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Protective roles of Cordyceps on lung fibrosis in cellular and rat models

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ABSTRACT

Ethnopharmacological relevance: *Cordyceps sinensis* is a fungus used in traditional Chinese medicine as a tonic to soothe the lung for the treatment of fatigue and respiratory diseases.

Idiopathic pulmonary fibrosis is a chronic, irreversible and debilitating lung disease showing fibroblast/myofibroblast expansion and excessive deposition of extracellular matrix in the interstitium leading to breathing difficulty. Our previous observation revealed a partial relief of lung fibrosis in patients suffering from severe acute respiratory syndrome (SARS). We hypothesize that Cordyceps has beneficial effects on lung fibrosis and the objective of this study is to explore the target(s) of Cordyceps in the relief of lung fibrosis in animal and cell models and to gain insight into its underlying mechanisms.

Material and methods: A rat model of bleomycin (BLM)-induced lung fibrosis and a fibrotic cell model with transforming growth factor beta-1 induction were employed in the studies.

Results: Reduction of infiltration of inflammatory cells, deposition of fibroblastic loci and collagen, formation of reactive oxygen species, and production of cytokines, as well as recovery from imbalance of MMP-9/TIMP-1, were observed in fibrotic rats after treatment with Cordyceps in preventive (from the day of BLM administration) and therapeutic (from 14 days after BLM) regimens. In a fibrotic cell model with transforming growth factor beta-1 induction, the human lung epithelial A549 acquired a mesenchymal phenotype and an increase of vimentin expression with a concomitant decrease of E-cadherin. This epithelial–mesenchymal transition could be partially reverted by cordycepin, a major component of Cordyceps.

Conclusion: The findings provide an insight into the preventive and therapeutic potentials of Cordyceps for the treatment of lung fibrosis.

1. Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, irreversible and debilitating lung disease showing progressive scarring of the alveolar tissue and leading to difficulty in breathing. The etiology of this disease is largely unknown but it is generally considered to be a result of exposure to occupational and/or environmental risk factors acting in concert with genetic susceptibility to fibrosis (Antoniou et al., 2007). There are more than 5 million people worldwide suffering from this disease, and there is no effective treatment or a cure. Most patients die from this lung disorder within 5 years of diagnosis (Verma and Slutsky, 2007).

IPF has a poor prognosis (Kinnula and Myllärniemi, 2008), and it normally initiates inflammation and oxidant production in the site of injury at early stage. It is characterized by transdifferentiation of

Abbreviations: BALF, bronchoalveolar lavage fluid; BLM, bleomycin; CE, Cordyceps powder extract in culture medium; CS, Cordyceps sinensis; CS1, low-dose CS powder suspension treatment; CS2, high-dose CS powder suspension treatment; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; IPF, idiopathic pulmonary fibrosis; MMP, matrix metalloproteinases; ROS, reactive oxygen species; TGF-β1, transforming growth factor beta-1; TIMP, tissue inhibitors of metalloproteinases

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the epithelial cells, accumulation of fibroblasts/myofibroblasts, formation of fibroblastic foci, and distortion of the lung architecture (Antoniou et al., 2007). The change of epithelial cobblestone-like morphology to myofibroblast phenotype through epithelial–mesenchymal transition (EMT), and the expression of mesenchymal marker vimentin with a concomitant disruption of E-cadherin junction are the hallmarks for IPF. Many patients, however, show little evidence of inflammation, and anti-inflammatory treatments have little impact on the disease (Willis et al., 2006).

The development and progression of IPF is associated with an activation of transforming growth factor beta-1 (TGF-β1), which induces excessive production and deposition of collagen and extracellular matrix (ECM) by the myofibroblasts, as well as disruption of the basement membrane by an imbalance of gelatinases (matrix metalloproteinases) and tissue inhibitors of metalloproteinases (MMP/TIMP) (Coward et al., 2010). The coupling of mitochondrial dysfunction with reactive oxygen species (ROS) production also aggravates the epithelial cell damage in idiopathic interstitial disorder (Kabuyama et al., 2010).

Cordyceps sinensis (Dong-Chong-Xia-Cao, abbreviated as CS) is an entomogenous fungus whose mycelia grow inside the pupae or larvae of Lepidoptera moth in the autumn, with its fruiting body protruding from the dead insect body during the summer. A renowned Chinese medicine to soothe the lung, CS is now often used for the treatment of respiratory diseases and strengthening immune responses. It has also been reported to be an antioxidant and an inhibitor of angiogenesis. Cordyceps extracts have been shown to relieve fibrosis in the liver (Das et al., 2010; Zhu et al., 1998a, 1998b), kidney (Chai et al., 2009), and lung (Wang et al., 2007; Xu et al., 2011) through an inhibition of TGF-β1 expression (Wang et al., 2007; Xu et al., 2011) and promotion of collagen degradation (Li et al., 2006a). The dried form of CS contains ergosterol, polysaccharides, glycoprotein and peptides. Adenosine and cordycepin (3′-deoxyadenosine) are the major components (Li et al., 2006b; Tsai et al., 2010) and they have been demonstrated to modulate cell activation and alter cell morphology (Shin et al., 2009). Several therapeutic strategies targeting various stages of fibrogenesis are being investigated (Selman et al., 2011), and it is the objective of the present study to explore the target(s) of CS in the relief of lung fibrosis and to gain insight into its underlying mechanisms.

