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Effects of curcumin in treatment of experimental pulmonary fibrosis: A comparison with hydrocortisone

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Abstract

To compare curcumin with hydrocortisone for treating bleomycin-induced pulmonary fibrosis (BLMPF), four groups of rats were injected with 1.5 mg/kg bleomycin intratracheally. Then the Group HC rats were treated with three injections of 2 mg/kg hydrocortisone i.p.; Group CH and CL rats, respectively, were orally given 500 or 250 mg/kg curcumin daily; and Group PC rats were given deionized water alone. After 28 days of treatment, lung samples were examined by H-E staining, Masson's staining and immunohistochemical analyses and pulmonary type I collagen (Col-I), inducible nitric oxide synthetase (iNOS) and transforming growth factor- β 1 (TGF- β 1) were determined by Western blotting and real-time RT PCR analyses. The results showed that (1) Group PC rats had histopathological characteristics of BLMPF with significant increase in their protein/mRNA expressions of Col-I (+114%/+173%), iNOS (+146%/+523%) and TGF- β 1 (+476%/+527%) ($P < 0.01$); (2) in Group HC, CH and CL rats, protein/mRNA expressions of Col-I (−39%/−52%, −31%/−57%, −33%/−58%), iNOS (−31%/−51%, −31%/−79%, −31%/−47%) and TGF- β 1 (−64%/−78%, −75%/−74%, −81%/−79%) were significantly lower than Group PC ($P < 0.05$); (3) except for levels of TGF- β 1 protein, there was no significant difference among Group CH, CL and HC rats ($P > 0.05$). It suggests that curcumin may play a similar role as hydrocortisone in preventing BLMPF.

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1. Introduction

Pulmonary fibrosis (PF) represents the end stage of pulmonary inflammation and injury with histopathological characteristics that include an absolute increase of collagen content, excessive number of inflammatory cells and remarkable abnormality in ultrastructural appearance of mesenchyme. The accumulation of collagen-rich extra cellular matrix (ECM), damage of alveolar wall and loss of functional capillary units can lead to respiratory failure within a few years followed diagnostic confirmation (Giri, 2003). Patients suffering from chronic diseases as well as acute damages, such as severe acute respiratory syndrome (SARS), may develop typical PF, and most patients eventually die (Cheung et al., 2004). Based on published reports, the estimated incidence of PF was 0.25‰ in USA and 0.07–0.18‰ in European countries during 1990s, and estimated

cost of healthcare was about US\$ 40,707 per patient (Raghu et al., 2004).

PF can result from many endogenous or exogenous causes, such as infection, chemical poisoning, radiation, etc. Bleomycin-induced pulmonary fibrosis (BLMPF) in rats has been widely used as a model to investigate the pathology of PF (Giri, 2003). In recent years, a number of studies showed significant up-regulation of inducible nitric oxide synthetase (iNOS) and transforming growth factor- β 1 (TGF- β 1) in the lung tissue of BLMPF rats that resembled clinical findings in PF patients (Khalil et al., 1993; Gurujeyalakshmi et al., 2000; Coker et al., 2001; Romanska et al., 2002). These similarities suggest that the active iNOS in inflammatory cells is responsible for excessive formation of nitric oxide (NO), and is involved in pulmonary injury (Hobbs et al., 1999). Meanwhile, these results further suggest that TGF- β 1 derived from fibroblasts is one of the key cytokines in fibrogenetic phase of PF, and may result in collagen overproduction and deposition in lungs (Coker et al., 2001). These two factors play important roles during the PF induced by bleomycin, and therefore have been regarded as pathological

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markers in researches and as potential therapeutic targets in treatments (Hobbs et al., 1999; Flanders and Burmester, 2003).

It is well known that PF is hard to treat clinically and few medications successfully relieve the condition. Corticosteroid therapy is the first-line choice to prevent and treat this disease (American Thoracic Society, 2000). Laboratory studies showed that corticosteroids might prevent the collagen over-expression (Dik et al., 2003) and might decrease up-regulated cytokines including TGF- β 1 in BLMPF models (Khalil et al., 1993). In other studies, corticosteroids might inhibit iNOS over-expression during acute cardiac allograft rejection, although this effect has not been reported in PF (Worrall et al., 1996). Since unacceptable adverse effects are associated with corticosteroid treatments, searching for more effective and safer therapies has been one of the hot topics in this field. In recent years, it has been reported that curcumin, a botanical compound with anti-inflammatory, anti-oxidant and anti-tumor effects, can ameliorate adverse pathological changes in BLMPF models (Venkatesan et al., 1997; Punithavathi et al., 2000), and thus appears to be a potentially valuable treatment for PF.

In view of the lack of knowledge on exact pharmacodynamic effects and pharmacological mechanisms of curcumin in PF treatment, comparing therapeutic effects of curcumin with current standard therapy such as corticosteroids, and examining regulative roles of curcumin on protein and mRNA expressions of iNOS and TGF- β 1 should be valuable. In the present study, BLMPF rats were used as a model; histopathological examinations including H-E staining, Masson's staining and immunohistochemical analysis were conducted for identifying characteristics of PF in rat lung tissues; expressions of pulmonary protein and mRNA of Col-I, iNOS and TGF- β 1 were quantitatively determined by Western blotting and real-time RT PCR analyses after treatment with curcumin or hydrocortisone.

