

Original Research Article

Chrysophanol administration alleviates bleomycin-induced pulmonary fibrosis by inhibiting lung fibroblast proliferation and Wnt/ β -catenin signaling

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Abstract

Purpose: To determine the functional effect of chrysophanol (CH) on bleomycin (BLM)-induced pulmonary fibrosis (PF) and reveal its mechanism of action.

Methods: A mouse model of PF was established by intratracheal instillation of BLM (5 mg/kg), prior to CH administration. Masson's trichrome staining was used to analyze interstitial fibrosis and collagen deposition. Hydroxyproline (HYP) content was measured, and lung fibroblast viability determined by MTT assay. Bronchoalveolar lavage fluid (BALF) was collected, and levels of tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, and interferon- γ (IFN- γ) were evaluated using enzyme-linked immunosorbent assays (ELISA). Expression of cell signaling, adhesion, and apoptotic proteins were determined by western blotting.

Results: Administration of CH reduced collagen deposition and HYP content, downregulated α -smooth muscle actin, upregulated E-cadherin, and decreased the levels of TNF- α , IL-1 β , IL-6, and IFN- γ in BLM-treated mice. The viability of lung fibroblasts was also reduced, and Bcl-2-associated X protein and cleaved caspase-3 were upregulated after CH treatment in BLM-treated mice. In addition, CH treatment in BLM-treated mice significantly increased levels of cytoplasmic β -catenin but decreased its expression in the nucleus.

Conclusion: Administration of CH alleviated BLM-induced PF by inhibiting lung fibroblast proliferation and nuclear translocation of β -catenin. Thus, this study provides a potential therapeutic strategy for PF.

Keywords: Chrysophanol, Bleomycin, Pulmonary fibrosis, Hydroxyproline, E-cadherin

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INTRODUCTION

Pulmonary fibrosis (PF) is a progressive disorder characterized by pathological destruction of lung

tissue architecture and extracellular matrix (ECM) accumulation in the interstitial and alveolar spaces [1]. Idiopathic PF (IPF), a diffuse lung condition with chronic progressive fibrosis,

is a fibroproliferative disease that manifests as chronic damage of alveolar epithelial cells and abnormal tissue repair, leading to fibroblastic foci formation [2]. However, the etiology and pathogenesis of IPF remain unclear, and effective treatments to prolong the survival of patients with IPF are lacking. Therefore, exploring effective and novel therapies for IPF patients is of great importance. Rhubarb (*Rheum palmatum L.*), a traditional medicine in China, is widely used for treating various diseases [3,4]. Chrysophanol (CH), an active ingredient of rhubarb, has potent anti-oxidative [5], anti-inflammatory [6], and anti-diabetic [7] properties. In addition, a recent study found that CH exerted a protective effect against paraquat-induced lung injury via activation of peroxisome proliferator-activated receptors (PPARs) [8]. Another study reported that CH could alleviate ovalbumin-induced chronic lung toxicity by inhibiting Th17 cell responses [9]. However, the role of CH in bleomycin (BLM)-induced PF is poorly understood. The Wnt signaling pathway plays a pivotal role in various biological processes, tissue and organ homeostasis, organogenesis, embryogenesis, and the development of human cancers [10]. β -catenin mediates the Wnt pathway, and accumulation of nuclear β -catenin contributes to systemic sclerosis-associated fibrosis [11]. A recent study reported that aberrant Wnt/ β -catenin signaling could promote organ fibrosis development [12]. Excessive expression of β -catenin was also observed in hepatic fibrosis, and inactivation of Wnt/ β -catenin signaling prevented the activation of hepatic stellate cells [13]. These findings raised the possibility that the role of CH in BLM-induced PF may be associated with Wnt/ β -catenin signaling. To verify this relationship, a mouse model of pulmonary fibrosis was established via BLM treatment, and the underlying mechanism of CH activity was investigated.

EXPERIMENTAL

Induction of PF model

Male C57BL/6 mice (n=18; 8 weeks of age; weight: 20 ± 2 g) were obtained from Charles River Laboratory Animal Co., Ltd. (Wilmington, MA, USA) and housed in standard laboratory cages for 3 days prior to experiments. The experimental protocol conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [14] and was approved by the institutional ethics committee of Guangdong Medical University (approval no. 2018065).

