Protective Effect of *Houttuynia cordata* Extract on Bleomycin-Induced Pulmonary Fibrosis in Rats

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Abstract: The present study aimed to examine the antioxidant properties of *Houttuynia cordata* (HC) and its protective effect on bleomycin-induced pulmonary fibrosis in rats. Results showed that aqueous extract of HC exhibited a different magnitude of antioxidant activities in all model systems tested. Although HC showed weaker free radical scavenging and xanthine oxidase inhibitory activity than vitamin E, its anti-lipid peroxidation activity in rat liver homogenate was close to that of vitamin E. In animal studies, HC significantly decreased the levels of superoxide dismutase, malondialdehyde, hydroxyproline, interferon- γ , and tumor necrosis factor- α . However, an increase in the concentration of catalase was noted in the bronchoalveolar lavage fluid. HC also remarkably improved the morphological appearance of the lung of bleomycin-treated rats. These results suggest that HC possesses a protective effect against bleomycin-induced pulmonary fibrosis. Interestingly, this protective effect was more pronounced than that of vitamin E. In conclusion, the protective effect of HC on pulmonary fibrosis could be partly associated with the reduction of oxidative damage caused by bleomycin.

Keywords: *Houttuynia cordata*; Antioxidant; Bleomycin; Pulmonary Fibrosis.

Introduction

Pulmonary fibrosis is characterized by the accumulation of extracellular matrix collagen, fibroblast proliferation and migration, and loss of alveolar gas exchange units. It is a devastating disease as it may lead to progressive lung destruction and death.

Houttuynia cordata (HC), a vegetable commonly consumed in Taiwan, has been widely used in the traditional Chinese medicine as anti-microbial, anti-pyretic, anti-purulent,

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diuretic and detoxicant (Zheng *et al.*, 1998). Studies have shown that HC possesses anti-viral (Hayashi *et al.*, 1995; Chiang *et al.*, 2003), anti-tumor (Kim *et al.*, 2001), anti-leukemia (Chang *et al.*, 2001), anti-inflammatory (Probstle and Bauer, 1992), antioxidant (Chen *et al.*, 2001; Cho *et al.*, 2003), and anti-mutagenic (Chen *et al.*, 2003) activities. During the outbreak of severe acute respiratory syndrome (SARS) in Taiwan in late 2002, as conventional drugs failed to treat or ameliorate the conditions of SARS infected patients, certain traditional preparations such as crude drug prepared from HC were proposed to be an alternative for treating this disease. However, its effectiveness against pulmonary fibrosis has never been evaluated.

Bleomycin (BLM), a family of glycopeptide antibiotics from *Streptomyces verticillus*, is a potent anti-neoplastic agent and is known to produce pulmonary fibrosis in humans as well as in experimental animals (Adamson, 1976; Ikezaki *et al.*, 1996). BLM-induced lung fibrosis in animals is a popular model for the study of human lung fibrosis (Wang *et al.*, 2002). Although the exact mechanisms by which BLM causes pulmonary fibrosis remain unclear, it is generally believed that reactive oxygen species (ROS) generated by BLM causes direct injury to the lung epithelial cells (Hay *et al.*, 1991; Wang *et al.*, 2002). In experiments, BLM was shown to induce diffuse alveolar damage and pulmonary fibrosis in mice (Chen *et al.*, 2001; Segel *et al.*, 2003), hamsters (Ikezaki *et al.*, 1996), and rats (Thrall *et al.*, 1979; Punithavathi *et al.*, 2000).

Previous studies on pulmonary fibrosis have mainly focused on the fibroproliferative process in the lung. Recently, increasing interests have been given to ROS generation in lung fibrosis (Castranova *et al.*, 2002; Shukla *et al.*, 2003). ROS, such as superoxide anions, hydrogen peroxides, and hydroxyl radicals have been demonstrated to be an important mediator of BLM-induced lung fibrosis (Arslan *et al.*, 2002; Chen and Stubbe, 2004). Excessive production of ROS is known to induce tissue damage or cell death, which could lead to several physiological and pathological processes.

In the search of potential treatment for SARS infected patients and for the prevention of lung fibrosis, the present study was conducted to examine the antioxidant activities of HC and its inhibitory effects on bleomycin-induced pulmonary fibrosis in rats.