2. Materials and methods

2.1. Preparation of Cordyceps

Dried *Cordyceps sinensis* (Berk.) Sacc. (containing the mycelia-filled insect body and the fungal fruiting body) originated from Tibet was donated by the Eu Yan Sang (Hong Kong) Ltd. The herbal materials were authenticated by Prof. Songming Liang of the School of Chinese Medicine (SCM), the Chinese University of Hong Kong, and voucher specimens have been deposited in the herbarium of the SCM. The herbal drug was ground to powder, passed through a sieve of aperture no. 6, and stored at 4° C until use. To mimic the traditional route of administration, the powder was suspended in distilled water and given to the animals intragastrically using a feeding tube.

2.2. Rat model for pulmonary fibrosis induced by bleomycin

All experiments were performed in accordance with the Hong Kong Guidelines for Animal Welfare and the protocols were approved by the Animal Research Ethics Committee of the Chinese University of Hong Kong (Ref. nos. 08/026/ERG and 461908).

Male Sprague-Dawley rats of 8–12 weeks old were housed in normal laboratory conditions at 21 ± 2° C under a 12/12 light–dark cycle. Pulmonary fibrosis was induced by intra-tracheal administration of a single dose of 2.5 mg/kg of bleomycin (BLM, Nippon Kayaku Co. Ltd., Japan) dissolved in 0.25 mL of physiological saline (Bonniaud et al., 2005) (Day 1). Pulmonary fibrosis developed in 2 weeks (Day 14), confirmed by morphological changes in the lungs and an accumulation of excessive interstitial collagen (Chaudhary et al., 2006). Intragastric administration of CS during the course of fibrosis development (Days 1–14 after BLM administration) was considered prophylactic/preventive, whereas administration of CS after fibrosis had already developed (Days 14–28) was considered therapeutic in the present protocol.

In order to determine an optimal dose of CS, Experiment 1 was performed in which 32 rats were equally and randomly divided into four groups, namely (a) bleomycin-induced fibrosis rats (BLM group), (b) low-dose CS treatment (CS1) (0.54 g/kg body weight, equivalent to 2-fold human dose of 0.27 g/kg) (Hui and Chan, 2006), (c) high-dose CS treatment (CS2) (1.35 g/kg body weight, 5-fold human dose), and (d) normal animals receiving vehicle (CTL) as sham controls. The CS1 and CS2 groups of animals received daily intragastric administration of CS1 or CS2 (each 0.5 mL) for 14 days before the lung tissues were collected for morphological and biochemical analyses.

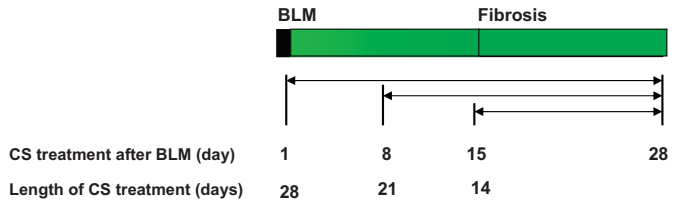
All rats survived BLM treatment. The results of Experiment 1 showed more potent antifibrotic activity in the CS2 group. A dose of CS at 1.35 g/kg was therefore used in the subsequent time course study in Experiment 2 (see Scheme 1). Thus, forty rats were divided into five groups: four groups received intra-tracheal administration of BLM, and the fifth group only received water as sham control (CTL). Among the four groups of BLM-treated rats, three groups received daily CS2 dose (1.35 g/kg) starting from Day 1, Day 8 and Day 15, respectively, and they were all sacrificed on Day 28. One group of fibrotic rats received water to serve as positive lung fibrosis control.

2.2.1. Microscopy

Rats were anaesthetized by 3.5% chloral hydrate (1 mL/100 g body weight) and the lungs were removed and divided into two halves: the right lung was used for differential alveolar cell counts by May-Grunwald–Giemsa stain, and the left lung was further separated into two portions: The upper portion fixed in 3% paraformaldehyde for histopathological and immunohistochemical analyses, and the lower portion frozen in liquid nitrogen for RNA analysis and histochemical analysis of ROS production (oxidative stress) in alveolar tissues.