Mice were randomly assigned into three groups (n=6 per group): sham operation (sham), BLM, and BLM+CH. BLM (5 mg/kg; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was dissolved in saline and intratracheally instilled in the mice. After BLM challenge, mice were fed with 10 mg/kg/day CH (Seebio Biotech Company Ltd, Shanghai, China) for 4 weeks. Sham mice received the same volume of saline instead of CH. At day 28, animals were sacrificed under general anesthesia by exsanguination. Pulmonary tissues were collected and frozen immediately in liquid nitrogen.

Histopathological examination

Pulmonary samples were fixed, embedded in paraffin, and sectioned into 5- μ m slices, followed by Masson's trichrome staining (Sangon Biotech, Inc., Shanghai, China). The slides were observed under a light microscope and analyzed using ImageJ software (NIH, Bethesda, MD, USA).

Hydroxyproline (HYP) assay

Assays were performed to quantify levels of HYP [15]. Briefly, pulmonary tissues were digested, diluted using papain solution to obtain a volume of 100 mL, and incubated with 625 μ L chloramine-T, followed by addition of Ehrlich's solution (625 μ L) and incubation at 65°C for 20 min. The reaction was quenched by immersion in cool water. Absorbance was measured at 550–565 nm with a spectrophotometer.

Cell culture

Mouse lung fibroblasts were obtained as reported previously [16]. The cells were maintained in F12K medium (MSKCC Media Facility, New York, NY, USA) containing 10% fetal calf serum (Gibco, Grand Island, NY, USA), 1 \times low-serum growth supplement, 1% penicillin and streptomycin (Thermo Fisher Scientific) at 37 °C and 5 % CO₂.

Cell viability

Cells (1×10^4 cells/well) were cultured in 48-well plates overnight. When cells reached 80% confluence, MTT assays were performed. Cell viability was assessed at 570 nm using a microplate reader (BioTek, Winooski, VT, USA).

Cytokine measurement

Bronchoalveolar lavage fluid (BALF) from each mouse was obtained by endotracheal intubation. After centrifugation, the cell-free supernatants

were collected for measurement. Levels of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, and interferon (IFN)- γ were determined using enzyme-linked immunosorbent assay (ELISA) kits (Abcam, MA, USA).

Western blot analysis

Pulmonary samples were homogenized, and proteins were extracted and quantified using a BCA Protein Assay kit (Thermo Fisher Scientific). Next, 10 % SDS-PAGE was used to separate equal amounts of proteins, which were then transferred onto polyvinylidene difluoride membranes. The membranes were incubated with primary antibodies against α -small muscle actin (α -SMA), E-cadherin, β -catenin, Bcl-2-associated X protein (Bax), and cleaved caspase-3 (BD Biosciences Franklin Lakes, NJ) overnight at 4°C. The horseradish peroxidase-conjugated secondary antibody was added. Finally, an ECL detection system (GE Healthcare, Chicago, IL, USA) was utilized. The loading controls for cytoplasmic and nuclear proteins were α -tubulin and TopoII β , respectively.

Statistical analysis

SPSS (Version 16.0) (SPSS Inc, Chicago, IL, USA) was utilized for all statistical analyses. The experimental data were reported as the mean \pm standard deviation (SD). One-way analysis of variance was utilized for comparison between multiple groups, followed by Tukey's multiple comparison tests. The cutoff for statistical significance was $p < 0.05$.

RESULTS

CH reduces ECM deposition in BLM-treated mice

An overview of this experiment is shown in Figure 1 A. Interstitial fibrosis and collagen deposition were visible in BLM-treated mice compared with sham mice ($p < 0.01$), while CH significantly reduced these effects (Figure 1 B; $p < 0.05$). BLM-induced HYP content also decreased after CH treatment ($p < 0.05$) (Figure 1 C). Western blotting analysis revealed that α -SMA levels were decreased, whereas E-cadherin expression was significantly increased in the CH+BLM group compared to the BLM group ($p < 0.01$) (Figure 1 D). These results indicated that CH reduced ECM deposition in mice with PF.