Materials and Methods

Chemicals

Vitamin E (Vit E; α -tocopherol), thiobarbituric acid (TBA), iron (II) chloride anhydrous, cytochrome c, xanthine and xanthine oxidase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Bleomycin hydrochloride (BLM) was purchased from Chiyoda-ku Co. (Tokyo, Japan). All other chemicals used were of analytical grade.

Animals

Male Wistar rats of age 5 weeks and weight 160–180 g were purchased from the National Laboratory of Animal Breeding and Research Center (Taipei, Taiwan). They were kept for

1 week under environmentally controlled conditions (room temperature $22 \pm 3^\circ\text{C}$, relative humidity $55 \pm 5\%$ and 12 hours light/dark cycle) with free access to a commercial diet and water. Animals were treated in accordance with the guidelines of the National and Institute's Animal Care Committee.

Plant Extract

The whole plant of *Houttuynia cordata* (HC) was purchased from the traditional Chinese pharmacy in Kaohsiung (Taiwan). It was then authenticated by Professor C.C. Lin, Kaohsiung Medical University, Taiwan. The dried HC sample was ground to powdered form and stored in an air-tight plastic bag until use.

To prepare the HC extract, 100 g of HC powder was extracted with 1 L of boiling water for 1 hour. After cooling, the sample was filtered and the residue was re-extracted under the same conditions twice. The filtrates collected from three extractions were combined, concentrated and lyophilized to dryness. The yield of dried HC extract was about 14.2%.

On the experimental days, this crude extract was dissolved in normal saline at a concentration of 0.1 g/ml and orally given to rats at a dose of 1 g/10 ml/kg body weight. The control group received a similar volume of normal saline solution.

Free Radical Scavenging Activity

The activity on superoxide anions scavenging were assayed by the cytochrome c method (McCord and Fridovich, 1969). In brief, 15 mg of samples were dissolved in 1 ml of distilled water and then further diluted to various concentrations (0.1 to 10.0 mg/ml), 20 μl of 0.07 units/ml xanthine oxidase, 300 μl of 100 μM xanthine and 15 μl of 50 μM cytochrome c were added to these samples. They were incubated for 3 min at room temperature and then read at 550 nm.

Xanthine Oxidase Inhibition Test

Xanthine oxidase inhibitory activity was estimated by the formation of uric acid from xanthine (Chang *et al.*, 1994). Briefly, 15 mg of samples were dissolved in 1 ml distilled water, and then diluted with 50 mM KH_2PO_4 buffer (pH 7.8) to various concentrations (0.1 to 10.0 mg/ml). After 350 μl of xanthine (100 μM) and 20 μl of xanthine oxidase (0.4 units) were added, samples were vigorously mixed and then incubated for 3 min at room temperature. The absorbance was spectrophotometrically measured at 295 nm.

FeCl₂-Ascorbic Acid Stimulated Lipid Peroxidation

The effect of HC extracts on lipid peroxidation in rat liver homogenate induced by FeCl_2 -ascorbic acid was determined by the method of Yoshiyuki *et al.* (1981). A mixture containing 0.5 ml of liver homogenate, 0.1 ml of Tris-HCl buffer (pH 7.2), 0.05 ml of

0.1 mM ascorbic acid, 0.05 ml of 4 mM FeCl₂ and 0.05 ml of various concentrations of crude extracts, were incubated for 1 hour at 37°C. After incubation, 0.9 ml of distilled water and 2 ml of 0.6 % TBA were added, and then shaken vigorously. The mixture was heated for 30 min in boiling water. After cooling, 5 ml *n*-butanol was added and the mixture was shaken vigorously. The *n*-butanol layer was separated by centrifugation at 3,000 × *g* for 10 min. The absorbance of the supernatant was read at 532 nm against a blank, which contained all reagents except liver homogenate. The protein content was determined by the method of Lowry *et al.* (1951).

