Int J Clin Exp Pathol 2017;10(8):8503-8509
www.ijcep.com /ISSN:1936-2625/IJCEP0055743

Original Article

Bone morphogenetic protein 7 alleviates paraquat-induced pulmonary fibrosis via TGF-β1/Erk1/2 pathway

Yongsheng Wang[1*], Shuang Ren[2*], Lin Liu[3*], Rong Yao[4], Xiaojuan Ma[1], Lei Chen[2]

[1]Department of Respiratory Medicine, Chengdu First People's Hospital, Chengdu, Sichuan, China; Departments of [2]Respiratory and Critical Care Medicine, [4]Emergency Medicine, West China Hospital, West China School of Medicine, Sichuan University, Chengdu, Sichuan, China; [3]Department of Respiratory Medicine, 363 Hospital, Chengdu, Sichuan, China. [*]Equal contributors.

Received April 19, 2017; Accepted June 23, 2017; Epub August 1, 2017; Published August 15, 2017

Abstract: Bone morphogenetic protein 7 (BMP-7) recently demonstrates an anti-fibrotic effect. To evaluate the role of BMP-7 in paraquat (PQ)-induced pulmonary fibrosis, PQ-exposed mice and lung fibroblasts (MRC-5) were treated with BMP-7. Our results showed that BMP-7 treatment could significantly reduce PQ-induced pulmonary fibrosis, accompanied by downregulation of transforming growth factor (TGF)-β1 and collagen I deposition in mouse lungs. Moreover, PQ-induced inviability, apoptosis, high level of collagen I, as well as phosphorylation of Erk1/2, in MRC-5 cells were significantly inhibited by BMP-7 treatment. These findings indicate BMP-7 alleviates PQ-induced pulmonary fibrosis partly via TGF-β1/Erk1/2 pathway, suggesting a promising therapeutic means for PQ-induced fibrotic lung injury.

Keywords: Bone morphogenetic protein, transforming growth factor-β1, mitogen activated protein kinase, paraquat, pulmonary fibrosis

Introduction

Pulmonary fibrosis is characterized by chronic inflammation and fibrotic changes in lungs, which dampens alveolar gas exchange, leading to pulmonary dysfunction and even death [1]. Paraquat (PQ) poisoning is a common cause of lung injury and pulmonary fibrosis especially in farmers. In this process, PQ-induced inflammatory reaction and excessive extracellular matrix (ECM) deposition contribute to the fibrotic changes in lungs, which is initiated and maintained by a variety of profibrotic cytokines. In PQ-activated profibrotic cytokine network, transforming growth factor-β 1 (TGF-β1) plays a central role [2]. TGF-β1 can activate fibroblast proliferation, differentiation, and then promote accumulation of collagens and other ECM in lungs, leading to occurrence and development of pulmonary fibrosis. Interestingly, in PQ-induced pulmonary fibrosis, TGF-β1 is related to not only its downstream Smad proteins [3, 4], but also mitogen activated protein kinase (MAPK) family signaling pathway [5].

Bone morphogenetic protein-7, molecular weight 35000, is a secreted multifunctional protein, belonging to the TGF-β superfamily [6]. It was reported transfection of BMP-7 gene can significantly inhibit the expression of collagen type I α2 chain mRNA induced by TGF-β1 in mouse pulmonary myofibroblasts [7]. In human lung fibroblasts, BMP-7 could also inhibit ECM accumulation and fibroblast differentiation stimulated by TGF-β1 [8]. These findings indicate a potential anti-fibrotic role of BMP-7 in TGF-β1-induced fibrosis in lungs. However, the effect of BMP-7 on PQ-induced pulmonary fibrosis has not been reported.

To investigate the effect of exogenous BMP-7 on PQ-induced pulmonary fibrosis and whether MAPK signaling pathway was involved in this process, a PQ-poisoning mouse model, as well

as an *in vitro* experiment was performed in the present study.