2. Materials and methods

2.1. Animal and experimental protocol

Fifty pathogen-free female Sprague–Dawley rats, with body weight 200–250 g were purchased from the Animal Centre of the Chinese University of Hong Kong (Hong Kong), and randomly divided into five groups, i.e. negative control (NC), positive control (PC), hydrocortisone (HC), high-dose curcumin (CH) and low-dose curcumin (CL) groups. Rats in the Group NC were injected with 0.3 ml physiological saline intratracheally, and rats in other groups were injected with 1.5 mg/kg bleomycin (Sigma) intratracheally to induce BLMPF. After bleomycin injection, rats in the Group HC were treated with 2 mg/kg hydrocortisone (Yangzhou Pharm. Ltd., China.) i.p. once per 2 days for three times total, while rats in other groups were injected with deionized water alone according to the same schedule. In the Group CH and CL, rats were orally given 500 or 250 mg/kg curcumin (Henan Guangye Natural Pigment Co., Ltd., China) once per day, respectively, while other rats were orally given deionized water alone. During experiments, all rats were housed in

our animal room with temperature/humidity control, and a 12-h light:12-h dark cycle, and allowed to take food and water ad libitum.

On the 28th day after intratracheal injection, all rats were anesthetized and sacrificed. The lung, heart, liver, kidney, brain, spleen and uterus were collected from each animal. Left lungs were cut into small pieces and stored at -80°C for Western blotting and real-time PCR analyses. Other organs were kept in 10% formaldehyde solution for histological analyses including H-E staining, Masson's staining and immunohistochemical analysis. On the 1st, 14th and 28th day after intratracheal injection, 2 ml blood samples from each rat were collected by the posterior venous plexuses of eye orbit, and serum creatinine (Cr) and glutamic-pyruvic transaminase (GPT) were analyzed by biochemical kits. The protocol of this research was approved by the Animal Experimentation Ethics Committee of Hong Kong Baptist University.

2.2. Histopathologic and immunohistochemical examination

2.2.1. H-E stain and Masson's stain

After being fixed in 10% formaldehyde solution for 12 h, the blocks of rat lung tissues were dehydrated and embedded in paraffin, cut into $5\ \mu\text{m}$ slices, incubated at 60°C overnight, dewaxed and finally stained with hematoxylin-eosin or Masson's trichrome. Samples of other organs were also prepared and stained with hematoxylin-eosin, similarly, for a toxicity assessment.

2.2.2. Immunohistochemical analysis of Col-I, iNOS and TGF- β 1

Slices for immunohistochemical analysis were treated at 58°C , dewaxed, rehydrated, and equilibrated in Tris buffered saline (TBS) containing 0.3% Triton X-100 (Sigma). Endogenous peroxidase activity was blocked using 0.3% H_2O_2 (Sigma) in methanol for 30 min. Then slices were treated with 0.1% trypsin in 0.05 M Tris, 0.02 M CaCl_2 (pH 8.0). Non-specific binding was eliminated by blocking with 1.5% normal goat serum (Santa Cruz) in TBS with 0.5% bovine serum albumin (BSA). Subsequently, slices were incubated overnight at 4°C with primary antibody (goat polyclonal IgG to rat Col-I, rabbit polyclonal IgG to rat iNOS, and rabbit polyclonal IgG to rat TGF- β 1, Santa Cruz) in TBS with 0.5% BSA, followed by incubation with secondary antibody (rabbit anti-goat IgG-HRP for Col-I, and goat anti-rabbit IgG-HRP for iNOS and TGF- β 1, Santa Cruz) in TBS with 0.5% BSA (1:2000 diluted), and then incubated with avidin biotin complex (Santa Cruz) in TBS with 0.1% BSA for 1 h. Development was conducted with 0.5% 3,3'-diaminobenzidine (Sigma) in 0.1% H_2O_2 , 0.05 M Tris, 0.85% NaCl (pH 7.4) for 5 min. Finally the slices were counterstained with Gill's haematoxylin, dehydrated and mounted with Permount (Sigma). In this study a negative control in which primary antibody was not added in the above incubation was used to verify results of immunohistochemical analyses.

2.3. Western blotting

Five lung samples from each group were randomly selected and protein expressions of Col-I, iNOS and TGF- β 1 were determined by Western blotting analyses. For cell lysate preparation, frozen lung tissues were homogenized at 4 °C in Protease Inhibitors and Phosphatase Inhibitors (Santa Cruz). After ice incubation, the homogenates were centrifuged at 10,000 \times g for 10 min at 4 °C and supernatant fluid was gathered as total cell lysate.

2.3.1. Electrophoresis and transmembrane

The 40 μ g whole cell lysate was mixed with equal volume of 2 \times electrophoresis sample buffer containing glycerol, 2-mercaptoethanol, sodium dodecyl sulfate, 1.0 M Tris–HCl pH 6.7 and bromophenol blue. After boiling for 3 min, 10 μ l of the lysate mixture was loaded on polyacryamide gel for electrophoresis (BioRad). Meanwhile, nitrocellulose membrane was pre-treated with dH₂O and blotting buffer. After electrophoresis, the polyacryamide gel was placed on a wick, covered with a nitrocellulose membrane and wet paper (Whatman), and left on an electro-blotting apparatus overnight for transferring of protein from gel to nitrocellulose membrane.

