

Research Paper

The Anti-Fibrotic Effect of Bone Morphogenic Protein-7(BMP-7) on Liver Fibrosis

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Abstract

Background/Aims: Transforming growth factor- β_1 (TGF- β_1) plays an important role in the pathogenesis of liver fibrosis and cirrhosis. Recombinant human bone morphogenic protein-7 (rhBMP-7) alleviates renal fibrosis and improves kidney function. However, the beneficial effect of BMP-7 on hepatic fibrosis and cirrhosis remains unknown. The purpose of this study was to investigate the prophylactic and therapeutic effects of rhBMP-7 on liver fibrosis and the underlying mechanisms.

Methods: Liver fibrosis in the rat model was induced by peritoneal injection of porcine-serum (0.5ml/kg body weight) twice a week over 8 weeks. The effect of rhBMP-7 on hepatic fibrosis was monitored in rhBMP-7 pre-treated and non-treated rats. Pathologic changes were determined by immunohistological staining. TGF- β_1 expression was investigated by immunohistological staining, western blotting, and real-time PCR. Collagen secretion was measured by enzyme-linked immunosorbent assay.

Results: Liver fibrosis was significantly reduced by rhBMP-7. The secretion of collagen type-I and -III was decreased by rhBMP-7 in hepatic stellate cells (HSCs) but not in hepatocytes. The anti-fibrotic effect of rhBMP-7 on liver fibrosis was resulted by blocking the nuclear accumulation of Smad2/3 or by inhibiting TGF- β_1 expression in HSCs or hepatocytes.

Conclusions: The anti-fibrogenic mechanism of rhBMP-7 in the rat liver fibrosis was depended on the reduction of TGF- β_1 overexpression and the inhibition of TGF- β_1 triggered intracellular signalling in hepatic cells.

Key words: liver fibrosis, hepatic stellate cell, hepatocytes, bone morphogenic protein-7, transforming growth factor beta 1, epithelial-to-mesenchymal transition, extracellular matrix.

Introduction

Chronic liver diseases are characterized by the gradual destruction of the parenchyma structure and the excessive accumulation of extracellular matrix (ECM). It is assumed that the increased synthesis and decreased degradation of ECM distorts the hepatic architecture and results in liver fibrosis and cirrhosis (1-3).

Several studies provided evidence of hepatic fibrogenesis and increased expression of transforming growth factor- β_1 (TGF- β_1), which resulted from inflammation and injury. The complex role of TGF- β_1 in the pathology of liver fibrosis includes the deregulation of cell proliferation, differentiation, apoptosis, increased cytokine expression, and the deregulated

synthesis and degradation of ECM (4-15). Overexpression of TGF- β_1 in transgenic mice induced hepatic fibrosis and apoptosis of hepatocytes (16), which could be inhibited by neutralizing TGF- β_1 (17). TGF- β_1 increased the accumulation of collagen type-I, a major pro-inflammatory ECM component and plasminogen activator inhibitor-1 (PAI-1), the inhibitor of ECM degradation. The TGF- β /Smad intercellular signaling pathway is regarded to be the major mediator in TGF- β_1 induced hepatic fibrosis (18-21). Smad2/3 associates with Smad4 is required for TGF- β_1 induced collagen type-I synthesis and epithelial mesenchymal transition (EMT) activation (22). In contrast, Smad7 inhibited collagen and alpha smooth muscle actin (α -SMA) expression and reduced the ECM content by inhibiting Smad2/3 phosphorylation (23). In addition, hepatic stellate cell (HSC) generated TGF- β_1 promoted the upregulation of ECM expression and the increase of chemokine and cytokine expression during liver fibrosis (1). Moreover, the inhibition of TGF- β_1 by siRNA transfection or shRNA significantly decreased the expression of tissue inhibitors of metalloproteinases-I (TIMP-1), α -SMA and collagen type-I and of inflammatory cytokines (7).