Materials and methods

Animals

Female C57BL/6 mice (eight-week-old, 21±3 g weight) were purchased from Dossy Biological Technology Co., Ltd. (Chengdu, China). All mice were maintained under specific pathogen-free conditions. The animals were cared for in accordance with the guiding principles described in our former article [2] and the animal study was approved by the Panel on Laboratory Animal Care of West China School of Medicine of Sichuan University.

Treatment of animals

C57BL/6 mice were divided into eight groups with five mice in each group. Experimental mice were treated with a single intraperitoneal injection of paraquat (10 mg/kg, Sigma) with dilution in PBS. Then, recombinant BMP-7 (100 or 500 μg/kg, Sigma) or vehicle was diluted in PBS and injected via the tail vein every day. All mice were killed on day 7 and day 21 after PQ treatment.

Lung histopathology

Histology was assessed following hematoxylin and eosin (H&E) staining and Masson's trichrome staining (MTS). The stained sections were coded and examined by two independent observers who were blinded to the groups. The extent of lung injury was estimated by morphometric assessment. The histological changes for pulmonary fibrosis were evaluated semiquantitatively by Ashcroft scoring detailedly described in our former article [9]. TGF-β1 was detected in lung tissues using immunohistochemistry. Goat-anti TGF-β1 polyclonal antibody (Santa Cruz) were used as the primary antibodies, and the secondary detection was performed using Horseradish Peroxidase (HRP) Anti-Goat DAB Detection kit (Golden Bridge International Inc., Mukilteo, WA) according to the manufacturer's instructions.

Treatment of MRC-5 cells

Human embryonic lung fibroblast (MRC-5) cells were stimulated with PQ (0.3 mmol/L) in 96-well microculture plates (at 10^4 cells/well) at different concentrations of BMP-7 (5, 10, 20 and 40 μg/mL) for 12, 24 and 48 h. After treatment, the culture supernatant and cells were collected.

Determination of cell viability and apoptosis

Cell viability was determined using Cell Counting Kit-8 (Beyotime, China) according to instructions. Briefly, 10 μl of CCK-8 (5 mg/ml) was added to each well and the cells were cultured for 1 h, and then the absorbance was determined at a wavelength of 450 nm using a microplate reader (Bio-Rad). The cell apoptosis rate was determined by the flow cytometry (FCM) assay by using the apoptosis detection kit (Keygen, China). Briefly, the cells were incubated with propidium iodide (PI, Sigma) and annexin V-FITC (annexin V) for 15 min at room temperature. Next, the samples were analyzed via FCM (Beckman). Dual-parameter FCM was used to determine the number of apoptotic cells (annexin V/PI double-positive cells).

Measurement of collagen I

The concentration of collagen I in mouse lungs and MRC-5 cells were quantified with the ELISA kits (R&D Systems) according to the manufacturers' instructions. Briefly, the cytoplasmic protein extracts from lung tissues and MRC-5 cells were applied to the wells. After washing, HRP-conjugated specific antibody was added to the wells. Following the next wash, color development was proportional to the protein concentration and calculated by comparison with a standard. After a colorimetric reaction, the samples were measured in a spectrophotometer at a wavelength of 450 nm.

Western blot analysis

The cytoplasmic and nuclear protein extracts from lung tissues and MRC-5 cells were resolved on 10% SDS-PAGE gels followed by electrophoretic transfer to nitrocellulose membranes. Then immunoblot analysis was performed with specific primary antibodies at a 1:500 dilution, including anti-TGF-β1, anti-Erk1/2, anti-phospho Erk1/2, anti-p38, anti-phospho p38, anti-JNK, anti-phospho JNK, and anti-GAPDH monoclonal antibodies (Santa Cruz), and the secondary antibody conjugated with HRP at a 1:1000 dilution. Membranes were visualized with substrate from ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech) in the Molecular Imager Gel Doc XR System (Bio-Rad).

