Int J Clin Exp Med 2016;9(3):6054-6061
www.ijcem.com /ISSN:1940-5901/IJCEM0019016

Original Article

Inhibition of bleomycin-induced pulmonary fibrosis by extract from Rosmarinus officinalis in rats

Liteng Yang[1], Xin Liu[2], Deyun Cheng[3], Xun Fang[3], Mao Mu[3], Xiaobo Hu[3], Li Nie[3]

[1]Department of Respiratory Medicine, Southern Medical University TCM-Integrated Hospital, Guangzhou 510315, Guangdong, China; [2]Zunyi Medical And Pharmaceutical College, Zunyi 563000, Guizhou, China; [3]Department of Respiratory Medicine, West-China Hospital, Sichuan University, Chengdu 610041, Sichuan, China

Received November 2, 2015; Accepted January 26, 2016; Epub March 15, 2016; Published March 30, 2016

Abstract: Pulmonary fibrosis is a fatal disease which is characterized by inflammation and formation of permanent scar tissue in lung. Current treatment for this disease is limited and mainly relies on slowing the progression of fibrosis by anti-inflammatory agent. In this study, we examined the application of diterpene phenol extract of *Rosmarinus officinalis* (DERO) as an anti-inflammatory agent for treating bleomycin (BLM) induced pulmonary fibrosis in rat model. After stimulating pulmonary fibrosis in Sprague-Dawley rats by endotracheal injection of bleomycin, oral administration of DERO was conducted with different dose (low, medium and high). Compared with NS group, infiltration of pro-inflammation lymphocytes and the collagen accumulation in the lung tissue of BLM group were significantly increased. However, with administration of DERO, cell infiltration and collagen deposition reduced significantly in medium dose and high dose groups, while little reduced was demonstrated in low dose group. Moreover, the expressions of Collagen-I, TGFBR2 and TGF-β_1 genes were decreased as well in medium and high dose group. In conclusion, our study demonstrated that DERO may play a regulatory role in BLM-induced pulmonary fibrosis by inhibiting over-depositing of Collagen I. DERO could inhibit the expression of TGF-β_1 and TGFBR2 to suppress the activation of pre-collagen I by TGFβ-Smad1 pathway as well.

Keywords: *Rosmarinus officinalis*, bleomycin, pulmonary fibrosis, TGF-β, Smad4

Introduction

Pulmonary fibrosis is an interstitial lung disease which is characterized by inflammation, proliferation of fibroblasts, and abnormal deposition of extracellular matrix (ECM) proteins [1]. Transforming growth factor-beta1 (TGF-β_1), a major regulator of the fibrotic process, has been considered to play a crucial role in the pathogenesis of pulmonary fibrosis. Various cell types in the lung tissue secret TGF-β_1, including alveolar macrophages, epithelia cells, endothelial cells and fibroblasts [2]. Activated TGF-β_1 binds to TGF-β type II receptor (*TGFB2R*) on target cells induced the phosphorylation of TGF-β type I receptor (*TGFB1R*) [3]. The receptor complex then causes the phosphorylation of Smad2 and Smad3 proteins [4]. The phosphorylated Smad proteins form heteromeric complexes along with Smad4 and then translocate into the nucleus to activate downstream genes. The activation of TGF-β_1-Smad pathway promotes the development of inflammation and induces the proliferation of the fibroblasts, leading to pulmonary fibrosis [5]. This makes TGF-β_1 and TGF-β_1-Smad pathway attractive therapeutic targets for pulmonary fibrosis.

Rosemary (Rosmarinus officinalis L.), belonging to the labiate family (Lamiaceae), is native in Mediterranean region and widely used as a flavor in foods. The mainly active components of Rosemary are phenolic diterpenes and triterpenes, such as carnosol, carnosic acid (CA), rosmanol, rosmarinic acid (RA) as well as others. The most abundant compounds, CA and RA, have been showed have a wide range of activities, including antioxidant, hepatoprotective and anti-inflammatory effects [6-8]. Tsai et al showed that ethanolic rosemary extract, mainly consisted by carnosol, CA and RA, significantly suppressed propionibacterium acnes-induced inflammatory responses [9].