2.2.2. Differential cell count

Alveolar cells were collected from right lungs of treated and untreated rats by bronchoalveolar lavage with physiological saline. Cells in the bronchoalveolar lavage fluid (BALF) were spread on clean glass slides using a Thermo Shandon Cytospin 2 centrifuge (Southern Products Ltd., Cheshire, UK), air-dried, fixed with ethanol, and stained with May Grünwald–Giemsa. Total cell number and percentages of macrophages, lymphocytes,



Scheme 1. Schematic diagram showing CS treatment schedules in Experiment 2.

neutrophils, and eosinophils in bronchoalveolar cells on cytospin slides were recorded.

2.2.3. Histopathological and immunohistochemical microscopy

Paraformaldehyde-fixed lung tissues were processed for paraffin embedding and sections of 5 μm thick were cut using a microtome, followed by staining with haematoxylin and eosin (H&E) for histopathological examination. Sections stained with Masson's trichrome were analyzed for collagen deposition in the lung tissues.

For immunohistochemical analysis, paraffin sections were dewaxed with xylene, and re-hydrated with graded ethanol before they were stained with antibodies against TGF-β1 (T1654 Sigma, St Louis, USA), MMP-9 (3309 Chemicon, Merck BioSciences, Darmstadt, Germany), and TIMP-1 (Ab-1 IM32 Calbiochem, Temecula, CA, USA) in the alveolar tissue.

Frozen lung sections of 15 μm thick were cut by using a Thermo cryotome (Shandon, Southern Products Ltd.). After incubation with intracellular fluorescent probe dihydroethidium (40 μM) (DHE; Molecular Probes, Eugene, USA) and NADPH (1 mM) in a light-protected humidified chamber at 37° C for 30 min, the oxidative signal (ROS) was analyzed using a fluorescence microscope. Sections incubated with 40 μM DHE without NADPH served as the "positive" control.

2.2.4. Biochemical analysis

Frozen left lung tissues were rinsed briefly with phosphate buffer and total proteins obtained by extracting with lysis buffer (50 mM Tris buffer, pH 8.0, containing 100 μg/mL PMSF, 1 μg/mL aprotinin, 1% triton and proteinase inhibitor cocktail). Protein array analysis was then performed using the Rat Antibody Array 1 (RayBiotech, Inc., GA, USA).

2.2.5. Measurement of collagen

Hydroxyproline was assessed as a measure of collagen deposited in the lung tissue (Reddy and Enwemeka, 1996). Briefly, hydroxyproline in the lung tissue homogenate was oxidized in 1 M acetate buffer containing 1.4% chloramine T (857319, Sigma, USA), 1 M Ehrlich's reagent, and 1 M 4-dimethylaminobenzaldehyde (D2004, Sigma, USA) at 65 °C for 15 min to develop chromophores. The optical density of the reaction product corresponding to the amount of collagen was measured at 550 nm using an ELISA microplate reader.

2.2.6. Transcription of fibrotic genes

Lung tissues were removed from the control and treated rats for total cellular RNA isolation using TRIzol reagents. One microgram of total RNA was directly reverse-transcribed at 42 °C for 50 min using a Superscript™ preamplification system (18089-011, GIBCO/BRL, Grand Island, NY, USA). Each RT product was subjected to semi-quantitative PCR using Thermoprime DNA polymerase and primers against TGF-β1, procollagen Iα, TIMP-1 and MMP-9 from GIBCO/BRL (Grand Island, NY, USA) (Table S1, Supporting information) in a 9700 Perkin Elmer thermal cycler. The PCR products were separated on a 1.2% agarose gel, and band intensity was measured by a Chemiluminescence Imaging Analysis System using FluorChem software (Alpha Innotech Corp. USA). The intensity of each band was normalized to that of the internal control, β-actin (Table S1, Supporting information) (Liu et al., 2005).

For the quantitative analysis of fibrotic proteins (TIMP-1, TGF-β1 and MMP-9) in Experiment 2, a quantitative real time RT-PCR method with Taqman probes from Applied Biosystems (USA) was used (Table S2, Supporting information).

2.3. Antifibrotic mechanism of Cordyceps extract and its components in a cell model cell culture

Human lung carcinoma epithelial A549 cells (ATCC CCL-185) were cultured in F-12K medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 incubator at 37 °C. Cells were seeded in 6 cm culture dishes at 0.5×10^6 for analysis of EMT protein expression.

2.3.1. Immunoblotting of marker proteins for EMT

Fibroblastic changes were induced in A549 cells by treatment with 5 ng/mL of TGF-β1 for 48 h, and the EMT phenotype was assessed by co-treatment of these cells with (1) *Cordyceps* extract (8 mg/mL), and (2) 80 μM cordycepin (Sigma C3394). For cell assay, *Cordyceps* powder was extracted with culture medium (8 mg/mL) at room temperature, centrifuged for 10 min at 700 *g* and the supernatant was collected as *Cordyceps* extract (CE) for cell assays. Cell lysates were collected after treatment with CE and cordycepin for 48 h and subjected to immunoblotting using antibody against epithelial marker protein, E-cadherin (3194 Cell Signaling Technol, Danvers, MA, USA), and a mesenchymal marker, vimentin (MS129 Lab Vision, Thermo Fisher Scientific, Fremont, CA). Band intensities were quantified by the software PD Quest (BioRad Laboratories, Hercules, CA, USA) and normalized by GAPDH (5174 Cell Signaling Technol.) (Liu et al., 2005).