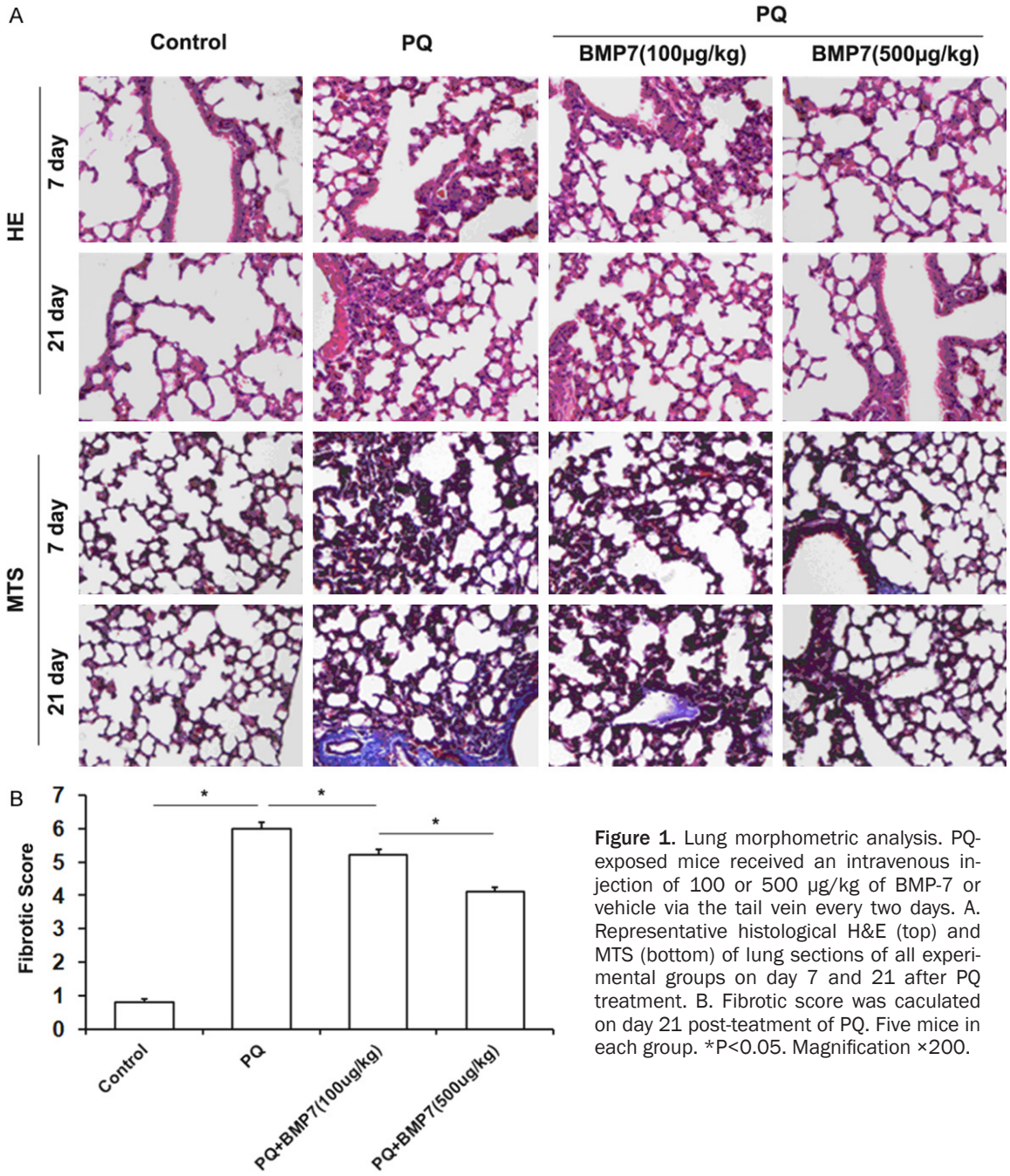

Figure 1. Lung morphometric analysis. PQ-exposed mice received an intravenous injection of 100 or 500 μg/kg of BMP-7 or vehicle via the tail vein every two days. A. Representative histological H&E (top) and MTS (bottom) of lung sections of all experimental groups on day 7 and 21 after PQ treatment. B. Fibrotic score was caculated on day 21 post-teatment of PQ. Five mice in each group. *P<0.05. Magnification ×200.