Bone morphogenetic protein-7 (BMP-7), a 35KDa homodimeric protein, was initially described for its ability to induce bone formation (24). Renal injury rat models suggested that BMP-7 prohibited tubulointerstitial fibrogenesis (25, 26) and reversed renal fibrosis by counteracting TGF- β_1 -induced EMT (26-30). In addition, the exogenous administration of BMP-7 antagonized the expression of collagen type-IV, fibronectin, thrombospondin, and connective tissue growth factor (CTGF) in murine mesangial cells (28). BMP-7 prevented the TGF- β -driven ECM overexpression associated with fibrogenesis in mesangial cells (29). Zeisberg et al demonstrated that in renal chronic fibrosis the mechanism underlying the inhibitory effect of BMP-7 on TGF- β dependent EMT involved Smad-1/-3 and -5, but not mitogen activated protein kinases (MAPK) Erk1/2, p38, and JNK (25-26, 31, 32). However the detailed mechanism of TGF- β_1 signaling in the pathophysiology of hepatic fibrosis and cirrhosis remains unclear.

Material and methods

Animal model

All experimental procedures have been approved by the Institutes Animal Care and Use committee and the local experimental ethics committee. Female Sprague-Dawley rats (body weight 150 \pm 10 g) were purchased from Dept. of Experimental Animals, Fudan University (Shanghai, China). Rats were ran-

domly divided into five groups: negative control (n=10), positive control (n=8), prophylactic group (n=9), two treatment groups: early-stage treatment group (n=10) and late-stage treatment group (n=8). Rats treated with intraperitoneal injection of 0.5 ml/kg weight of saline twice a week served as negative control. Rats injected with porcine serum (0.5 ml/kg weight) represented the fibrotic positive control. In the prophylactic group, rats were pretreated with rhBMP-7 (100 μ g/kg weight) 1 week before porcine serum injection. In the two treatment groups, rats were intraperitoneal injected with rhBMP-7 (100 μ g/kg weight) every 48 h starting at week 2 and week 4 after porcine serum injection respectively. In the period of 8 weeks, rats were sacrificed and livers were collected.

Cell isolation and cell culture

Rat HSCs were isolated as described previously (30). Briefly, livers were perfused with D-Hanks buffer via the portal vein followed by perfusion for 10 min with DMEM medium (Shanghai Qibio Science and Technologies Co., Ltd., China) containing type-IV collagenase (0.05% for negative control and 0.1% for fibrotic rats respectively) and 0.1% protease E (Shanghai Joy Biopharm. Co., Ltd., China). Livers were cut into 2 mm³ pieces which were incubated in digesting solution (0.05% type-IV collagenase, 0.02% protease E and 0.01% DNase in DMEM) for 10 min at 37°C with frequent vortex. The supernatant was centrifuged (25,000*g, 20 min) over a 9.2% Nycodenz density gradient and the top pale cell ring was gently collected. After two washes with D-Hanks buffer, HSCs were collected by centrifuge (800*g, 5 min) and then resuspended in DMEM supplemented with 20% of fetal calf serum (Shanghai Qibio Science and Technologies Co., Ltd., China) for culture.

Rat hepatocytes were isolated and purified with the modified two-step procedures method as described by Seglen (32, 33). Rat livers were perfused with D-Hanks buffer (contains 10 units/ml heparin) via the portal vein and were dissected. Isolated livers were incubated 30 min at 25 °C after a perfusion with D-hanks buffer containing 0.0025% collagenase type-I and 0.075% CaCl₂.

The tissue digestion was dispersed through an 80 micron sieve and the digestion was neutralized with an equal volume of DMEM with 20% FBS. Cells were pelleted (800*g, 5 min) and washed with D-Hanks buffer. Cells were purified by a centrifugation through Nycodenz D-hanks solution (18% w/v) for 30 min at 1200*g to avoid the contamination of stellate cells. Obtained hepatocytes were washed with 10 ml DMEM containing 20% FBS three times.