Bleomycin-Induced Lung Fibrosis in Rats

Rats were randomly divided into 4 groups of 6 animals each; there were (1) Control group (Control), which received intragastrically (i.g.) 1 ml/kg of sterile physiological saline; (2) Bleomycin-induced (BLM) group, which received 15 mg/kg of BLM only; (3) Bleomycin + vitamin E (BLM + Vit E) group, which was treated with BLM and 10 mg/kg vitamin E; (4) Bleomycin + HC (BLM + HC) group, which was challenged with BLM and 1 g/kg HC. Rats were intraperitoneally (i.p.) injected with BLM at a dose of 15 mg/ml/kg to induce lung fibrosis three times per week for a period of 5 weeks. Thirty minutes before receiving BLM, rats were subjected to their respective assigned treatments.

After 35 days of treatment, rats were anaesthetized and sacrificed. The thorax was opened by a median incision and the trachea was cannulated with a plastic catheter attached to a 10 ml syringe. The bronchoalveolar lavage fluid (BALF) was obtained by washing the lung across the trachea with 10 ml saline. It was then centrifuged at 300 × *g* at 4°C for 10 min to obtain the supernatant for biochemical assays. The yield of BALF was about 90%. Lung lobes of rats was collected, weighed and stored at -70°C until analysis.

Biochemical Assays

The supernatant of BALF was analyzed for superoxide dismutase (SOD), interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) activities using commercially available assay kits (Nebot *et al.*, 1993). Catalase (CAT) activity was assayed according to the method described by Aebi (1984). The lipid peroxidation (malondialdehyde) was estimated by the method of Ohkawa *et al.* (1979). The hydroxyproline content of lung was measured according to procedures described by Woessner (1961).

Histopathological Observation

Lung tissues were fixed by 10% formalin solution for 24 hours. They were then dehydrated with a sequence of ethanol solution, embedded in paraffin and cut into 3 μ m thick sections, followed by staining with haematoxylin-eosin before subjecting to photomicroscopic assessment.

Statistical Analysis

Data were expressed as means $\pm$ standard deviations (SD). Statistical analysis was performed by one way analysis of variance (ANOVA), followed by Least Significant Differences (LSD) test using SPSS software (SPSS Inc., USA). $p < 0.05$ was considered as statistically significance.

Results

Antioxidant Activities of H. cordata (HC)

Results on free radical scavenging, xanthine oxidase inhibition and anti-lipid peroxidation activities of HC are shown in Fig. 1. The superoxide radical scavenging and xanthine oxidase inhibition activities of HC were moderate, with IC_{50} values 3.98 mg/ml and 6.97 mg/ml, respectively. However, the anti-lipid peroxidation activity of HC ($IC_{50} = 1.02$ mg/ml) was similar to vitamin E ($IC_{50} = 0.94$ mg/ml).

Effect of H. cordata (HC) on Bleomycin-Induced Lung Injury

The body weight of BLM-treated group was significantly ($p < 0.05$) lower than animals of other treatments, whereas its lung weight was significantly heavier (Table 1). Results showed that HC and vitamin E treatments provide significant protective effect against BLM-induced changes in body and lung weight. Although HC possessed a weaker antioxidant activity, it exhibited the best protection against BLM-induced body weight loss and lung weight gain.

Compared to the control group, the SOD activity of the BLM-induced group was significantly ($p < 0.05$) increased. However, when rats received HC treatment (BLM + HC group), the level of SOD was decreased to the normal level as the control group (Table 1). CAT activity was significantly increased in the BLM + Vit E and BLM + HC groups as compared to BLM group ($p < 0.01$). The lipid peroxidation in BLM-treated group was significantly higher than other treatments. Interestingly, the MDA level between the control and BLM + HC groups was almost the same. These results suggest that HC and vitamin E are able to inhibit lipid peroxidation in lung, while BLM significantly caused an increase in the MDA content of lung tissue.

In BLM-induced animals, the concentrations of IFN- γ and TFN- α in the BALF were significantly higher than other treatments (Table 1). This result suggests that BLM-induced pulmonary toxicity is associated with enhanced production of IFN- γ and TFN- α .

Lung fibrosis was assessed by measuring hydroxyproline content in lungs as an index of collagen accumulation. BLM produced almost twofold increases in hydroxyproline content; however, it was completely prevented by HC. HC treatment significantly decreased the BLM-induced increase in the lung collagen content (Table 1). These results revealed that oral HC may prevent the development of lung fibrosis caused by BLM injection via the antioxidant action and anti-lipid peroxidation.