Statistical analysis

All data were expressed as Mean ± standard deviation (SD). The differences among groups were determined by the Student's t test (singular comparisons) and one-way ANOVA (multiple comparisons). P<0.05 was accepted as a statistically significant difference. SPSS 13.0 software package (SPSS, Inc., Chicago, IL) was used for statistical analysis.

Results

BMP 7 dose-dependently inhibited PQ-induced fibrotic lung injury in mice

PQ-exposed mice developed a markedly lung injury and fibrotic response. Histological changes were assessed with H&E and MTS, respectively. As illustrated in **Figure 1A**, inflammatory cell infiltration and collagen accumulation were

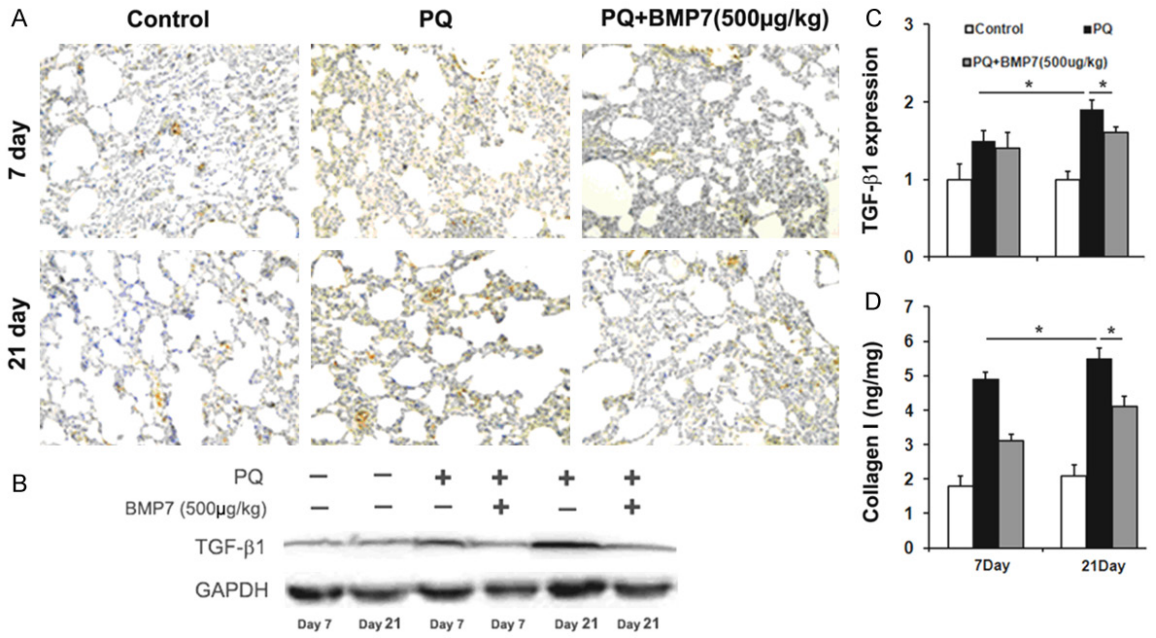

Figure 2. BMP7 suppressed TGF-β1 overexpression and collagen I deposition in mouse lungs. PQ-exposed mice received an intravenous injection of 500 μg/kg of BMP-7 or vehicle via the tail vein per two day. The expression of TGF-β1 in lungs was examined by immunohistochemical study (A) and western blot analysis (B), and the relative expression levels of TGF-β1 (C) were normalized to GAPDH. The concentration of collagen I in mouse lungs was assessed by ELISA (D). Five mice in each group. *P<0.05. Magnification ×200.

significantly increased in lungs after PQ exposure. Meanwhile, BMP-7 dose-dependently reduced PQ-induced fibrotic lung injury on day 21, with greater effects at the higher dose of 500 μg/kg of BMP-7 (**Figure 1B**).