2.4. Statistical treatment of data

Data were analyzed by one-way analysis of variance, followed by Duncan's multiple range test to detect intra-group (inter-group for 2-way analysis) differences using GraphPad Prism sofeware 5.0. Significant difference was considered when $p < 0.05$.

3. Results

3.1. Experiment 1: dose-dependent antifibrotic activity of CS

3.1.1. Morphological observations and fluorescence microscopy

The lungs of untreated Sprague-Dawley rats showed normal alveolar pattern with broncheoli surrounded by alveolar sacs and alveoli separated by alveolar septa (Fig. 1a). Pathological features in rat lungs of BLM-induced fibrosis with and without CS2 treatment are summarized in Table 1.

Significant increase ($^{***}p < 0.01$) of infiltrated inflammatory cells, including macrophages, neutrophils, eosinophils, lymphocytes (Table S3, Supporting information), and presence of peribronchial and interstitial fibrosis in foci or regions was prominent in alveolar tissues of rats after treatment with BLM for 14 days (Fig. 1b). In these lungs, an intense staining for collagen fibers was demonstrated by Masson's trichrome stain (Fig. 1e–h), and about 3-fold increase (0.024 mg/mg protein vs 0.008 mg/mg protein for BLM and untreated sham control, respectively) was observed by hydroxyproline analysis for collagen fibers (Fig. 2). However, the fibrogenic remodeling intensity was significantly reduced in rats treated with CS for 2 weeks, which was associated with decreased numbers of inflammatory cells ($^{***}p < 0.01$) (Table S3, Supporting information) and fibroproliferative foci (Fig. 1c and d; g and h). The levels of collagen were reduced by 37.5% and 45% in CS1 and CS2 groups, respectively (Fig. 2), indicating a dose-dependent attenuation of lung fibrosis in BLM-rats after CS treatment for two weeks.

Since reactive oxygen species (ROS) and tissue remodeling regulators, e.g., metalloproteinases (MMPs) and their inhibitors (TIMPs), are mediators for the development of BLM-mediated

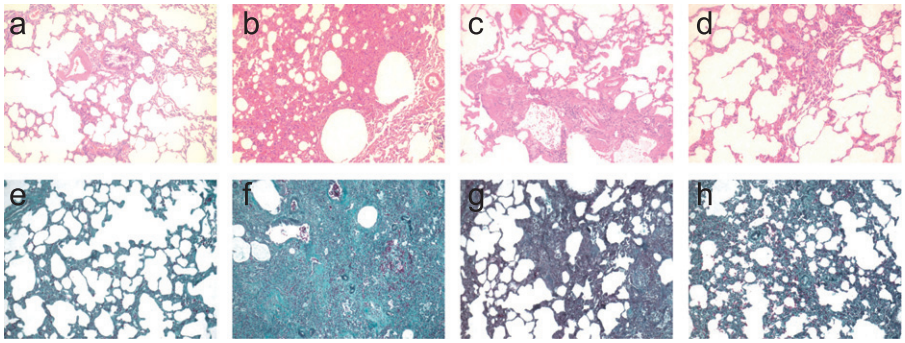


Fig. 1. Morphology of lungs and collagen content from rats of (a) and (e) normal untreated, or treated with (b) and (f) bleomycin, (c) and (g) bleomycin + 0.54 g/kg CS (CS1), and (d) and (h) bleomycin + 1.53 g/kg CS (CS2) for 14 days. Left lung of untreated and treated rats was fixed in 3% paraformaldehyde, embedded in paraffin, and sections of 5 μm were stained with Haemoatoxylin and Eosin dyes. Morphological changes and collagen deposition in alveolar tissues, particularly in fibrotic areas stained with Masson's trichrome, were observed under a Zeiss Axioskop (200x).

Table 1
Pathological features in rat lungs of BLM-induced fibrosis with and without CS2 treatment.