Figure 1. Antioxidant activities of *H. cordata* (HC). IC₅₀ values are the concentration of HC extract that produced 50% of antioxidant activity. Cytochrome c: superoxide scavenging activity in cytochrome c test; XOI: anti-superoxide formation effect in xanthine oxidase inhibition test; TBA: anti-lipid peroxidation effect in FeCl₂-ascorbic acid induced lipid peroxidation assay.

Table 1. Protective Effects of *H. cordata* on Bleomycin (BLM)-Induced Lung Injury in Rats

	Control	BLM	BLM + Vit. E	BLM + HC
Body Weight (g)	295.8 ± 11.6 ^a	165.8 ± 9.0 ^c	234.2 ± 15.2 ^b	244.2 ± 0.4 ^b
Lung Weight (g)	3.28 ± 0.21 ^b	4.86 ± 0.51 ^a	3.56 ± 0.47 ^b	2.71 ± 0.10 ^b
SOD (U/ml)	25.18 ± 1.39 ^c	52.24 ± 10.02 ^a	32.26 ± 0.96 ^b	28.32 ± 3.80 ^{bc}
CAT (U/ml)	10.83 ± 0.94 ^a	3.54 ± 0.20 ^c	7.50 ± 0.64 ^b	7.39 ± 0.47 ^b
MDA (nmol/mg protein)	1.16 ± 0.15 ^c	4.68 ± 0.53 ^a	2.29 ± 0.84 ^b	1.10 ± 0.14 ^c
Hydroxyproline (mg/g)	10.45 ± 0.27 ^c	20.40 ± 1.67 ^a	16.71 ± 0.71 ^b	12.15 ± 1.04 ^c
IFN-γ (pg/ml)	56.88 ± 1.21 ^c	140.06 ± 22.2 ^a	92.94 ± 6.24 ^b	61.27 ± 4.85 ^c
TNF-α (pg/ml)	53.05 ± 2.47 ^c	127.57 ± 16.6 ^a	73.31 ± 6.86 ^b	64.46 ± 5.62 ^{bc}

Data were presented as means ± SD from six independent analyses. Values within the same row with the different superscript letters were significantly different at $p < 0.05$ as analyzed by Least Significant Differences (LSD) test.

Morphological Appearance of Lung

In the histological studies, lungs of the BLM group showed a diffuse and marked increased alveolar wall thickness (Fig. 2). In contrast, lungs from BLM + Vit E and BLM + HC groups showed fewer fibrotic lesions. Interestingly, lungs of BLM + HC group appeared to have a relatively similar appearance as that of the control group. These results revealed that HC and vitamin E treatments could attenuate the histopathological changes in lungs after BLM treatment.

Figure 2. Histopathological observation of lung on 35th day after *H. cordata* extract (HC) and vitamin E (Vit E) treatment. (A) Normal control group: arrows show alveolar spaces and alveolar septum; (B) BLM group: collapse of alveolar spaces as marked by thickening alveolar septa; (C) BLM + Vit E group: histological observation shows some protection against BLM-induced fibrosis; (D) BLM + HC group: a relative normal lung morphology as compared to the BLM group.

Discussion

The present study showed that HC exhibited a different magnitude of antioxidant activities in both *in vivo* and *in vitro* studies. HC also demonstrated an ability to inhibit pulmonary fibrosis caused by BLM as determined by biochemical and histopathological analyses of the lung.

Tissue injury caused by ROS may include DNA damage, lipid peroxidation (Halliwell, 1991), protein damage (Bartold *et al.*, 1984), and oxidation of important enzymes in the human body (Varani *et al.*, 1985), and consequently, lead to a number of chronic diseases. Antioxidants, which protect against oxidative damage induced by free radicals, can prevent the onset and progression of these diseases. ROS are believed to be involved in the early stages of lung fibrosis (Sogut *et al.*, 2004), and play an important role in the BLM-induced lung injury (Borok *et al.*, 1991).