BMP 7 suppressed PQ-induced TGF-β1 expression and collagen I accumulation in mouse lungs

Immunohistochemical study revealed the changes of TGF-β1-positive staining in mouse lungs. PQ-exposed mice developed more significant positive-stained areas of TGF-β1 in lung tissues than those in controls, which was decreased after treatment of 500 μg/kg of BMP-7 on day 7 and 21 (**Figure 2A**). Consistent with the immunohistochemical study, western blot quantitatively indicated overexpression of TGF-β1 in PQ-exposed mice on day 7 and 21, and BMP-7 (500 μg/kg) significantly suppressed PQ-induced TGF-β1 overexpression (**Figure 2B**, **2C**) as well as collagen I accumulation (**Figure 2D**) in lung tissues.

BMP-7 dose-dependently attenuated PQ-induced inviability in MRC-5 cells

To test the effects of BMP-7 on PQ-induced injury in fibroblasts, MRC-5 cells were stimulated by PQ (0.3 mmol/L) *in vitro* for 12, 24 and 48 h in the presence or absence of BMP-7 (1 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL or 40 μg/mL). The cells viability was determined by CCK-8 assay. Stimulation with PQ (0.3 mmol/L) strongly decreased the number of viable cells. However, treatment with BMP-7 could dose-dependently increase the number of viable cells (**Figure 3A**), especially at the higher doses of 20 ug/mL or 40 ug/mL.

BMP-7 alleviated PQ-induced fibrotic injury via Erk1/2 dephosphorylation in MRC-5 cells

To explore the underlying mechanisms of the protective effects of BMP-7 on PQ-induced fibrotic injury, MRC-5 cells were treated with PQ (0.3 mmol/L) *in vitro* for 48 h in the presence or absence of BMP-7 (20 μg/mL). BMP-7 significantly reduced PQ-induced apoptotic rate and collagen I deposition in MRC-5 cells (**Figure 3B**, **3C**). Meanwhile, PQ markedly induced phosphorylation of Erk1/2, JNK and p38, however, only Erk1/2 phosphorylation, but not JNK and p38, was alleviated by BMP-7 treatment (**Figure 4A**, **4B**).

Discussion

In the present study, BMP-7 dose-dependently attenuated PQ-induced pulmonary fibrosis in mice. Meanwhile, PQ-induced TGF-β1 overex-