HSCs and hepatocytes (1×10^4 cells/cm²) were seeded in DMEM supplemented with 20% FBS and were grown under regular cell culture condition until 60-70% confluence. Cells were then starved in serum free DMEM medium for 24 h before treatment.

Histological staining

Rat liver tissue was fixed in 10% formaline and then embedded in paraffin following standard procedures. Tissue sections were stained with Hematoxylin-Eosin and Masson's trichrome. The extent of liver fibrosis was estimated by Knodell scores (34).

Real-time (RT)-PCR

Total RNA was obtained by the phenolic-acid guanidium method (35). RNA (1 µg) was reverse transcribed using AMV First Strand cDNA Synthesis kit and random oligodeoxynucleotide primers (Roche Diagnostics Corporation, Indianapolis, IN). Quantitative real-time PCR amplifications were performed for TGF-β₁ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was employed as an internal control. Forward and reverse primer sequences for TGF-β₁ were 5'-ACTACACGCGAAGGCACAGC-3' and 5'-CTGCTCCTTCTGTCTCCCGA-3' (TOYOBO Biotech Co., Ltd., JPN) respectively. The TGF-β₁ probe was fam-5'-CTGGAGCCCGCGGTGATTGTAGAGC-3'-tamara 73. RT-PCR (Icycler, Bio-Rad laboratories, USA) was performed using 60 cycles as follows: 20s at 95°C thermal cycling and 20s at 60°C detection.

Western blots

The expression of TGF-β₁ by HSCs was evaluated by western blots (36). Total protein was extracted and an equal amount of protein was size fractionated in a 12% poly-acrylamide SDS-gel. Standard transfer to PVDF membranes (Millipore Corp., Billerica, MA, USA) was processed and monitored by Ponceau's staining. Membranes were blocked in blocking buffer (0.2% casein in 0.1% TBS-Tween, pH 7.5) for 1 h and then were incubated with the primary anti-TGF-β₁ antibody (1:200, SC-146, Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. After 3 washes (10 min each) with 0.1% TBS-Tween, membranes were incubated with a species specific horseradish-peroxidase linked secondary antibody (1:5000, SC-2004, Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature. Following 3 washes with 0.1% TBS-Tween, membranes were soaked in enzyme-linked chemiluminescence detection reagent (GE Healthcare, NJ, USA) and protein bands were visualized on x-ray films.

Enzyme-Linked Immunosorbent Assay (ELISA)

Serum starved cells were treated with rhBMP-7 (10-100ng/ml) for 24 h or 96 h. The culture medium was collected and cell debris was discarded after centrifugation (2,000*g, 5min). The quantity of collagen type-I, -III and -IV in supernatant was measured with ELISA kit (BP-E30413, BP-E30458 and BP-E30178, Shanghai Lengtong Biological Technology, China) following the manufacturer's instructions.

Immunohistostaining

HSCs and hepatocytes were grown to 80% confluence in cell culture chambers. Quiescent cells were incubated with rhBMP-7 for 24 h or 96 h respectively. Cells were fixed and permeabilized with cold methanol/acetone (1:1) for 10 min followed by 2 washes with phosphate buffered saline (PBS)-Triton x-100 (0.1%) for 10 min. Slides were blocked with 10% FBS in PBS for 1 h at room temperature and then incubated with the primary anti-Smad 2/3 antibody (1:200, SC-6033 Santa Cruz Biotechnology, CA, USA) over night at 4°C. After 3 washes with PBS-Triton x-100, slides were incubated with a species specific secondary antibody (1:1000, SC-2361 Santa Cruz Biotechnology, CA, USA) for 2 h at room temperature and nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI).

Statistical analysis

To assess intergroup differences: variance analysis and Chi²-square test were performed with SPSS 10.0 for windows. $P < 0.05$ was considered as statistical significance. The null hypothesis was: no difference between groups.