2.3.2. Immunoblotting

Nitrocellulose membrane was subsequently treated with 5% blocking reagent for 45 min, incubated with primary antibody (goat polyclonal IgG to rat Col-I, rabbit polyclonal IgG to rat iNOS, rabbit polyclonal IgG to rat TGF- β 1, and mouse monoclonal IgG to β -actin, Santa Cruz) in phosphate buffered solution (PBS) for 1 h and then with secondary antibody (rabbit anti-goat IgG-AP for Col-I, goat anti-rabbit IgG-AP for iNOS and TGF- β 1, and goat anti-mouse IgG-AP for β -actin, Santa Cruz) in PBS for 30 min, developed in a solution (a mixture of 24 ml dH₂O with 1 ml of 25 \times buffer concentrate, 0.25 ml of nitroblue tetrazolium solution, and 0.25 ml of 5-bromo-4-chloro-3-indolyl phosphate solution) for 30 min, and finally rinsed with dH₂O to stop the reaction. In order to control inter- and intra-assay variations, β -actin was used as an internal control and the levels of Col-I, iNOS and TGF- β 1 proteins were represented as a ratio between β -actin and Col-I or iNOS or TGF- β 1, respectively.

2.4. Real-time RT PCR

Five lung samples from each group were randomly selected and their mRNA expressions of Col-I, iNOS and TGF- β 1 were determined by real-time RT PCR analyses. The total RNA of lung samples was extracted using a RNA isolation and purification kit (Qiagen) following its instructions. The RNA concentration was determined with a U2001 UV/vis spectrophotometer (Hitachi, Japan) at 260 nm.

2.4.1. Reverse-transcription

The 3 μ l total RNA were denatured at 70 °C for 5 min, and then mixed into 20 μ l reaction system containing 5 \times buffer solution (50 mM Tris–HCl (pH8.0), 50 mM KCl, 4 mM MgCl₂, 10 mM DTT), 10 mmol/L dNTP, M-MLV reverse transcriptase,

and DEPC-treated water. The system was allowed to react at 42 °C for 1 h and terminated at 95 °C for 3 min to complete reverse-transcription course and obtain cDNA solution.

2.4.2. Real-time RT PCR and quantification

The 2 μ l cDNA were added to 50 μ l reaction volume containing 1.5 μ l Taq DNA polymerase, 0.3 mmol/L dNTP, 2.0 mmol/L MgCl₂, 0.25 mmol/L target primers. The gene sequences are listed in Table 1. The PCR amplification and quantification were conducted by a Thermocycler PE 7000 (Perkin Elmer, USA) according to the following protocol: solutions were pre-denatured at 93 °C for 120 s, denatured at 93 °C for 45 s, annealed at 55 °C for 60 s, extended at 72 °C for 90 s, and amplified by 42 cycles. The levels of Col-I, iNOS or TGF- β 1 mRNA in samples were displayed as copy numbers of target mRNA per microgram of total RNA.

2.5. Biochemical analysis

Serum glutamic-pyruvic transaminase (GPT) and creatinine (Cr) were analyzed using biochemical kits (Nanjing Jiancheng BioEng. Ins., China) and a U2001 UV/vis spectrophotometer (Hitachi, Japan) at 505 and 510 nm, respectively.

2.6. Statistical analysis

Data were analyzed with the Statistical Package for Social Science (SPSS, Version 10.0) for Windows. One-way analysis of variance (ANOVA) was used to determine whether an intra-group difference existed; significance between two groups was evaluated with Fisher's least-significant-difference (LSD) comparison procedure of the post hoc Scheffe analysis. A *P*-value of less than 0.05 in a two-tailed test was accepted as significant.

Table 1
Primers and probes of Col-I, iNOS and TGF- β 1

Genes	Sequence of primers and probes	Length (bp)
Col-I	Forward: 5'- CCCAACCCCAAAAACG-3' Reverse: 5'-CTGCGTCTGGTGATACATATT- CTTCT-3' Probe: 5'-FAM-AGGGCGAGTGCTGTCC- TTCT-TAMRA-3'	66
iNOS	Forward: 5'-GTCTTGGTGAAAGCGGTGTC-3' Reverse: 5'- ACGCGGGAAGCCATGAC-3' Probe: 5'-FAM- TTGCTTCTGTGCTAATGCGGAA- TAMRA-3'	71
TGF- β 1	Forward: 5'-GCTGCTGACCCCACTGAT-3' Reverse: 5'-TGCCGGACAACCTCCAGTGA-3' Probe: 5'- FAM-CGCCTGAGTGGCTGTCTTTG- TAMRA-3'	63