Table 1. Primer used for qPCR detection

Target gene	Forward	Reverse	TaqMan probe
TGF-β_1	5'-actactgcttcagctccaca-3'	5'-gtgtccaggctccaaatgt-3'	5'-ccaagggctaccatgccaac-3'
GAPDH	5'-tgggtgtgaaccacgagaa-3'	5'-ggcatggactgtggtcatga-3'	5'-ctgcaccaccaactgcttagc-3'

Accordingly, the purpose of the present study was to investigate the possible effects of diterpene phenol extract of Rosmarinus officinalis (DERO) on collagen metabolism in pulmonary fibrosis rats induced by Bleomycin (BLM), especially on TGF-β_1 signal transduction pathway.

Materials and methods

Ethics statement

The animal protocols used in this study were approved by the Ethics Committee of the Sichuan University and followed the instruction of the Guidelines for Experimental Animals which is issued by the Ministry of Science and Technology (Beijing, China). All procedures were performed according to recommendations proposed by the Sichuan University, and all efforts were made to minimize suffering of mice. Moreover, the mice were housed in a temperature-controlled room with proper darkness-light cycles, fed with a regular diet, and maintained under the care of the Experimental Animal Center of Sichuan University.

Animals

Sprague-Dawley (SD) rats, weighing 150-250 g, aged 6-7 weeks, were purchased from Experimental Animal Center of West China Medical College of Sichuan University (Chengdu, China) (No. SCXK 2005-09).

Chemicals

Bleomycin hydrochloride was purchased from Nippon Kayaku (Tokyo, Japan). PCR primers were synthesized by Sangon Biotech (Shanghai, China). In situ hybridization Kit of transforming growth factor-beta type II receptor (TGFβRII) and Smad4 mRNA were purchased from Boster Biological Technology (Wuhan, China). Diterpene phenol extract of Rosmarinus officinalis (DERO) was purchased from Guizhou Institute of Biology (Guiyang, China).

Experimental model and study group

Fifty rats were divided into control (normal saline group, NS) and BLM-treated group (BLM). The effect of DERO was examined by giving three different dosages of DERO to the BLM group: the low dose group or DERO1 (50 mg/kg.d of DERO), the medium dose or DERO2 (100 mg/kg) and the high dose or DERO3 (200 mg/kg). In total there were five groups with 10 rats in each group. Pulmonary fibrosis was established in rats by an intratracheal instillation of BLM (5 mg/kg in saline). The rats were rotated after instillation to make sure that BLM was evenly distributed in both lungs. DERO were administrated by oral gavage for consecutive 28 days since day 2. Rats in NS and BLM groups were given an equal volume of PBS following the same schedule.

Tissue specimens and microarray construction

All rats were euthanatized in day 29 and lung tissues were harvested. The weight of lungs was measured. The lower lobe of the left lung was removed and fixed with 4% paraformaldehyde. Inferior lobe of the right lung was kept in liquid nitrogen for future experiments.

The lung tissue microarray was constructed as previously described [10]. After the construction of the array block, multiple 6-μm sections were cut for H&E staining, Van-Gieson staining, immunohistochemistry staining of collagen type I and *in situ* hybridization of TGF-βRII and Smad4 mRNA.

Reverse Transcription and real-time PCR

Total RNA was extracted from the lung sample by using TRIzol® Reagent (Invitrogen) according manufacturer's instruction. DNase treatment was conducted to remove genome DNA carryover. For the detection of gene expression in cellular RNA level, reverse transcription was conducted by using AMV reverse transcriptase(Promega) and a combination of random primer and oligo (dT) as previously reported [11]. Real-time PCR with SYBR Green detection was done as described previously [12]. Transcripts of house-keeping gene GAPDH were also amplified from the same samples to serve as an internal control for cellular mRNA normal-