Results

Establishment of animal model

As shown in Fig. 1, the liver fibrosis was established by intraperitoneal injection of porcine serum. The severity of fibrosis in the animal model was time dependent or increased over time. Livers of the negative control group (panel A) showed normal hepatic lobule structure with the terminal hepatic venule at the centre and the portal tracts around the lobule. In the positive fibrosis control group, the pathological changes induced by porcine serum injection were observed. After 2 weeks, a significant increase of hepatic interstitial proliferation and fibrotic structures in form of enriched deposition of collagen (panel B-1: 2 weeks) was observed. After 6 weeks the typical septal fibrosis structure was observed, in which the hepatic lobules were clipped by complete and incomplete

fibrotic septa (panel B-2: 6 weeks). Late stages of liver fibrosis were found 8 weeks after porcine serum injection as massive fibrous proliferation, pseudo-lobules, and the start of nodular cirrhosis (panel B-3: 8 weeks). HSCs and hepatocytes were isolated.

The effect of rhBMP-7 on liver fibrosis development in rats

Liver tissue sections sampled from different groups were stained with Masson-trichrome to measure collagen accumulation and to score fibrosis (Fig.1). The degree of liver fibrosis was decreased in animals treated with rhBMP-7 in a time dependent

manner. The highest density of collagen in the liver was observed in the porcine serum treated group and the lowest expression of collagen was found in animals treated with rhBMP-7 for 6 weeks. Semi-quantitative analysis of the collagen contents showed a significant inhibitory effect of rhBMP-7 on porcine serum induced liver fibrosis scored by Masson's Trichrome-stain (Table 1). Notably, the level of liver fibrosis was significantly alleviated in rats that were pre-treated with rhBMP-7 comparing to those injected with rhBMP-7 after the initiation of fibrosis (Table 1).