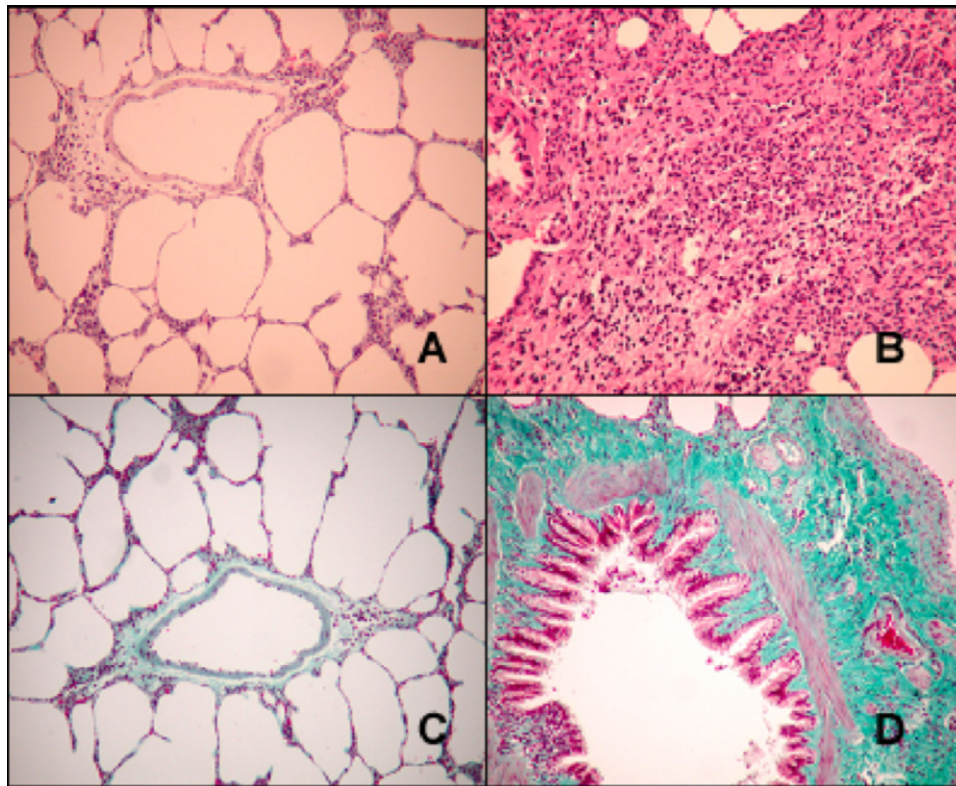


Fig. 1. Histo-morphological appearances of lung samples from the Group NC (A and C, magnification $\times 100$) and Group PC (B and D, magnification $\times 200$) were stained with hematoxylin-eosin (A and B) or Masson's trichrome (C and D). Under the light microscope, few inflammatory cells appeared to be distributed around bronchiole, and alveolar structure was integrated in samples of Group NC (A). In contrast, in the samples of Group PC, there were large amounts of inflammatory cells distributed in wider mesenchyme, collapsed alveoli with lots of inflammatory exudates, and pulmonary hyaline membranes. Telangiectasis, hyperemia and thrombogenesis were also found in the alveolar wall (B). Meanwhile, compared with the Group NC (C), Masson's stain showed an excessive collagen deposition (green staining) in abnormal mesenchymal tissue in samples of Group PC (D) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

3. Results

3.1. Histo-morphological changes in lung

Fig. 1 displays four images of H-E stain and Masson's stain, showing that lung tissue samples in the Group NC had a normal pulmonary architecture with thin-lined alveolar septa, normal cellularity and only a few alveolar macrophages, while the samples in Group PC had typical fibrotic changes including infiltration with excessive inflammatory cells (Fig. 1B), large

collagen depositions, large fibrous areas and collapsed alveolar spaces (Fig. 1D). In addition, telangiectasis hyperemia, thrombogenesis, bronchiole stenosis and wider mesenchyme also were found in the samples of Group PC viewed under a light microscope.

Fig. 2 shows results of immunohistochemical analyses of Col-I, iNOS and TGF- $\beta 1$ expressions and distributions in rat lung samples. Compared with the Group NC in which no abnormal change was found, Group PC samples showed a significantly strong expression of all three proteins. Negative controls

Table 2
Pulmonary protein/mRNA expression of Col-I, iNOS and TGF- $\beta 1$ in 5-group rats (Mean \pm S.D.)

	NC	PC	HC	CH	CL
Protein					
Col-I	0.62 \pm 0.12	1.34 \pm 0.11***	0.81 \pm 0.19###	0.92 \pm 0.27##	0.90 \pm 0.09##
iNOS	0.74 \pm 0.12	1.83 \pm 0.23***	1.27 \pm 0.41##	1.26 \pm 0.42##	1.25 \pm 0.16##
TGF- $\beta 1$	0.74 \pm 0.35	4.26 \pm 0.43***	1.52 \pm 0.21###	1.07 \pm 0.28###	0.81 \pm 0.23###
mRNA					
Col-I	225.15 \pm 153.30	614.87 \pm 208.70**	295.56 \pm 158.34#	262.68 \pm 126.00#	255.22 \pm 293.67##
iNOS	7.62 \pm 1.70	47.46 \pm 18.20***	23.30 \pm 3.28##	9.87 \pm 3.88###	24.97 \pm 21.70#
TGF- $\beta 1$	19.55 \pm 6.58	122.68 \pm 39.10***	27.39 \pm 11.33###	31.61 \pm 8.80###	25.85 \pm 4.04###

ANOVA indicated intra-group differences for the proteins of Col-I ($F = 12.261$, $P < 0.001$), iNOS ($F = 8.388$, $P < 0.001$) and TGF- $\beta 1$ ($F = 112.979$, $P < 0.001$), and mRNA of Col-I ($F = 3.329$, $P < 0.05$), iNOS ($F = 7.605$, $P < 0.001$) and TGF- $\beta 1$ ($F = 26.258$, $P < 0.001$). LSD comparison showed a significant difference between two groups: *** $P < 0.001$ and ** $P < 0.01$ vs. the Group NC, respectively; ### $P < 0.001$, ## $P < 0.01$ and # $P < 0.05$ vs. the Group PC, respectively.