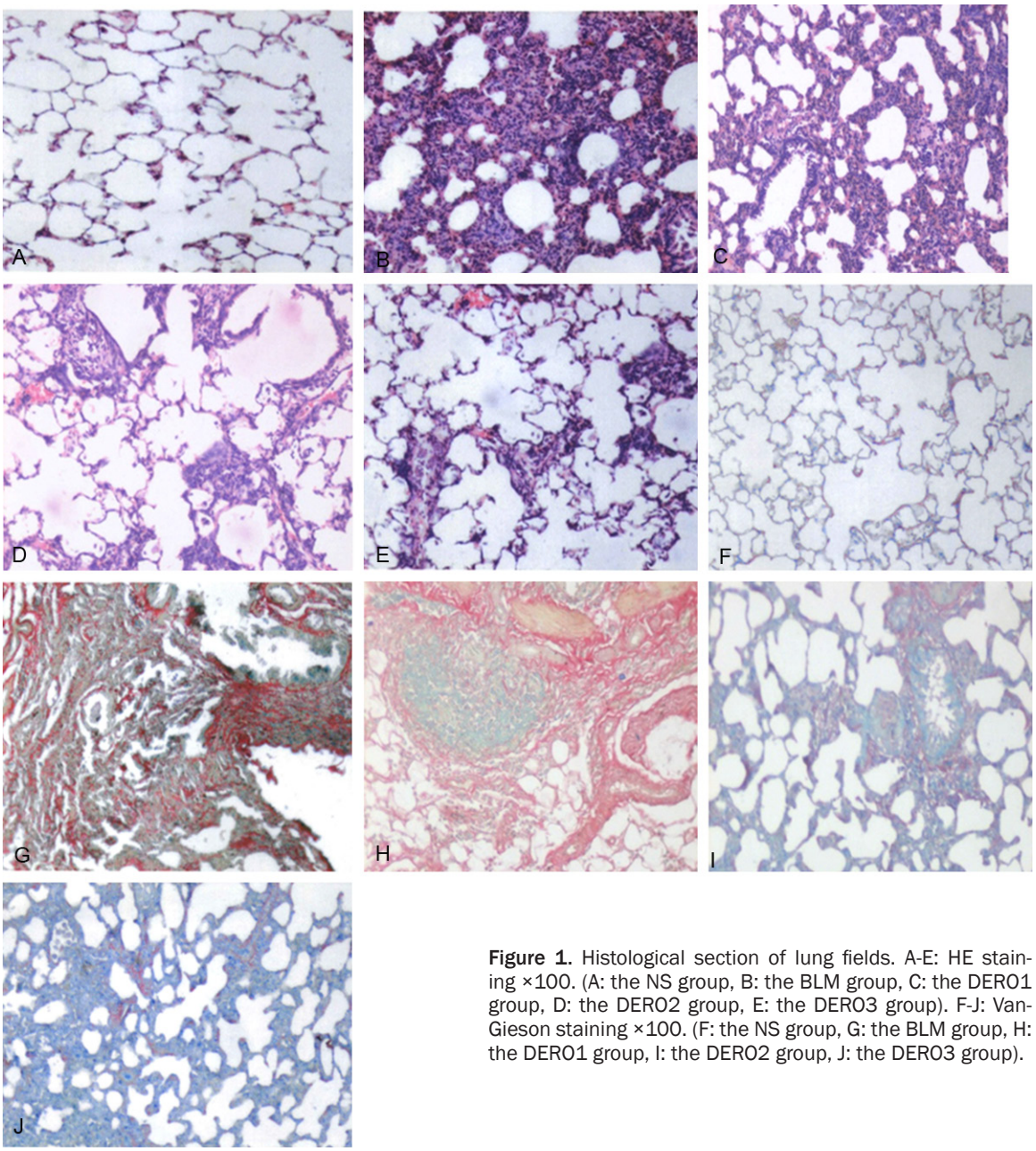

Figure 1. Histological section of lung fields. A-E: HE staining ×100. (A: the NS group, B: the BLM group, C: the DERO1 group, D: the DERO2 group, E: the DERO3 group). F-J: Van-Gieson staining ×100. (F: the NS group, G: the BLM group, H: the DERO1 group, I: the DERO2 group, J: the DERO3 group).

ization. Expression of indicated genes was quantified by $2^{-\Delta\Delta CT}$ method as previously described [13]. The primers used in RT-PCR were listed as **Table 1**.

Immunohistochemistry

The TMA sections were deparaffinized in xylene and rehydrated in graded series of ethanols followed by heat-induced epitope retrieval in citrate buffer (pH 6.0). Sections were incubated overnight at room temperature with polyclonal antibodies to collagen I diluted 1:500 for immunohistochemical analysis. The slides were finally counterstained with hematoxylin, dehydrated and mounted.

In situ hybridization

In situ hybridization of TGFβRII and Smad4 mRNA were performed according to the manufacturer's instruction.