	Sham	BLM	BLM + 14d CS2	BLM + 21d CS2	BLM + 28d CS2
Mucosal lymphoid tissue	+	+	+	+/+++	+
Alveolar hemorrhage	No	Focal	No	No	No
Hemosiderin deposit	Absent	Yes	No	No	Focal
Alveolar macrophages	Absent	Yes	Absent	Absent	Yes
Interstitial inflammation	Absent	Minimal	Absent	Absent	Absent
Peribronchial fibrosis	Absent	Present	Absent	Absent	Present
Type 2 pneumocytes	Absent	Focal	Absent	Absent/focal	Absent

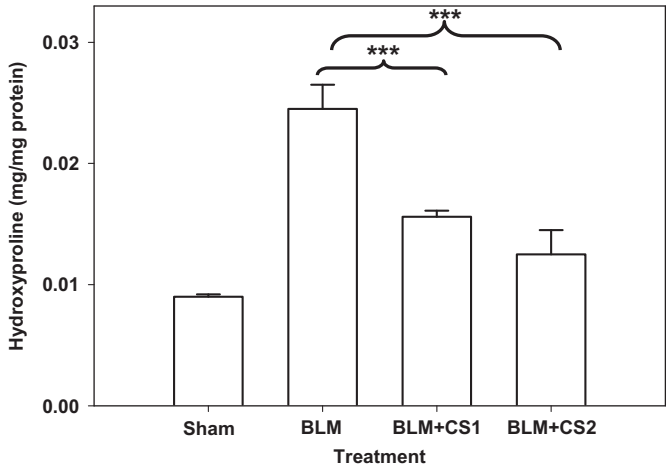


Fig. 2. Hydroxyproline assessment as a measure of collagen deposition in lungs from normal, bleomycin treatment, bleomycin treatment with CS1, and bleomycin treatment with CS2. *Cordyceps* powder suspension significantly ($^{***}p < 0.05$) alleviated collagen deposition in lungs of both CS1 and CS2 treatment groups comparing to BLM treatment group. Data are presented as mean and standard deviation of 8 samples ($n = 8$).

lung fibrosis (Liu et al., 2007), an *in situ* detection of ROS (superoxide) and RT-PCR analysis of regulators at transcriptional levels was performed. A mild emission of red fluorescence from DHE, a superoxide anion-specific indicator, was found to be present in the lung tissue of untreated rats, but it increased intensively in the lung tissue of BLM-treated rats (Fig. S1, Supporting information), indicating a strong ROS production in the fibrotic tissues (Fig. S1b, Supporting information). The fluorescence returned to control levels in CS1 rats (Fig. S1c, Supporting information), and further to an invisible level in CS2 rats (Fig. S1d, Supporting information).

3.1.2. Measurement of production of procollagen and tissue remodeling regulators

MMP-9, TIMP-1, TGF-β1 and collagen are pro-fibrotic genes that were activated in lung fibrosis, and thus no MMP-9 and procollagen transcripts but only mild levels of TGF-β1 and TIMP-1 transcripts were found in normal lung tissues by RT-PCR analysis. All these fibrogenic genes were expressed dramatically in the lungs of BLM-treated rats, but they were suppressed by CS treatments in a dose-dependent manner (Fig. S2, Supporting information). This finding was partially confirmed by protein array analysis; out of 16 cytokine proteins analyzed TIMP-1 protein in lung tissues from BLM-rats was significantly suppressed by CS2 treatment (Fig. S3, Supporting information).

3.2. Experiment 2: time-dependent antifibrotic activity of CS

Cordyceps treatment (CS1 and CS2 groups) for two weeks had attenuated BLM-induced lung fibrosis in a dose-dependent manner in Experiment 1 (Figs. 1 and 2) and thus the high-dose (CS2) was used in subsequent time-dependent experiments. In order to study the preventive/prophylactic and the therapeutic effects of CS treatments, CS2 was given before, during and after lung fibrosis was developed (i.e., immediate, 1-week and 2-week after intragastric BLM injection, respectively). These rats received CS2 treatments for 28 day, 21 day and 14 day, respectively, and the lung tissues were subjected to morphological and biochemical analysis.

Focal alveolar hemorrhage with hemosiderin deposition, and mild interstitial inflammation with macrophages, eosinophils, neutrophils and lymphocytes (Table S3, Supporting information), as well as peribronchonchial fibrosis, were observed in the lung tissue of BLM-rats (Table 1). Results shown in Fig. 3 further confirm an increase of lung hydroxyproline content in BLM-rats by 1.72 folds in comparison to the untreated sham controls, which was consistent with the findings in Experiment 1. The hydroxyproline

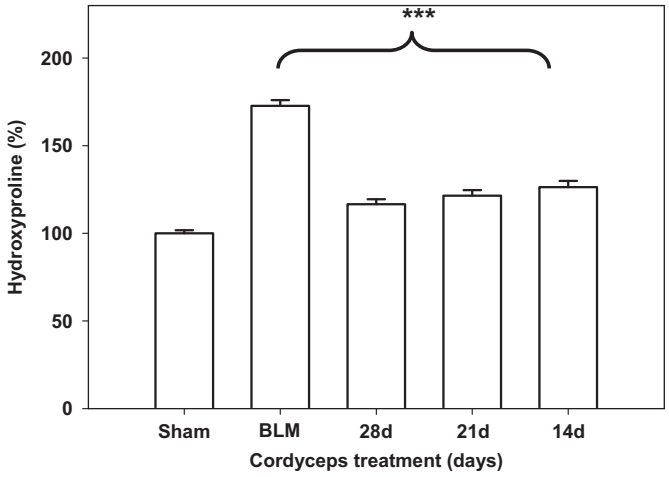


Fig. 3. Hydroxyproline assessment as a measure of collagen deposition in lungs from sham control, bleomycin treatment, and bleomycin treatment with CS2 for 14, 21 and 28 days. *Cordyceps* extracts significantly (***$p < 0.05$) alleviated collagen deposition in all CS2 treatment groups comparing to the BLM-treated group. Data are presented as mean and standard deviation of 8 samples ($n=8$).