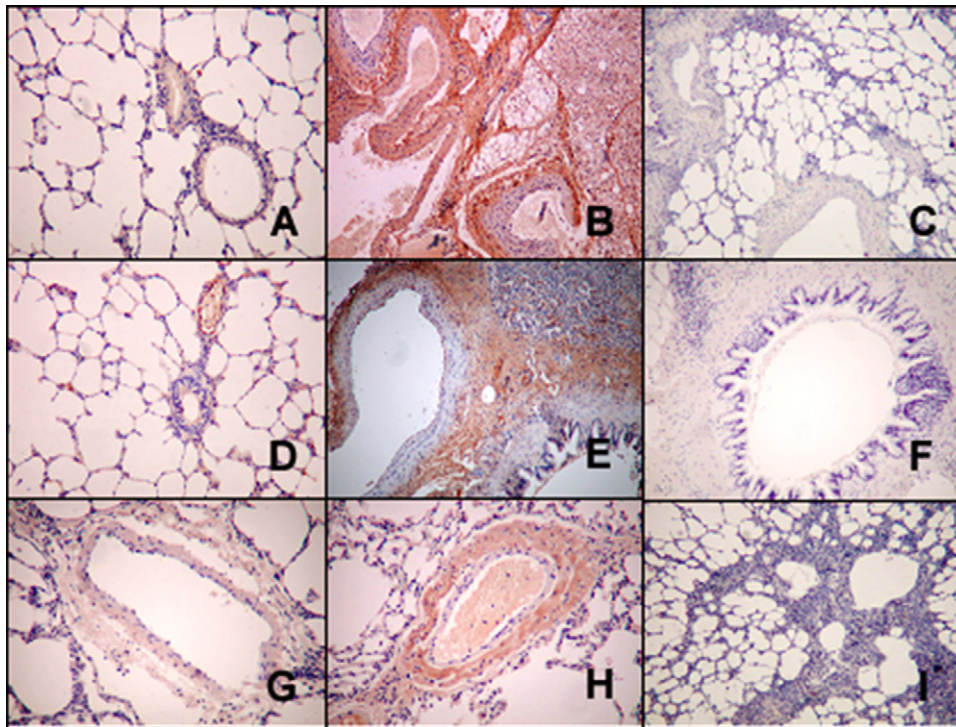


Fig. 2. Immunohistochemical analysis of the Col-I (A–C), iNOS (D–F) and TGF- β 1 expressions (G–I) in lung samples of rats (magnification $\times 100$). Compared with the Group NC (A, D and G), Group PC (B, E and H) showed a significantly strong expression (orange staining) of Col-I (B), iNOS (E) and TGF- β 1 (H) in areas of fibrotic tissues. Negative controls (C, F and I) without the addition of primary antibodies of Col-I, iNOS and TGF- β 1 were shown for the same samples of Group PC (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article).

did not present any apparent non-specific coloration of target proteins.

3.2. Protein expression

Figs. 3 and 4 and Table 2 show a result of Western blotting analysis that was a further quantitative determination of all three proteins following immunohistochemical analysis. Compared with the Group NC, protein expressions of Col-I, iNOS and

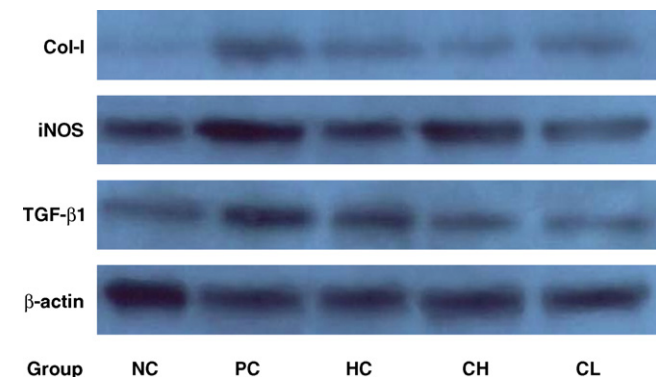


Fig. 3. Western blotting analysis of Col-I, iNOS and TGF- β 1 proteins in lung samples of 5-group rats. Results showed bleomycin-induced over-expression of pulmonary Col-I, iNOS and TGF- β 1 proteins in the Group PC, and a down-regulation in Group HC, CH and CL after treatment with hydrocortisone, high-dose and low-dose curcumin, respectively. The outcomes were determined from five samples for each group and three replicates for each sample. β -actin was used as an internal control to normalize the expressions of Col-I, iNOS and TGF- β 1 in different groups.

TGF- β 1 in Group PC had an average increase of 114, 146 and 476%, respectively ($P < 0.001$). While in the Group HC, CH and CL, Col-I expression was 39, 31 and 33% lower than Group PC ($P < 0.01$), respectively; iNOS expression was 31, 31 and 31% lower than Group PC ($P < 0.01$), respectively; and TGF- β 1 expression was 64, 75 and 81% lower than Group PC ($P < 0.001$), also, respectively. Except that the TGF- β 1 protein expression in Group CH and CL was lower than Group HC ($P < 0.05$), there was no significant difference in any protein expression among these three treatment groups ($P > 0.05$).