Western blot analysis

Total protein was isolated from the lung tissue by homogenization with a buffer as previously

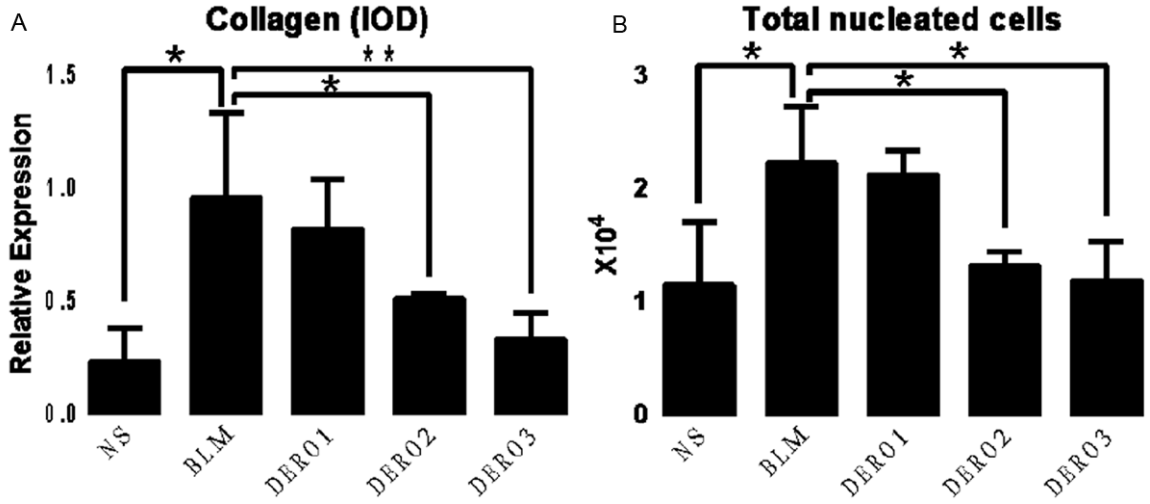

Figure 2. Inflammation and collagen deposition of each group. A: Collagen deposition measured by Van-Gieson staining. B: Inflammation level measured by counting total nucleated cells from HE staining. Data are presented as mean ± SEM, **P*<0.05, ***P*<0.01.

described [14]. Then the homogenized proteins were further lysed in Laemmli sample buffer. The whole proteins in the lysate were separated and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described previously, respectively [15, 16]. Antibodies against Smad2/3 (Santa Cruz Biotechnology, Santa Cruz, CA), Smad7 (Santa Cruz Biotechnology) and actin (Sigma-Aldrich, St. Louis, MO) were used in the blotting. The chemiluminescence signal was recorded digitally using a ChemiDoc XRS imaging system (Bio-Rad Laboratories, Hercules, CA). Digital signal acquisition and densitometry analyses were conducted by using the QuantityOne Program, Version 4.6 (Bio-Rad).

Image process and analysis

Image-pro plus software were used to process and quantitatively analyze the images. A 100× image of left, right, superior and inferior field of each site in tissue microarray were taken for quantitative analysis. Average value of four images from these four fields was considered as value in this tissue microarray. Average value of same measurement of four sites from four sets of tissue microarray was considered as value of this measurement in this rat.

Total nucleated cells were automatically counted using the software after H&E staining. Each nucleus represented a single cell, including lung tissue cell and inflammatory white cell. The total nucleated cells numbers of experimental groups were compared to NS group. The difference between the cells numbers represented the severity of inflammation. The more the difference of cells numbers, the worse the inflammatory was.

The quantity of collagen fiber staining, immunohistochemical analysis of collagen-I, in situ hybridization of TGFβRII and Smad4 mRNA were expressed as the integrated optical density (IOD).

Statistical analysis

The results are expressed as mean ± SD. Homogeneity test was performed for quantitative data at the first place. Categorical data were converted to quantitative data. Statistical analysis was performed using one-way ANOVA and q test. Data analysis was carried out using SPSS 13.0. *P* value of 0.05 was considered statistically significant.