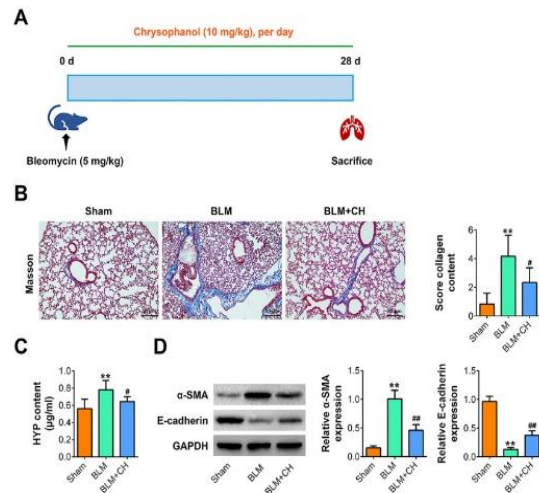


Figure 1: Effect of CH on ECM deposition in BLM-treated mice. (A) Experimental design overview. (B) Masson's trichrome staining of pulmonary samples. Scale bar = 50 μ m. (C) HYP content measurement. (D) Western blotting and quantitation of protein expression; HYP, hydroxyproline; Sham, the sham operation group; BLM, the bleomycin group; BLM + CH, the bleomycin plus chrysothanol group; ** $p < 0.01$, vs. sham; # $p < 0.05$, ## $p < 0.01$ vs. BLM group

CH attenuates BLM-induced pro-inflammatory cytokine expression in BALF from mice

Compared with the sham group, BLM mice showed significantly increased levels of TNF- α , IL-1 β , IL-6, and IFN- γ levels in BALF ($p < 0.01$). The administration of CH significantly reduced expression of these inflammatory cytokines (Figure 2; $p < 0.01$ or $p < 0.05$).

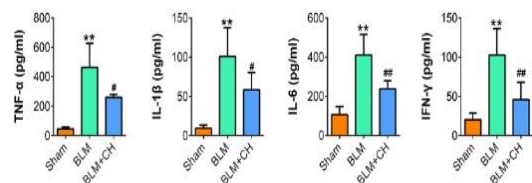


Figure 2: Effect of CH on inflammatory responses in BALF from BLM-treated mice. TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin 1; IL-6, interleukin 6; IFN- γ , interferon- γ . Sham, the sham operation group; BLM, the bleomycin group; BLM + CH, the bleomycin plus chrysothanol group; ** $p < 0.01$ vs. sham; # $p < 0.05$, ## $p < 0.01$ vs. BLM group

CH inhibits BLM-induced nuclear translocation of β -catenin in mice

To investigate the mechanism of CH action, levels β -catenin of in the cytoplasm and nuclei of mouse lung fibroblasts was determined. BLM inhibited cytoplasmic expression of β -catenin but

enhanced its nuclear expression. Treatment with CH increased levels of cytoplasmic β -catenin but decreased its levels in the nucleus (Figure 3).

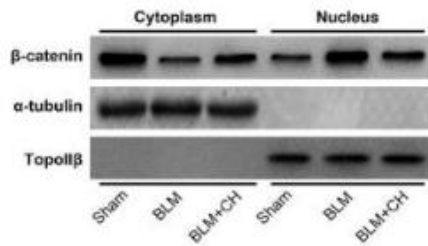


Figure 3: CH inhibits BLM-induced nuclear translocation of β -catenin in mice. Western blotting was used to determine the levels of β -catenin in the cytoplasm and nuclei of mouse lung fibroblasts. Sham, the sham operation group; BLM, the bleomycin group; BLM + CH, the bleomycin plus chrysophanol group

CH inhibits viability of and induces apoptosis in BLM-treated lung fibroblasts

Stimulation with BLM significantly increased the viability of and reduced protein levels of Bax and cleaved caspase-3 in lung fibroblasts ($p < 0.01$). After CH treatment, we observed a significant reduction in cell viability (Figure 4A). Western blotting analysis showed that CH treatment remarkably elevated the protein levels of Bax and cleaved caspase-3 (Figure 4 B–D).