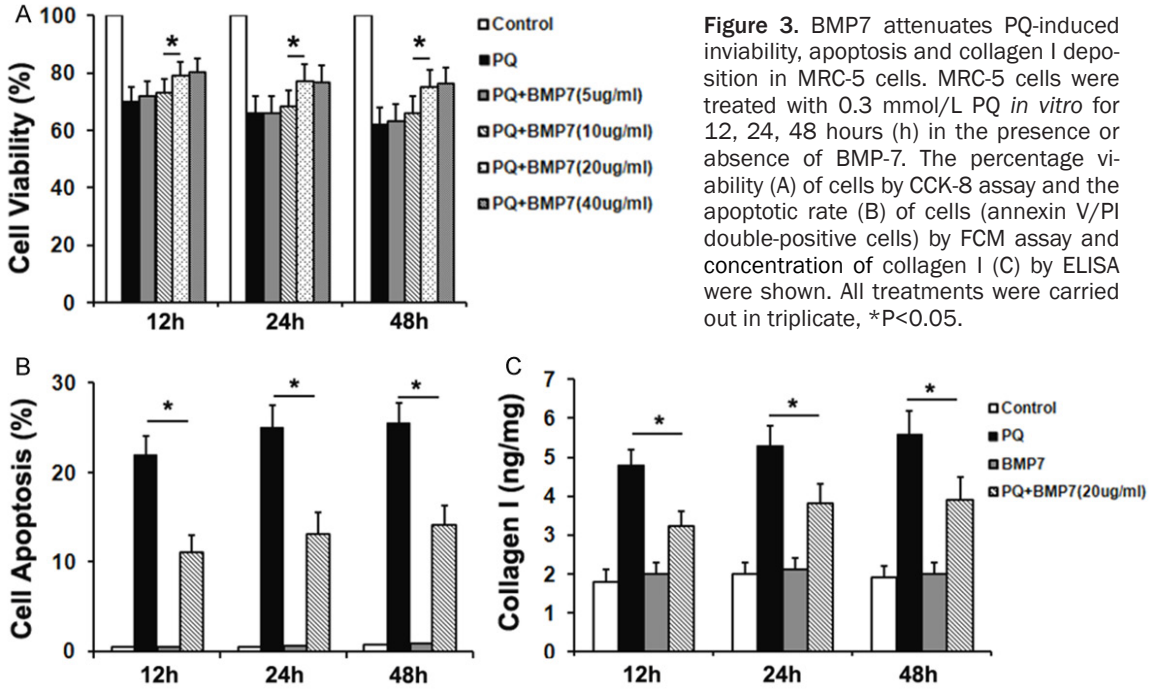

Figure 3. BMP7 attenuates PQ-induced inviability, apoptosis and collagen I deposition in MRC-5 cells. MRC-5 cells were treated with 0.3 mmol/L PQ *in vitro* for 12, 24, 48 hours (h) in the presence or absence of BMP-7. The percentage viability (A) of cells by CCK-8 assay and the apoptotic rate (B) of cells (annexin V/PI double-positive cells) by FCM assay and concentration of collagen I (C) by ELISA were shown. All treatments were carried out in triplicate, *P<0.05.

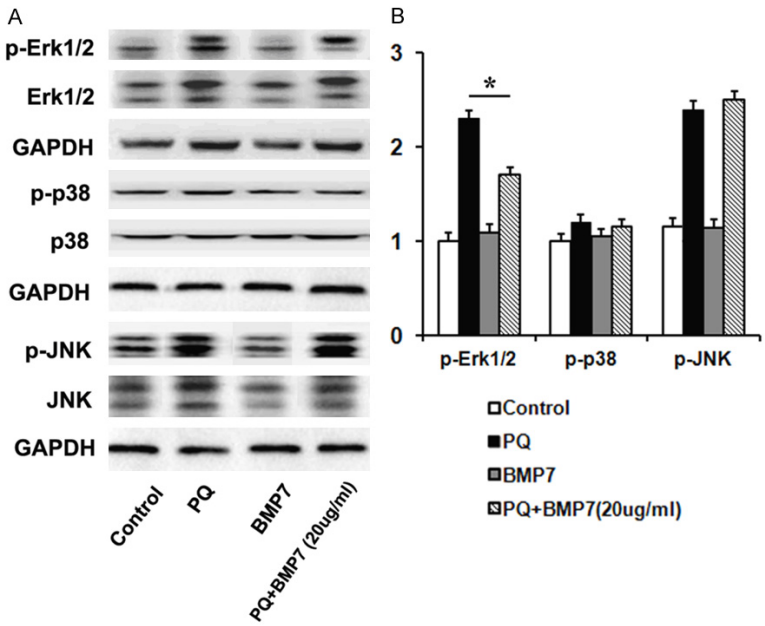

Figure 4. BMP7 inhibited PQ-induced phosphorylation of Erk1/2, but not p38 and JNK, in MRC-5 cells. MRC-5 cells were treated with PQ (0.3 mmol/L) *in vitro* for 48 h in the presence or absence of BMP-7 (20 ug/mL). The phosphorylation of Erk1/2, p38 and JNK were evaluated by Western blotting at 48 h after PQ treatment (A). Levels of phosphor(p)-Erk1/2, p-p38 and p-JNK were normalized to total Erk1/2, p38 and JNK (B). All treatments were carried out in triplicate, *P<0.05.

pression and collagen I deposition in mouse lungs was also downregulated by BMP-7 treatment. In vitro study further suggested PQ-induced apoptosis and accumulation of collagen I, as well as Erk1/2 phosphorylation in MRC-5 cells were significantly alleviated by BMP-7 treatment, which indicated an important role of Erk1/2 phosphorylation, but not JNK and p38 pathways in PQ-induced fibrotic lung injury.