Results

Pulmonary fibrosis rat model

BLM is a chemotherapeutic antibiotic could induce fibrosis as its major adverse effects [17]. Therefore, BLM is a commonly used drug to induce pulmonary fibrosis in animal model [17]. After endotracheal injection of BLM in

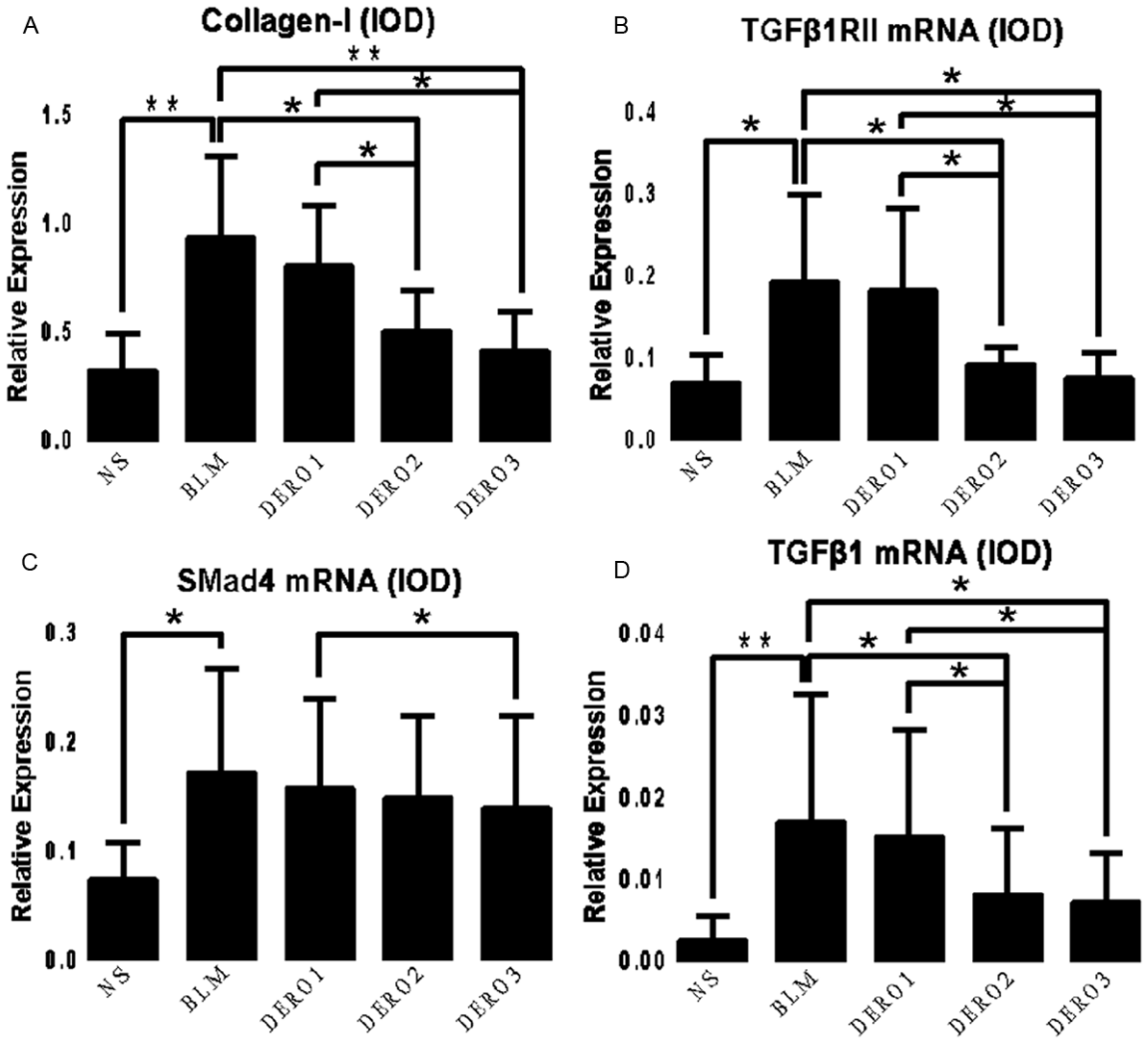

Figure 3. Effects of DERO on the expression of Collagen-I detected by immunohistochemical staining, and the mRNA levels of $TGF\beta_1RII$, Smad4 and $TGF\beta_1$ in lung tissue of rats. A: Collagen-I expression detected by immunohistochemical staining. B, C: mRNA levels of $TGF\beta_1RII$ and Smad4 detected by *in Situ Hybridization*. D: mRNA level of $TGF\beta_1$ detected by RT-qPCR. Data are presented as mean ± SEM, $*P<0.05$, $**P<0.01$.