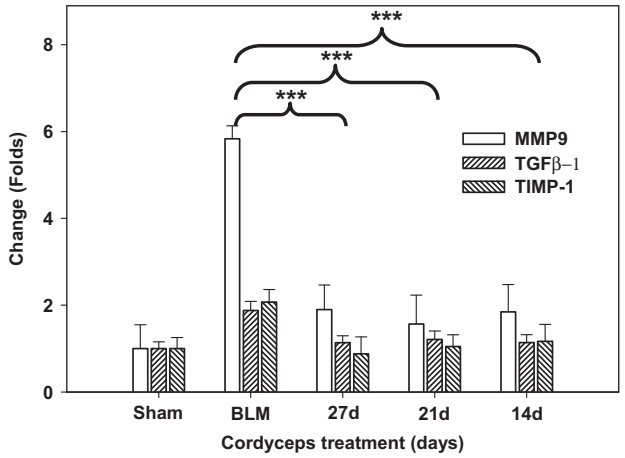


Fig. 4. Analysis of profibrotic gene transcription by quantitative real time-PCR. Total RNA was isolated from lung tissues of untreated and BLM-treated rats, and reverse transcribed into cDNA for PCR using Taqman probes for MMP-9, TGFβ-1 and TIMP-1 genes ($n=6$). Increase of fibrotic gene transcription, including TIMP-1, procollagen I, TGF-β1, and MMP-9, in lung tissues from BLM-treated rats, but the transcripts returned to normal levels after time-course CS2 treatments.

production was significantly suppressed to the untreated control levels after CS treatments in a time-independent manner (Fig. 3).

To quantify the changes of fibrogenic genes after CS2 treatment at various time points, total RNAs were collected for measurement by quantitative real time RT-PCR. The results suggested a 6-fold increase of MMP-9 in lung tissues of BLM-rats, which was gradually reduced to about 2-folds after CS2 treatment. Similar suppression of fibrogenic gene expression, i.e., TGF-β1 and TIMP-1, was observed in lung tissues with all three time-course treatments (Fig. 4), indicating no significant difference among different treatment times (14, 21 or 28 day), and before or after the development of fibrosis (28-day and 14-day treatment groups, respectively).

3.3. Anti-EMT effect of Cordyceps extract and its active ingredients

Alveolar epithelial A549 cells normally joined to one another at cell junctions to form a layer of cobblestone-like appearance (Fig. 5a) with a high expression of E-cadherin in cell culture, but they changed into spindle fibroblast-like phenotype in the

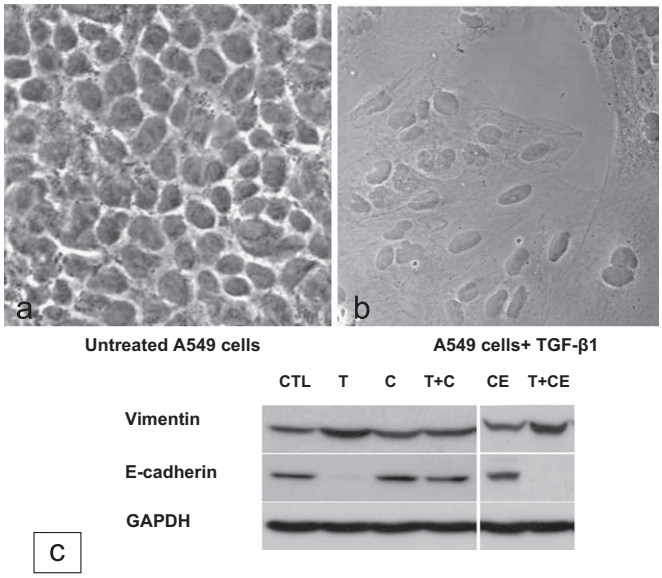


Fig. 5. Phenotypic change of cobblestone-like A549 cells (a) altered to spindle-shaped myofibroblasts (arrows, (b)) in the presence of TGF-β1 for 48 h. Immunoblotting (c) reveals a TGF-β1-mediated (T) decrease of epithelial E-cadherin (3% of CTL) with a concomitant up-regulation of vimentin (175% of CTL) in A549 cells. The changes were reversed (133% and 119% for vimentin and E-cadherin, respectively) by 80 μM cordycepin (C) but not by 8 mg/mL *Cordyceps* extract (CE) (152% and 2% for vimentin and E-cadherin, respectively). No significant change in expression of vimentin (103%) and E-cadherin (99%) in CE group was compared with those of untreated controls (CTL) (100% for both vimentin and E-cadherin).