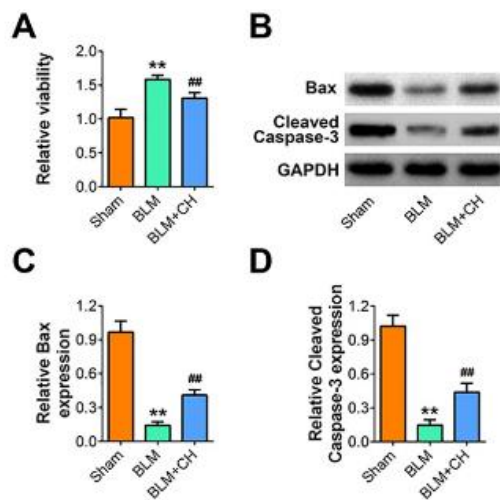


Figure 4: Effects of CH on the proliferation and apoptosis of lung fibroblasts. (A) MTT assays were used to measure the viability of lung fibroblasts. (B–D) Western blotting was used to measure levels of Bax and cleaved caspase-3. Sham, the sham operation group; BLM, the bleomycin group; BLM + CH, the bleomycin plus chrysophanol group; Bax, Bcl-2-associated X protein; ** $p < 0.01$ vs. sham; ## $p < 0.05$, ### $p < 0.01$ vs. BLM group

DISCUSSION

Idiopathic PF is a life-threatening lung disease with increasing incidence and prevalence [17]. Patients with IPF usually suffer from chronic and progressive exertional dyspnea and cough upon clinical presentation. Unfortunately, limited treatment options are available for IPF, highlighting the urgency to develop an optimal strategy for treating IPF. Increasing evidence shows that CH can alleviate lung injury by regulating PPAR or inflammatory responses [8,9]. However, few studies have discussed the possibility of treating IPF with CH administration. Thus, the current study was conducted to investigate the protective role and underlying mechanisms of CH action during IPF.

CH reportedly alleviates ovalbumin-induced expression of inflammatory cytokines, including TNF- α , IL-4, IL-5, and IL-13, in the airways [18]. In addition, CH could ameliorate high-fat diet-induced injury and fibrosis in the heart by reducing collagen deposition and inflammatory responses [19]. Here, CH reduced ECM deposition and levels of inflammatory cytokines in a mouse model of PF.

Glioma cell apoptosis can be induced by tubeimoside-1 through regulation of Bax and Bcl-2 expression and the reactive oxygen species/cytochrome *c*/caspase-3 pathway [20]. Matrine exerted anti-proliferative activity against cardiac fibroblasts by upregulating Bax and cleaved caspase-3 [21]. Doxorubicin decreased the viability of breast cancer cells and upregulated Bax, while downregulating Bcl-xL [22]. In a human lung adenocarcinoma cell line, 13-chlorine-3,15-dioxy-gibberellic acid methyl ester inhibited cell proliferation and induced cell apoptosis, accompanied by regulation of the mitochondrial apoptosis pathway through Bax upregulation [23]. In the present study, CH exerted an inhibitory effect on lung fibroblast proliferation. Treatment with BLM downregulated Bax and caspase-3, while CH increased Bax and caspase-3 expression levels, implying that CH attenuates PF by inhibiting cell proliferation.

Abnormal regulation of Wnt signaling is linked with the pathogenesis of various human fibrotic diseases in different organs. Hyperactive Wnt/ β catenin signaling contributes to systemic sclerosis with observed elevated levels of nuclear β -catenin [24]. Aberrant activation of Wnt/ β -catenin signaling lead to IPF and accumulation of nuclear β -catenin in fibroblast foci [25]. William and colleagues [26] reported that nuclear β -catenin accumulation indicates activated Wnt signaling in IPF, which is

attenuated by inhibiting the Wnt/ β -catenin/cyclic AMP response element binding protein pathway.

The results in this study were consistent with those of previous studies. In the current investigation, accumulation of nuclear β -catenin was observed after BLM challenge but decreased after CH treatment. These findings indicated that CH reversed BLM-induced IPF by inhibiting Wnt/ β -catenin signaling. Although these findings revealed that CH alleviates BLM-induced IPF *in vitro*, additional *in vivo* studies are needed. In addition, how CH inhibits PF via the Wnt/ β -catenin pathway warrants further investigation.

CONCLUSION

CF administration alleviates BLM-induced PF in mice via inhibition of Wnt/ β -catenin signaling pathway and suppression of lung fibroblast proliferation, thus providing a potential therapeutic strategy for IPF.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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