Sprague-Dawley rats following administration of DERO, the effects of DERO was examined by analysis of the lungs on the 29th day after injection. Compared to NS group, feeding and defecation of rats in BLM group and DERO1 group were decreased, while they were not affected in DERO2 and DERO3 group (data not shown). One rat in DERO1 group died on day 3 due to acute lung injury induced by BLM.

Histopathological manifestations

BLM administration resulted in the development of extensive fibrosis in the lung after 28 days of BLM treatment. Results showed that the lungs of rats in NS group were elastic with pink color and had a smooth surface. However, compared with the NS group, the lungs of rats in BLM group were less elastic and dilated in size with uneven dark red color and had a rough surface. The lungs of rats in DERO groups were elastic with even dark red color and had a smooth surface.

Moreover, compared to NS group (**Figure 1A**), H&E staining showed that the lung tissues of rats in BLM group presented severe epithelial proliferation, alveolitis, edema, inflammatory cell infiltration and interstitial fibrosis (**Figure 1B**). Histopathological manifestations of DERO1 group were similar to BLM group. Infla

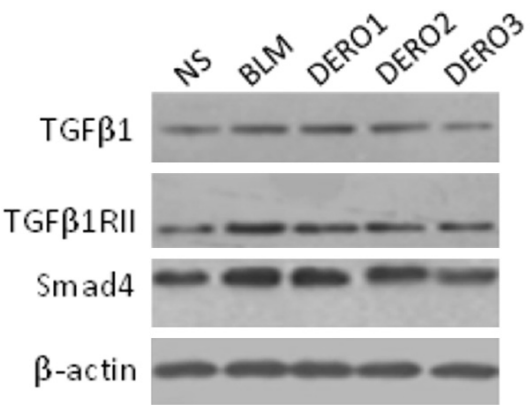

Figure 4. Effects of DERO on the expression of TGF-β_1, TGFβ_1RII and Smad4 detected by western blot.

mmation manifestations of DERO2 and DERO3 (**Figure 1C-E**).

Compared with NS group, collagen deposition and inflammation infiltration of BLM group were significantly increased. Collagen deposition and inflammation infiltration of DERO1 group were similar to BLM group. However, compared to BLM group, collagen deposition and inflammation infiltration of DERO2 and DERO3 groups were significantly decreased. There was no difference between DERO2 group and DERO3 group (**Figure 2**).

The expression of collagen-I detected by immunohistochemical staining

Compared with NS group, the expression of collagen-I detected by immunohistochemical staining were significantly increased in BLM group. Compared with BLM group, the expression of collagen-I of DERO1 group has no statistical significantly difference, while the expression of collagen-I of DERO2 and DERO3 group were significantly decreased. There was no difference between DERO2 and DERO3. All the results were given by comparison of the average IOD value (**Figure 3A**).

The expression of TGFβ_1RII and Smad4 detected by in situ hybridization

Compared with NS group, the expression of TGFβ_1RII and Smad4 detected by in Situ Hybridization were significantly increased in BLM group. Compared with BLM group, neither expression of TGFβ_1RII or Smad4 of DERO1 group showed any difference. Compared with BLM group, the expression of TGFβ_1RII in DERO2 and DERO3 group were significantly decreased, however, expression of Smad4 of DERO2 and DERO3 group showed no difference. There was no difference between DERO2 and DERO3. All the results were given by comparison of the average IOD value (**Figure 3B**, **3C**).

The expression of TGF-β_1 detected by qRT-PCR

Compared with NS group, the expression of TGF-β_1 detected by qRT-PCR was significantly increased in BLM group. Compared with BLM group, the expression of TGF-β_1 of DERO1 group showed no difference, while the expression of TGF-β_1 of DERO2 and DERO3 group were significantly decreased. There was no difference between DERO2 and DERO3 (**Figure 3D**).

The expression of TGF-β_1, TGFβ_1RII and Smad4 detected by western blot

Compared with NS group, the expression of TGF-β_1, TGFβ_1RII and Smad4 was increased in BLM group. Compared with BLM group, the expression of TGF-β_1 and TGFβ_1RII was reduced in DERO2 and DERO3 group, and the expression of Smad4 was reduced in DERO3 group (**Figure 4**).