presence of 5 ng/mL of TGF-β1 for 48 h (Fig. 5b). The E-cadherin protein decreased to undetectable level (100%–3% for untreated control and TGF-β1 groups, respectively) but the expression of vimentin increased dramatically, typical features of epithelial–mesenchymal transition. In order to determine if EMT was inhibited by active ingredients of *Cordyceps* (e.g., cordycepin), A549 cells were co-treated with TGF-β1 and *Cordyceps* extract (CE, 8 mg/ml) or 80 μM cordycepin for 48 h. The expression of vimentin and E-cadherin was found to reverse back to those in epithelial phenotype by cordycepin but not by CE (Fig. 5c), implicating that TGF-β1-induced EMT had been inhibited. On the other hand, the low level of cordycepin in crude *Cordyceps* extract may be insufficient to reverse the EMT process.

4. Discussion

Idiopathic pulmonary fibrosis is a chronic, irreversible and debilitating lung disease with fibroblast expansion and excessive deposition of extracellular matrix in the interstitium that causes breathing difficulty. There are more than 5 million people worldwide suffering from this disease, but all current treatments are not effective, and patients usually die from this lung disorder within 3 years of diagnosis (Antoniou et al., 2007). *C. sinensis* (Dong-Chong-Xia-Cao) is a folk medicine in China to soothe the lung for the treatment of fatigue and respiratory diseases, as well as modulation of immune response, redox balance, inhibition of angiogenesis and the relief of liver fibrosis. Clinically a partial relief of lung fibrosis has been achieved in patients suffering from severe acute respiratory syndrome (SARS) (Hui and Chan, 2006). As a continuous study on the protective/therapeutic mechanisms of *Cordyceps* against lung fibrosis, we analyzed the morphological changes of lung tissues and the expression of fibrogenic factors in a rat model of bleomycin-induced lung fibrosis in order to gain insight into the treatment of this hampering disease.

Lung fibrosis is characterized by abnormal proliferation of alveolar mesenchymal cells, e.g. myofibroblasts, and activation of inflammatory cells, e.g. neutrophils and macrophages (Chaudhary et al., 2006; Dunkern et al., 2007). All these cells under stress produce TGF-β1 which activates characteristic accumulation of matrix proteins, mainly type I collagen and matrix metalloproteinases (MMPs) in IPF or in animal models of BLM-induced fibrosis (Cutroneo et al., 2007). MMPs are a family of ECM degrading enzymes from inflammatory cells which play important roles in inflammation and tissue remodeling (Yoshimura et al., 2006). MMP-9, a member of the MMP family commonly associated with IPF, binds non-covalently to its specific inhibitor, tissue inhibitor of metalloproteinase 1 (TIMP-1), in a 1:1 M ratio. About 2-fold increase of TGF-β1 and TIMP-1, and 5.8-fold increase of MMP-9 were found in lung tissues of BLM-rats, making MMP-9/TIMP-1 imbalance and accumulation of alveolar ECM commonly found in human fibrotic lungs (Kelly and Jarjour, 2003; Yoshimura et al., 2006). An increase in MMP/TIMP favors tissue injury, while the reverse may be associated with anti-fibrosis (Kelly and Jarjour, 2003). The quantitative RT-PCR was only performed in Experiment 2, in which lung fibrosis is alleviated at a decrease of MMP-9/TIMP-1 in a time-independent manner as shown in all CS treatment groups. It is, however, noteworthy that MMP-9 transcription was suppressed to a greater extent than that of TIMP-1, resulting in a significant reduction of MMP-9/TIMP-1 imbalance (from 5.8 to around 1.6–1.8 for all CS treatment groups), and thus attenuating the fibrotic injury of the BLM-rats in Experiment 2.

Imbalance of MMP-9/TIMP-1 was induced by oxidative stress (Yoshimura et al., 2006), but alleviated by antioxidants, e.g., epigallocatechin-3-gallate (EGCG) (Sriram et al., 2008) and resveratrol (Sener et al., 2007). Although antioxidative activity was demonstrated in *Cordyceps*, it is, however, unlikely the cause for its antifibrotic activity (Yu et al., 2006) because even *N*-acetylcysteine (NAC), a strong oxidant scavenger, can only slightly alleviate fibrogenesis when it is prescribed in association with corticosteroid for this incurable lung disease (Cottin and Cordier, 2008; Moeller et al., 2008). In addition, cordycepin, the active ingredient isolated from Cordyceps, did not show significant antioxidative activity (Yu et al., 2006), implicating current information about pharmacological activities of *Cordyceps* is insufficient to explain its underlying anti-fibrotic mechanism.