3.3. mRNA expression

Fig. 4 and Table 2 also show results of real-time RT PCR analysis. Compared with the Group NC, mRNA expression of Col-I, iNOS and TGF- β 1 in Group PC had an average increase of 173, 523 and 527%, respectively ($P < 0.01$). While in the Group HC, CH and CL, Col-I expression was 52, 57 and 58% lower than Group PC ($P < 0.05$); iNOS expression was 51, 79 and 47% lower than Group PC ($P < 0.05$); and TGF- β 1 expression was 78, 74 and 79% lower than Group PC ($P < 0.001$), all, respectively. There was no significant difference in mRNA expression among these 3 treatment groups ($P > 0.05$).

3.4. Toxicity assessment

Biochemical analysis showed that there was no significant difference in serum GPT and Cr among the five groups. In addition, no significant histological abnormality could be found in

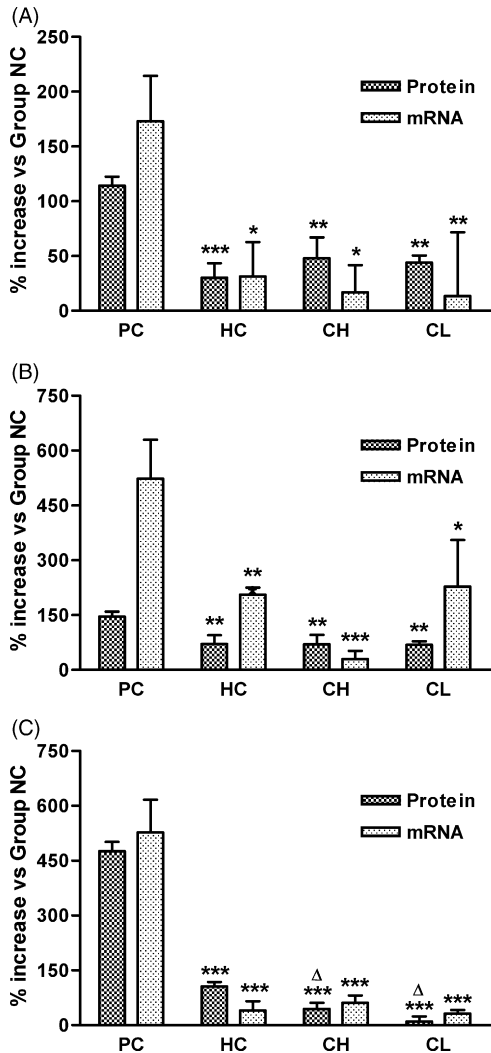


Fig. 4. Western blotting and real-time RT PCR analyses showed a bleomycin-induced over-expressions of pulmonary Col-I (A), iNOS (B) and TGF- β 1 (C) at both protein and mRNA levels in the Group PC, and a down-regulation in Group HC, CH and CL after treatment with hydrocortisone, high-dose and low-dose curcumin, respectively. The values were presented as a percentage increase (Mean \pm S.E.) compared with Group NC by measuring five samples for each group and three replicates for each sample (** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ vs. the Group PC, respectively; $\Delta P < 0.05$ vs. the Group HC).

the heart, liver, kidney, brain, spleen and uterus tissues of any rat in this study.

4. Discussion

Histopathological characteristics of human PF include both fibrogenetic and inflammatory features that are usually seen in BLMPF animal models (Giri, 2003; Gong et al., 2005). In this study, pulmonary fibrotic foci in rat models had formed on the 28th day after bleomycin injection. Other fibrogenetic changes such as a significant fibroblast proliferation and excessive collagen deposition in mesenchyme were found in lung samples of these rats. Meanwhile, many inflammatory cells and much exudate infiltrated to mesenchyme and alveoli were found, representing typical inflammatory changes in the samples. The

histological findings were in accordance with other previous studies (Gong et al., 2005) and constituted evidence that ours was a reliable pathological rat model with regard to pulmonary inflammatory injury and fibrosis endings.

PF results from increased inflammation related to cytokines, fibroblasts and collagen syntheses. Using immunohistochemical techniques, we could determine an over-expression and abnormal distribution of the Col-I, iNOS and TGF- β 1 proteins in lung tissue of BLMPF rats. The disorders of these molecular markers related to pulmonary inflammation and fibrosis were quantitatively identified by Western blotting analysis and were further confirmed by synchronous analysis at transcriptional level in this study. A clear coupling between the over-expression of three proteins and their mRNA in results of this study hinted that the key pathological changes in BLMPF rats could result in an excessive up-regulation of Col-I, iNOS and TGF- β 1 genes.

The over-expression of iNOS may increase synthesis of NO, an important toxic molecule regarding with tissue injury. While NO may stimulate transcription factor nuclear factor- κ B (NF- κ B) to promote TGF- β 1 gene expression and consequently increase TGF- β 1 production (Gossart et al., 1996). Thus, it was postulated that a regulatory network including iNOS and TGF- β 1 may be involved in the development of pulmonary fibrosis. Although an abnormal increase of iNOS in lung tissue may peak on the 5th day after bleomycin injection (Gurujeyalakshmi et al., 2000), we found that pulmonary expressions of both iNOS protein and mRNA on the 28th day were 146 and 523% higher in BLMPF rats than that in normal rats. These abnormal iNOS levels indicated that an inflammatory injury existed in the lungs of experimental rats to the end of our experiments. Meanwhile, the over-expressions of both protein and mRNA of pulmonary TGF- β 1 were shown on the 28th day in BLMPF rats. TGF- β 1 may be another important causative factor, and play a key role during the process of pulmonary fibrosis by increasing the synthesis and secretion of ECM protein through stimulation of its gene transcription, and by decreasing degradation of ECM through inhibition of secretion of protein kinases (Robert et al., 1990). It is well known that the Col-I expression is regulated by TGF- β 1; therefore, depressing over-expression of iNOS and TGF- β 1 may inhibit Col-I increase and collagen synthesis, and should be important therapeutic targets in preventing PF.