Discussion

Under normal conditions, the metabolism of pulmonary fibroblasts is under a dynamic equilibrium, which is important in maintaining the structure and function of the lung. Pulmonary fibrosis is characterized by aberrant wound healing of the interstitial lung tissue following lung injury. The major change in pulmonary fibrosis is the disturbance of the collagen metabolism, mainly Collagen-I and III. It has been shown that the over-deposition of Collagen III is reversible, however, the over-deposition of Collagen I leads to irreversible pulmonary fibrosis [18]. TGF-β_1 is so far the most effective stimulator for collagen synthesis. Therefore, TGF-β_1 is considered as a key therapeutic target for regulating excessive repair of interstitial lung tissue.

Rosemary (Rosmarinus officinalis L.) is a culinary as well as medicinal herb which was widely used all over the world. Previous studies have shown the biological effects of diterpene phe-

nol extract of Rosmarinus officinalis, mainly CA and RA, processing multiple functions such as antioxidant, anticancer, hepatoprotection and anti-inflammation [6-8]. Tsai et al showed that CA and RA, significantly suppressed propionibacterium acnes-induced inflammatory responses [9]. However, whether DERO has anti-fibrotic effects is not well studied.

BLM is a chemotherapeutic antibiotic could induce fibrosis as its major adverse effects. Therefore, BLM is a commonly used drug to induce pulmonary fibrosis in animal model [17]. In this study, we examined the anti-inflammatory and anti-fibrotic effects of DERO in BLM induced pulmonary fibrosis in rats by histopathological detection as well as measuring expression of collagen-I, TGF-β_1, TGFβ_1RII and Smad4.

Our data demonstrated the BLM treatment caused significant inflammation and fibrosis in rat lung tissue while medium to high dose of diterpene phenol extract of Rosmarinus officinalis can suppress the collagen deposition and inflammation infiltration of BLM rats, but low dose DERO had no such effects. We also found that expression of TGF-β_1, TGFβ_1RII and Smad4 were up-regulated in BLM group while the medium and high dose DERO can reverse the up-regulation of TGF-β_1 and TGFβ_1RII.

Pulmonary fibrosis is a fatal disease characterized by inflammation, proliferation of fibroblasts, and abnormal deposition of extracellular matrix proteins. As strongest pro-fibrotic cytokine, TGF-β_1 promotes fibroblast proliferation, drives the differentiation of fibroblasts to myofibroblasts and increases the synthesis of type I collagen. Alveolar macrophages and fibroblasts from fibrotic lungs secrete higher amounts of active TGF-β_1 protein compared with normal lungs [19, 20]. Previous data suggested overexpression of TGF-β_1 inducing fibrosis in the rat lung [21]. Therefore, it is reasonable that the regulation of TGF-β_1 activity will be a target for the treatment of pulmonary fibrosis. Our results showed that DERO can suppress the fibrotic reaction induced by BLM in rats, as well as suppressing the up-regulation of TGF-β_1 and TGFβ_1RII. These findings indicated that the anti-fibrotic effect of DERO was achieved by suppressing synthesis of collagen-1 via regulating TGF-β_1 pathway, suggesting that DERO may have potential benefits in pulmonary fibrosis treatment. Moreover, we found that medium and high dose of DERO had anti-fibrotic effects but low did not have anti-fibrotic effects, which indicated that the anti-fibrotic effects of DERO had a threshold dose. Our results showed that there were no significant differences in anti-fibrotic effect between medium and high dose of DERO. Further study will be needed to confirm whether there is a dose dependent effect of DEPO as an anti-fibrotic agent.

In conclusion, our study demonstrates that diterpene phenol extract of Rosmarinus officinalis significantly inhibits BLM-induced pulmonary fibrosis. Our data suggested that DERO have the potential as an effective anti-fibrosis agent for future drug development.

Disclosure of conflict of interest

None.

Address correspondence to: Liteng Yang, Department of Respiratory Medicine, Southern Medical University TCM-Integrated Hospital, No. 13 Shiliugang Rd., Haizhu District, Guangzhou 510315, Guangdong Province, China. Tel: +86-18620058677; Fax: +86-020-61650488; E-mail: litengyang@yeab.net

References

[1] Lota HK and Wells AU. The evolving pharmacotherapy of pulmonary fibrosis. Expert Opin Pharmacother 2013; 14: 79-89.