Among the wide range of biomedical properties of *Cordyceps*, antifibrotic activities reported in animal fibrosis models of kidney (Zhao and Li, 1993), liver (Zhang et al., 2004), and lung (Wang et al., 2007; Xu et al., 2011) receive pharmaceutical attention because fibrosis is an irreversible and debilitating disease. In those studies, *Cordyceps* was given orally in either suspension or aqueous extract (Nan et al., 2001; Won and Park, 2005), ranging from 0.3 g/kg/day for 9 weeks (Liu et al., 2003) to 0.66 g/kg/day for two weeks (Wang et al., 2007). We have also demonstrated a partial relief of lung fibrosis in patients suffering from severe acute respiratory syndrome (SARS) following an oral administration of *C. sinensis* powder at 0.27 g/day for 12 weeks (Hui and Chan, 2006). *C. sinensis* is the authentic Dong Chong Xia Cao, but other species, e.g., *C. militaris,* and *C. martialis* are occasionally used as substitutes because of their similar biological activities (Li et al., 2006b) and lower market values. Interestingly, similar antifibrotic results, including decreased production of TGF-β1 and collagen, and restoration of MMP/TIMP imbalance, were claimed in the treatment of liver or lung fibrosis with extracts from different *Cordyceps* species (i.e., *C. sinensis* or *C. militaris*) (Liu and Shen, 2003; Nan et al., 2001; Wang et al., 2007, Won and Park, 2005; Xu et al., 2011). Some common bioactive ingredients across *Cordyceps* species may attribute to such activity.

A dose-dependent antifibrotic activity of *Cordyceps* suspension was demonstrated in Experiment 1, in which *Cordyceps* was administered during the development of lung fibrosis. Similar conclusion was recently made by Xu et al. (2011), who showed a decrease in TGF-β1 by cultured CS. The significance of the present study is perhaps the finding of alleviation of lung fibrosis after the fibrosis had developed (Experiment 2). The results of inhibition of critical fibrosis mediator (TGF-β1), reduction of leukocyte infiltration and collagen production, and the recovery from imbalance of tissue degradation and remodeling (MMP-9/TIMP-1) do not show significant difference among groups of CS treatments for 14, 21 and 28 day. These results implicate both preventive and therapeutic potential of CS in the rat model of lung fibrosis induced by BLM (Hübner et al., 2008). Further studies are required to identify active components in *Cordyceps* responsible for its antifibrogenic mechanisms.

In addition over-expression of MMP/TIMPs by fibrogenic cells, phenotypic alteration of cobblestone-shaped epithelial cells to more mobile myofibroblasts (i.e., EMT) and the formation of fibroblastic foci in IPF lungs are prominent features of experimental and human lung fibrosis (Antoniou et al., 2007). These cells lose their cell–cell junctional protein, E-cadherin, and express mesenchymal markers such as vimentin and α-smooth muscle actin (α-SMA). EMT is thus critical to the development and progression of fibrosis, and its inhibition is considered a chemotherapeutic target for fibrosis. A preliminary study did show reverted EMT by cordycepin at 80 μM but not by crude *Cordyceps* extract in cultured A549 cells in the present study, probably attributed to relatively low level of cordycepin (40–60 μg/g) and high cytotoxicity of other ingredients in the crude *Cordyceps* extract (unpublished data). A combined pharmacokinetic and pharmacological analysis of *Cordyceps* extract or its active ingredients (e.g., cordycepin, adenosine and others) in the prevention of EMT may provide an insight into the antifibrogenic properties of *Cordyceps*.

There is increasing evidence for pro-fibrotic roles of growth factors [e.g. platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF)] (Chaudhary et al., 2006; Moeller et al., 2008), cytokines (e.g., tumor necrosis factor (TNF-α) and interferon-gamma (IFN-γ)) (Moeller et al., 2008), and soluble IL-2R (Hui and Chan, 2006). Scientific research in laboratories and clinical screening for therapeutic agents targeting these factors are extensively studied globally (Moeller et al., 2008). It is interesting to find that CS not only down-regulates pro-fibrotic factors, such as TGF-β1 and MMP-9, in the present study, but also suppresses soluble IL-2R (Hui and Chan, 2006), VEGF, inflammatory cytokines (TNF-α and IFN-γ) (Li et al., 2006a), and anti-apoptotic activity (Shahed et al., 2001). Such a wide range of activities warrants further investigation for the potential development of CS as a therapeutic agent for lung fibrosis.

5. Conclusion

Our animal results suggest that *C. sinensis* not only possesses preventive but also therapeutic potentials in the treatment of lung fibrosis, and that one of its active ingredient, cordycepin, can partially reverse TGF-β1-induced epithelial–mesenchymal transition in A549 alveolar epithelial cells.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2012.06.033.

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