Endogenous BMPs are synthesized and secreted from a variety of cell types and play important roles in regulating cell proliferation, apoptosis and differentiation [10, 11]. Previous studies indicated exogenous BMP-7 could reduce ECM accumulation and fibroblast activation in several fibrotic models with a high therapeutic potency [12, 13]. The present study further confirmed a protective effect of BMP-7 on a PQ-exposed mouse model of pulmonary fibrosis. Noticeably, in PQ-induced pulmonary fibrosis, TGF-β1 plays a key role in fibroblast proliferation, differentiation, and collagens and other ECM accumulation in

lungs. BMP-7, as a TGF-β antagonist, could inhibit TGF-β1-induced epithelial-to-mesenchymal transition and collagen deposition in hepatocytes, myocardial cells and renal tubular cells [14-16]. In lungs, it was recently demonstrated that BMP-7 could suppress collagen deposition and fibroblast differentiation induced by TGF-β1 in mouse lung fibroblasts and human lung fibroblasts [7, 8]. In our study, BMP-7 treatment dose-dependently reduced PQ-induced TGF-β1 overexpression and deposition of collagen I in mouse lungs, which indicated BMP-7 alleviated PQ-induced pulmonary fibrosis possibly via downregulating TGF-β1-dependent collagen deposition.

To further explore the underlying mechanisms, PQ-induced apoptosis and fibrotic response in fibroblasts (MRC-5 cells) treated by BMP-7 were investigated. Our data firstly suggested BMP-7 ameliorated PQ-induced apoptosis and deposition of collagen I in MRC-5 cells. Interestingly, recent studies demonstrated smads was not the only downstream molecules for PQ-induced TGF-β1 in fibrotic response, but MAPK proteins (for example p38) was implicated in this process [17, 18]. In this study, we reported PQ enhanced Erk1/2, JNK and p38 phosphorylation in MRC-5 cells. However, only PQ-induced Erk1/2 phosphorylation could be inhibited by BMP-7, indicating BMP-7 attenuated PQ-induced fibrotic injury in MRC-5 cells at least partly via Erk1/2 pathway. Overall, these results suggested Erk1/2, but not JNK and p38, contributed to the protective effects of BMP-7 on PQ-induced fibrotic injury in MRC-5 cells.

In summary, exogenous BMP-7 protects against PQ-induced fibrotic injury in mouse lungs and fibroblast cells (MRC-5 cells) dependent on a TGF-β1/Erk1/2 pathway. More importantly, our study could provide a promising therapeutic means for PQ-induced pulmonary fibrosis, and ultimately improve the prognosis of patients with PQ poisoning.

Acknowledgements

This study was supported in part by grant 2017SZ0120 from the Key Research and Development Program of Sichuan Province and grant 2014SZ0220 from the Science and Technology Support Program of Sichuan Province.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lei Chen, Department of Respiratory and Critical Care Medicine, West China Hospital, West China School of Medicine, Sichuan University, Guoxue Street 37#, Chengdu, Sichuan 610041, China. Tel: 86-28-85422380; Fax: 86-28-85422380; E-mail: lchens@126.com

References

[1] Wilson MS, Wynn TA. Pulmonary fibrosis: pathogenesis, etiology and regulation. Mucosal Immunol 2009; 2: 103-21.

[2] Yao R, Cao Y, He YR, Lau WB, Zeng Z, Liang ZA. Adiponectin attenuates lung fibroblasts activation and pulmonary fibrosis induced by paraquat. PLoS One 2015; 10: e0125169.

[3] Han YY, Shen P, Chang WX. Involvement of epithelial-to-mesenchymal transition and associated transforming growth factor-β/Smad signaling in paraquat-induced pulmonary fibrosis. Mol Med Rep 2015; 12: 7979-84.

[4] Chen J, Zeng T, Zhao X, Xie K, Bi Y, Zhong Z, Zhao X. Docosahexaenoic acid (DHA) ameliorates paraquat-induced pulmonary fibrosis in rats possibly through up-regulation of Smad 7 and SnoN. Food Chem Toxicol 2013; 57: 330-7.

[5] Liu MW, Su MX, Zhang W, Wang YQ, Chen M, Wang L, Qian CY. Protective effect of Xuebijing injection on paraquat-induced pulmonary injury via down-regulating the expression of p38 MAPK in rats. BMC Complement Altern Med 2014; 14: 498.