[2] Fukuda K, Kawata S, Tamura S, Matsuda Y, Inui Y, Igura T, Inoue S, Kudara T and Matsuzawa Y. Transforming growth factor-beta1-induced degradation of activated Src tyrosine kinase in rat fibroblasts. Oncogene 1998; 16: 3349-56.

[3] Wrana JL, Attisano L, Wieser R, Ventura F and Massague J. Mechanism of activation of the TGF-beta receptor. Nature 1994; 370: 341-7.

[4] Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, Oeda E, Tamaki K, Hanai J, Heldin CH, Miyazono K and ten Dijke P. TGF-beta receptor-mediated signalling through Smad2, Smad3 and Smad4. Embo J 1997; 16: 5353-62.

[5] Goodwin A and Jenkins G. Role of integrin-mediated TGFbeta activation in the pathogenesis of pulmonary fibrosis. Biochem Soc Trans 2009; 37: 849-54.

[6] Aruoma OI, Halliwell B, Aeschbach R and Loligers J. Antioxidant and pro-oxidant properties of active rosemary constituents: carnosol and carnosic acid. Xenobiotica 1992; 22: 257-268.

[7] Zheng W and Wang SY. Antioxidant activity and phenolic compounds in selected herbs. J Agric Food Chem 2001; 49: 5165-70.
[8] Chang CH, Chyau CC, Hsieh CL, Wu YY, Ker YB, Tsen HY and Peng RY. Relevance of phenolic diterpene constituents to antioxidant activity of supercritical CO(2) extract from the leaves of rosemary. Nat Prod Res 2008; 22: 76-90.
[9] Tsai TH, Chuang LT, Lien TJ, Liing YR, Chen WY and Tsai PJ. Rosmarinus officinalis extract suppresses Propionibacterium acnes-induced inflammatory responses. J Med Food 2013; 16: 324-333.
[10] Kononen J, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G and Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 1998; 4: 844-7.
[11] Nan Y, Wang R, Shen M, Faaberg KS, Samal SK and Zhang YJ. Induction of type I interferons by a novel porcine reproductive and respiratory syndrome virus isolate. Virology 2012; 432: 261-70.
[12] Patel D, Opriessnig T, Stein DA, Halbur PG, Meng XJ, Iversen PL and Zhang YJ. Peptide-conjugated morpholino oligomers inhibit porcine reproductive and respiratory syndrome virus replication. Antiviral Res 2008; 77: 95-107.
[13] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-8.
[14] Chen M, Lv Z and Jiang S. The effects of triptolide on airway remodelling and transforming growth factor-beta(1)/Smad signalling pathway in ovalbumin-sensitized mice. Immunology 2011; 132: 376-384.
[15] Zhang YJ, Wang KY, Stein DA, Patel D, Watkins R, Moulton HM, Iversen PL and Matson DO. Inhibition of replication and transcription activator and latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus by morpholino oligomers. Antiviral Res 2007; 73: 12-23.
[16] Kannan H, Fan S, Patel D, Bossis I and Zhang YJ. The hepatitis E virus open reading frame 3 product interacts with microtubules and interferes with their dynamics. J Virol 2009; 83: 6375-82.
[17] Szapiel SV, Elson NA, Fulmer JD, Hunninghake GW and Crystal RG. Bleomycin-induced interstitial pulmonary disease in the nude, athymic mouse. Am Rev Respir Dis 1979; 120: 893-9.
[18] Weller SA, Elphinstone JG, Smith NC, Boonham N and Stead DE. Detection of Ralstonia solanacearum strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Appl Environ Microbiol 2000; 66: 2853-8.
[19] Coker RK, Laurent GJ, Shahzeidi S, Lympany PA, du Bois RM, Jeffery PK and McAnulty RJ. Transforming growth factors-beta 1, -beta 2, and -beta 3 stimulate fibroblast procollagen production in vitro but are differentially expressed during bleomycin-induced lung fibrosis. Am J Pathol 1997; 150: 981-91.
[20] Coker RK, Laurent GJ, Jeffery PK, du Bois RM, Black CM and McAnulty RJ. Localisation of transforming growth factor beta1 and beta3 mRNA transcripts in normal and fibrotic human lung. Thorax 2001; 56: 549-56.
[21] Sime PJ, Xing Z, Graham FL, Csaky KG and Gauldie J. Adenovector-mediated gene transfer of active transforming growth factor-beta1 induces prolonged severe fibrosis in rat lung. J Clin Invest 1997; 100: 768-76.