition of p38 MAPK had beneficial effects on bleomycin-induced lung injury. The specific inhibitor of p38 MAPK suppressed augmented expression of TNF-\(\alpha\), connective tissue growth factor, and apoptosis of lung cells induced by bleomycin.\(^22\) And previous reports showed that p38 MAPK played an important role in fibrosis disease acting downstream of TGF-\(\beta\).\(^22\)\(^23\) In this study p38 MAPK were activated after bleomycin administration and treatment with HSYA significantly suppressed the phosphorylation of p38 MAPK. These results were in agreement with our previous study in the LPS-induced ALI mice model.

Recently, HSYA has been found to alleviate carbon tetra-chloride (CCl\(_4\))-induced liver fibrosis in rats.\(^24\) Our previous study demonstrated that SY had the beneficial effect against bleomycin-induced pulmonary fibrosis in rats.\(^25\) In this study we also tested the mRNA level of the pro-fibrogenic cytokine TGF-\(\beta\). As a link between inflammation and fibrosis, TGF-\(\beta\) is a key cytokine in the process of fibrogenesis.\(^26\) It is well known that TGF-\(\beta\) promotes the proliferation and differentiation of fibroblasts into activated myofibroblasts, and it also promote epithelial mesenchymal transition which is another source of myofibroblasts, at the same time it enhances collagen and fibronectin production and reduces extracellular matrix degradation.\(^27\) Our results manifested that HSYA evidently attenuated the increased mRNA level of TGF-\(\beta\) induced by bleomycin, this regulation of TGF-\(\beta\) can be partly explained by the reduction of the above two relevant cytokines TNF-\(\alpha\) and IL-1\(\beta\).

In summary our present study found that HSYA could significantly alleviate bleomycin-induced early pulmonary inflammation by suppressing the activation of NF-\(\kappa B\), phosphorylation of p38 MAPK and inhibiting the augmentation of pro-inflammatory and pro-fibrogenic cytokines expression. The early inflammatory response to bleomycin partly accounts for the following development of lung fibrosis. Our data provide some of the good evidence that HSYA may be effective to attenuate the subsequent fibrosis response. HSYA is highly water-soluble, so it may not be well cell membrane-permeable. We speculate that HSYA may target the cell membrane and interfere with the interaction of receptors and their specific ligands (such as microbial ligands, proinflammatory cytokines, growth factors, etc.) to exert its effects by regulating downstream signal transduction pathways. However the concrete mechanism by which HSYA alters intracellular signaling still needs further study.

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**REFERENCES**