Several animal models have been used to elucidate the mechanisms involved in the effects of BLM in lung fibrosis, as well the role of ROS in this damage (Ozyurt *et al.*, 2004). Although HC has been used for treating various diseases for many years in China, our understanding of its inhibitory effect on the BLM-induced lung fibrosis has never been studied. The present study showed that HC significantly inhibited the BLM-induced lung fibrosis in rats, and its protective effect was found to be more potent than vitamin E (a well-known natural antioxidant agent), suggesting that HC is potent against lung fibrosis.

The protective effect derived from HC on BLM-induced lung fibrosis could be demonstrated by an increase in the hydroxyproline content of the lung, one of the main biological markers of pulmonary fibrosis (Wang *et al.* 2002). An increase in CAT level in the BLM + Vit E and BLM + HC groups suggests that HC and vitamin E might act as an antioxidant against the oxidative action of BLM, and that the consumption of HC and vitamin E could be partly related to the inhibition of lung inflammation and reduced collagen deposition in the lung. SOD activity was significantly increased in the BLM-induced group, suggesting an increase in the oxidative stress in the rats. These findings suggest that antioxidant such as HC and vitamin E may play a crucial role in protection against BLM-induced oxidation in the lung.

Previous studies have shown that orally administrated antioxidants such as N-acetyl-L-cysteine (Hagiwara *et al.*, 2000), taurine (Wang *et al.*, 1989), bilirubin (Wang *et al.*, 2002), vitamin E (Kilinc *et al.*, 1993), BHA, and BHT (Ikezaki *et al.*, 1996) would protect and ameliorate BLM-induced pulmonary fibrosis. This protective effect was reported to be partly attributable to the radical scavenging action of these dietary antioxidants. In this study, HC was found to be more protective against BLM-induced alveolar damage in rats than vitamin E, since the antioxidant activities of vitamin E was better than HC, it is therefore suggested that the protective effect of HC against BLM-induced lung toxicity might be related to factors other than its scavenging of ROS.

T cells were shown to play an important role in the development of BLM-induced pulmonary toxicity in the rodent models (Chen *et al.*, 2001). IFN- γ was suggested to mediate, in part, BLM-induced pulmonary inflammation and fibrosis in mice. TNF- α was reported to be involved in the development of pulmonary fibrosis (Fujita *et al.*, 2003). The levels of IFN- γ and TNF- α were found to increase in the BLM-induced mice (Chen *et al.*, 2001; Fujita *et al.*, 2003) and rats (Punithavathi *et al.*, 2000). In this study, BLM significantly increased the levels of IFN- γ and TNF- α in rats, whereas after HC treatment, the levels of IFN- γ and TNF- α were significantly suppressed. It is possible that HC could block the initiation and progression of the BLM-induced inflammatory response through the suppression of IFN- γ and TNF- α release by activated macrophages.

BLM has been shown to generate toxic free radicals, which have been involved in the formation of hydroxylated proline (Bhatnager and Liu, 1972). Consistent with previous studies, the present study showed that HC and vitamin E, which possess antioxidant activity, are capable of reducing the BLM-induced pulmonary fibrosis as shown by a decrease in the hydroxyproline content in BALF. Furthermore, histological observation also showed that HC and vitamin E exert protective effects against BLM-induced lung fibrosis as indicated by a reduced hyperplastic lesion, with HC being more effective in reducing BLM-induced pulmonary histopathological changes.

BLM, a potent and efficacious anticancer agent, is widely used for treating certain cancers (Chen and Stubbe, 2004). However, its clinical use is limited because of its side-effects, such as lung fibrosis. It was thought to produce therapeutic or toxic effects by altering the normal balance between oxidants (active oxygen radicals) and antioxidant systems. Based on these findings, our data suggests that HC could be a promising new

therapeutic agent for lung fibrosis or for preventing the development of BLM-induced lung fibrosis during anti-neoplastic therapy.

In conclusion, the present study indicates that HC possesses protective effect against BLM-induced pulmonary fibrosis in rats as demonstrated by biochemical and histopathological analyses. Although other yet to be identified compounds could contribute to this activity, the antioxidant action of HC may partly explain the inhibitory effect of pulmonary fibrosis in the present model of study. Further studies on its bioactive components and mechanism(s) of action may provide a scientific basis for the possible application of HC in preventing the BLM-induced lung fibrosis.

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