Corticosteroids as a classic category of anti-inflammatory reagents have shown their effectiveness for PF treatment in animal studies (Khalil et al., 1993; Dik et al., 2003). Our data indicated that hydrocortisone could decrease the over-expression of pulmonary Col-I, iNOS and TGF- β 1 to normal levels on the 28th day after bleomycin injection. Especially, our work here is the first observation to show a down-regulation of increased iNOS expression caused by hydrocortisone treatment, and provide new evidence to explain the anti-inflammatory mechanism and therapeutic significance of corticosteroids in PF treatment. Furthermore, a parallel down-regulation of Col-I, iNOS and TGF- β 1 at both protein and mRNA levels after hydrocortisone treatment suggests that the amelioration of Col-I may at least partly result from inhibition of iNOS expression and consequent decrease of TGF- β 1 expression.

Curcumin treatment by daily gavage at 500 and 250 mg/kg could also significantly down-regulate excessive Col-I, iNOS and TGF- β 1 expressions in BLMPF rats. Except for levels of TGF- β 1 protein, there was no significant difference in rats receiving hydrocortisone treatment or high- and low-dose curcumin treatments. These findings suggest that curcumin may play a similar role as hydrocortisone in preventing development of PF induced by bleomycin. Some previous studies found that curcumin, like corticosteroids, could decrease the production of tumor necrosis factor- α and interferon- γ (IFN- γ), down-regulate iNOS synthesis and inhibit infiltration of inflammatory cells in BLMPF rats (Li et al., 2004; Punithavathi et al., 2000; Chu et al., 1998). The evidence indicated that curcumin and corticosteroids might regulate the same key cytokines in inflammatory response and play a similar role in PF treatment.

Several studies have presented more evidence to explore the mechanisms of curcumin in treatment of PF. For instance, nuclear factor κ B (NF- κ B) is a transcription factor which can conjugate to the 5' flanking sequence of iNOS gene, and contribute greatly to the iNOS up-regulation in response to various pro-inflammatory stimuli (Chu et al., 1998). Curcumin can abolish the activation of NF- κ B induced by pro-inflammatory factors such as interleukin (IL) 1 α , TNF- α and lipopolysaccharide (Xu et al., 1997–1998). These data suggest that decrease of the up-regulation of pulmonary iNOS in BLMPF rats may partly be due to curcumin's effects on inhibition of NF- κ B.

Furthermore, other studies showed that TGF- β receptor type II (T β R II) was necessary for the activation of TGF- β 1, and curcumin could reduce T β R II expression in a fibrotic model using renal fibroblasts (Gaedeke et al., 2004; Branton and Kopp, 1999). Curcumin may inhibit the DNA-binding activity of activator protein-1 (AP-1), then inhibit T β R II as AP-1 binding sites located in T β R II promoters (Gaedeke et al., 2004; Xu et al., 1997–1998), therefore it is reasonable to presume that the down-regulation of TGF- β 1 after curcumin administration, as in our current study, may involve T β R II reduction, and partly result from curcumin's suppression of AP-1 activation. Although the precise mechanisms by which curcumin regulates cytokines involved in pulmonary inflammatory injury and fibrosis remain uncertain and may be different from corticosteroids, our data in this study provide some new clues for further investigations.

Corticosteroids are commonly blamed for their adverse effects in clinical and experimental studies, but no abnormal changes in biochemical or histopathological parameters were found after hydrocortisone injection in this rat study. The dose and duration of hydrocortisone designed for this study may have been too low and too short to display its toxicity. It has been reported that administration of curcumin at 500 mg/kg/day continually for 80 days did not produce any adverse effect in rats (Wo et al., 2000). Our data confirmed that this dose was safe for rats in a 28-day treatment. Even so, a daily dose of 250 mg/kg should be recommended for future studies as its pharmacodynamic effects in prevention of BLMPF were similar to the daily dose of 500 mg/kg.

In conclusion, we compared the efficacy and safety of curcumin with hydrocortisone in a BLMPF rat model. Our data indicated that curcumin possesses therapeutic effects

comparable to hydrocortisone in treating BLMPF rats. Oral administration of curcumin at a daily dose of both 500 mg/kg and 250 mg/kg significantly inhibited bleomycin-induced over-expressions of Col-I, iNOS and TGF- β 1 at transcriptional and translational levels, and reduced pulmonary inflammatory injury and fibrosis. There was no significant difference between treatments with these two dosages. Curcumin, widely used as a food additive, did not show any adverse effect in our study. Therefore, it could be a valuable herbal component for PF treatment.