[6] Weiskirchen R, Meurer SK, Gressner OA, Herrmann J, Borkham-Kamphorst E, Gressner AM. BMP-7 as antagonist of organ fibrosis. Front Biosci (Landmark Ed) 2009; 14: 4992-5012.

[7] Izumi N, Mizuguchi S, Inagaki Y, Saika S, Kawada N, Nakajima Y, Inoue K, Suehiro S, Friedman SL, Ikeda K. BMP-7 opposes TGF-beta1-mediated collagen induction in mouse pulmonary myofibroblasts through Id2. Am J Physiol Lung Cell Mol Physiol 2006; 290: L120-6.

[8] Pegorier S, Campbell GA, Kay AB, Lloyd CM. Bone morphogenetic protein (BMP)-4 and BMP-7 regulate differentially transforming growth factor (TGF)-beta1 in normal human lung fibroblasts (NHLF). Respir Res 2010; 11: 85.

[9] Chen L, Wang T, Wang X, Sun BB, Li JQ, Liu DS, Zhang SF, Liu L, Xu D, Chen YJ, Wen FQ. Blockade of advanced glycation end product formation attenuates bleomycin-induced pulmonary fibrosis in rats. Respir Res 2009; 10: 55.

[10] Miyazono K, Kusanagi K, Inoue H. Divergence and convergence of TGF-beta/BMP signaling. J Cell Physiol 2001; 187: 265-76.
[11] Morrell NW, Yang X, Upton PD, Jourdan KB, Morgan N, Sheares KK, Trembath RC. Altered growth responses of pulmonary artery smooth muscle cells from patients with primary pulmonary hypertension to transforming growth factor-beta1 and bone morphogenetic proteins. Circulation 2001; 104: 790-5.
[12] Kinoshita K, Iimuro Y, Otogawa K, Saika S, Inagaki Y, Nakajima Y, Kawada N, Fujimoto J, Friedman SL, Ikeda K. Adenovirus-mediated expression of BMP-7 suppresses the development of liver fibrosis in rats. Gut 2007; 56: 706-14.
[13] Saika S, Ikeda K, Yamanaka O, Flanders KC, Ohnishi Y, Nakajima Y, Muragaki Y, Ooshima A. Adenoviral gene transfer of BMP-7, Id2, or Id3 suppresses injury-induced epithelial-to-mesenchymal transition of lens epithelium in mice. Am J Physiol Cell Physiol 2006; 290: C282-9.
[14] Zeisberg EM, Tarnavski O, Zeisberg M, Dorfman AL, McMullen JR, Gustafsson E, Chandraker A, Yuan X, Pu WT, Roberts AB, Neilson EG, Sayegh MH, Izumo S, Kalluri R. Endothelial-to-mesenchymal transition contributes to cardiac fibrosis. Nat Med 2007; 13: 952-61.
[15] Zeisberg M, Yang C, Martino M, Duncan MB, Rieder F, Tanjore H, Kalluri R. Fibroblasts derive from hepatocytes in liver fibrosis via epithelial to mesenchymal transition. J Biol Chem 2007; 282: 23337-47.
[16] Zeisberg M, Bottiglio C, Kumar N, Maeshima Y, Strutz F, Müller GA, Kalluri R. Bone morphogenic protein-7 inhibits progression of chronic renal fibrosis associated with two genetic mouse models. Am J Physiol Renal Physiol 2003; 285: F1060-7.
[17] Liu Z, Xue L, Liu Z, Huang J, Wen J, Hu J, Bo L, Yang R. Tumor necrosis factor-like weak inducer of apoptosis accelerates the progression of renal fibrosis in lupus nephritis by activating SMAD and p38 MAPK in TGF-β1 signaling pathway. Mediators Inflamm 2016; 2016: 8986451.
[18] Ge A, Ma Y, Liu YN, Li YS, Gu H, Zhang JX, Wang QX, Zeng XN, Huang M. Diosmetin prevents TGF-β1-induced epithelial-mesenchymal transition via ROS/MAPK signaling pathways. Life Sci 2016; 153: 1-8.