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References

- American Thoracic Society, 2000. Idiopathic pulmonary fibrosis: diagnosis and treatment. International consensus statement. American Journal of Respiratory and Critical Care Medicine 161, 646–664.
- Branton, M.H., Kopp, J.B., 1999. TGF-beta and fibrosis. Microbes and Infection/Institut Pasteur 1, 1349–1365.
- Cheung, O.Y., Chan, J.W., Ng, C.K., Koo, C.K., 2004. The spectrum of pathological changes in severe acute respiratory syndrome (SARS). Histopathology 45, 119–124.
- Chu, S.C., Marks-Konczalik, J., Wu, H.P., Banks, T.C., Moss, J., 1998. Analysis of the cytokine-stimulated human inducible nitric oxide synthase (iNOS) gene: characterization of differences between human and mouse iNOS promoters. Biochemical and Biophysical Research Communications 248, 871–878.
- Coker, R.K., Laurent, G.J., Jeffery, P.K., du Bois, R.M., Black, C.M., McAnulty, R.J., 2001. Localisation of transforming growth factor beta1 and beta3 mRNA transcripts in normal and fibrotic human lung. Thorax 56, 549–556.
- Dik, W.A., McAnulty, R.J., Versnel, M.A., Naber, B.A.E., Zimmermann, L.J.I., Laurent, G.J., Mutsaers, S.E., 2003. Short course dexamethasone treatment following injury inhibits bleomycin induced fibrosis in rats. Thorax 58, 765–771.
- Flanders, K.C., Burmester, J.K., 2003. Medical applications of transforming growth factor-beta. Clinical Medicine and Research 1, 13–20.
- Gaedeke, J., Noble, N.A., Border, W.A., 2004. Curcumin blocks multiple sites of the TGF-beta signaling cascade in renal cells. Kidney International 66, 112–120.
- Giri, S.N., 2003. Novel pharmacological approaches to manage interstitial lung fibrosis in the twenty-first century. Annual Review of Pharmacology and Toxicology 43, 73–95.
- Gong, L.K., Li, X.H., Wang, H., Zhang, L., Chen, F.P., Cai, Y., Qi, X.M., Liu, L.L., Liu, Y.Z., Wu, X.F., Huang, C.G., Ren, J., 2005. Effect of Feitai on bleomycin-induced pulmonary fibrosis in rats. Journal of Ethnopharmacology 96, 537–544.
- Gossart, S., Cambon, C., Orfila, C., Seguelas, M.H., Lepert, J.C., Rami, J., Carre, P., Pipy, B., 1996. Reactive oxygen intermediates as regulators of TNF- α production in rat lung inflammation induced by silica. Journal of Immunology (Baltimore, Md.: 1950) 156, 1540–1548.
- Gurujeyalakshmi, G., Wang, Y., Giri, S.N., 2000. Suppression of bleomycin-induced nitric oxide production in mice by taurine and niacin. Nitric Oxide: Biology and Chemistry/Official Journal of the Nitric Oxide Society 4, 399–411.
- Hobbs, A.J., Higgs, A., Moncada, S., 1999. Inhibition of nitric oxide synthase as a potential therapeutic target. Annual Review of Pharmacology and Toxicology 39, 191–220.
- Khalil, N., Whitman, C., Zuo, L., Danielpour, D., Greenberg, A., 1993. Regulation of alveolar macrophage transforming growth factor-beta secretion

- by corticosteroids in bleomycin-induced pulmonary inflammation in the rat. *The Journal of Clinical Investigation* 92, 1812–1818.
- Li, H.P., Li, X., He, G.J., Yi, X.H., Kaplan, A.P., 2004. The influence of dexamethasone on the proliferation and apoptosis of pulmonary inflammatory cells in bleomycin-induced pulmonary fibrosis in rats. *Respirology (Carlton, Vic.)* 9, 25–32.
- Punithavathi, D., Venkatesan, N., Babu, M., 2000. Curcumin inhibition of bleomycin-induced pulmonary fibrosis in rats. *British Journal of Pharmacology* 131, 169–172.
- Raghu, G., Nyberg, F., Morgan, G., 2004. The epidemiology of interstitial lung disease and its association with lung cancer. *British Journal of Cancer* 91, S3–S10.
- Robert, A.B., Flanders, K.C., Heine, U.L., Jakowlew, S., Kondaiah, P., Kim, S.J., Sporn, M.B., 1990. Transforming growth factor- β : multifunction regulator of differentiation and development. *Philosophical Transactions of the Royal Society of London. Series B. Biological Sciences* 327, 145–154.
- Romanska, H.M., Polak, J.M., Coleman, R.A., James, R.S., Harmer, D.W., Allen, J.C., Bishop, A.E., 2002. iNOS gene upregulation is associated with the early proliferative response of human lung fibroblasts to cytokine stimulation. *The Journal of Pathology* 197, 372–379.
- Venkatesan, N., Punithavathi, V., Chandrakasan, G., 1997. Curcumin protects bleomycin-induced lung injury in rats. *Life Sciences* 61, PL51–PL58.
- Wo, X.D., Hong, X.Q., Gao, C.X., Jin, M.M., Li, W.L., Ding, Z.S., Tang, L.H., 2000. Long-period virulent test of curcumin. *Journal of Zhejiang College of Traditional Chinese Medicine* 24, 61–65.
- Worrall, N.K., Misko, T.P., Sullivan, P.M., Hui, J.J., Rodi, C.P., Ferguson Jr., T.B., 1996. Corticosteroids inhibit expression of inducible nitric oxide synthase during acute cardiac allograft rejection. *Transplantation* 61, 324–328.
- Xu, Y.X., Pindolia, K.R., Janakiraman, N., Chapman, R.A., Gautam, S.C., 1997–1998. Curcumin inhibits IL1 alpha and TNF-alpha induction of AP-1 and NF-kB DNA-binding activity in bone marrow stromal cells. *Hematopathology and Molecular Hematology* 11, 49–62.