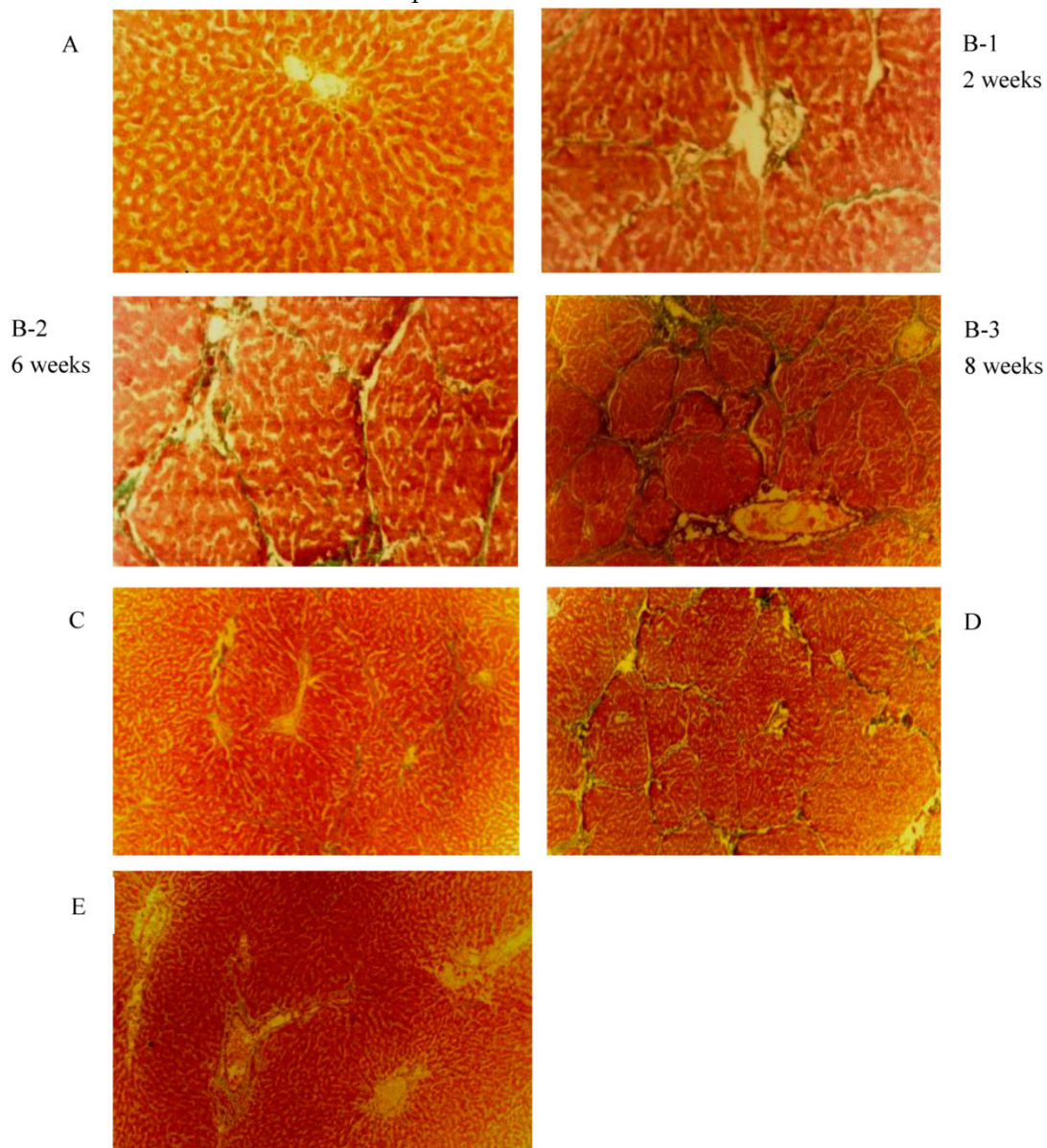


Fig. 1. Masson Trichrome and Hematoxylin-Eosin staining observed in rat liver tissue. (original magnification 40 X). (A) Negative control: Normal liver; (B) Positive control: liver fibrosis was induced by intraperitoneal injection with 0.5ml/kg weight porcine serum twice weekly for 8 weeks. Pictures were taken under at different time points. Week 2: Hepatic interstitial proliferation and fibrosis; Week 6: Septal fibrosis; Week 8: Massive fibrous proliferation in liver, pseudo-lobules and early nodular cirrhosis; (C) early-stage rhBMP-7 treatment; (D) late-stage treatment; (E) prophylactic.

Table I. The Knodell scores in liver tissue sections from different groups. The density of collagen was obtained by Masson-trichrome stain. Data were means ± SEM of independent experiments. Animal numbers with different Knodell classification was presented. *P<0.05, compared with group B (Positive control); + P<0.05, compared with group D (late-stage treatment).

Group	Knodell classification					n
	0	I	II	III	IV	
A: Negative control	10					10
B: Positive control				2	6	8
C: early-stage treatment*			2	6	2	10
D: late-stage treatment*			1	5	2	8
E: prophylactic*†		2	3	3	1	9

* P<0.05, compared with group B (Positive control); † P<0.05, compared with group D (late-stage treatment).

The effect of rhBMP-7 on the collagen secretion by primary HSCs and hepatocytes

The regulatory effect of rhBMP-7 on collagen secretion was examined by dose and time course experiments. In HSCs, the expression of collagen type-I and -III was significantly and dose dependently decreased by rhBMP-7 (Fig 2A, 2B, 2C), in which this effect also presented as a time dependent manner. Interestingly, the expression of collagen type-IV was not affected by rhBMP-7 at any experimental concentration in the first 24 h, but a higher concentration of rhBMP-7 (100 ng/ml) with a longer treatment (96 h) reduced the collagen type-IV expression. In contrast to HSC cells, the expression of three kinds of collagen in hepatocytes was not modified by rhBMP-7 (Fig 2D, 2E, 2F).

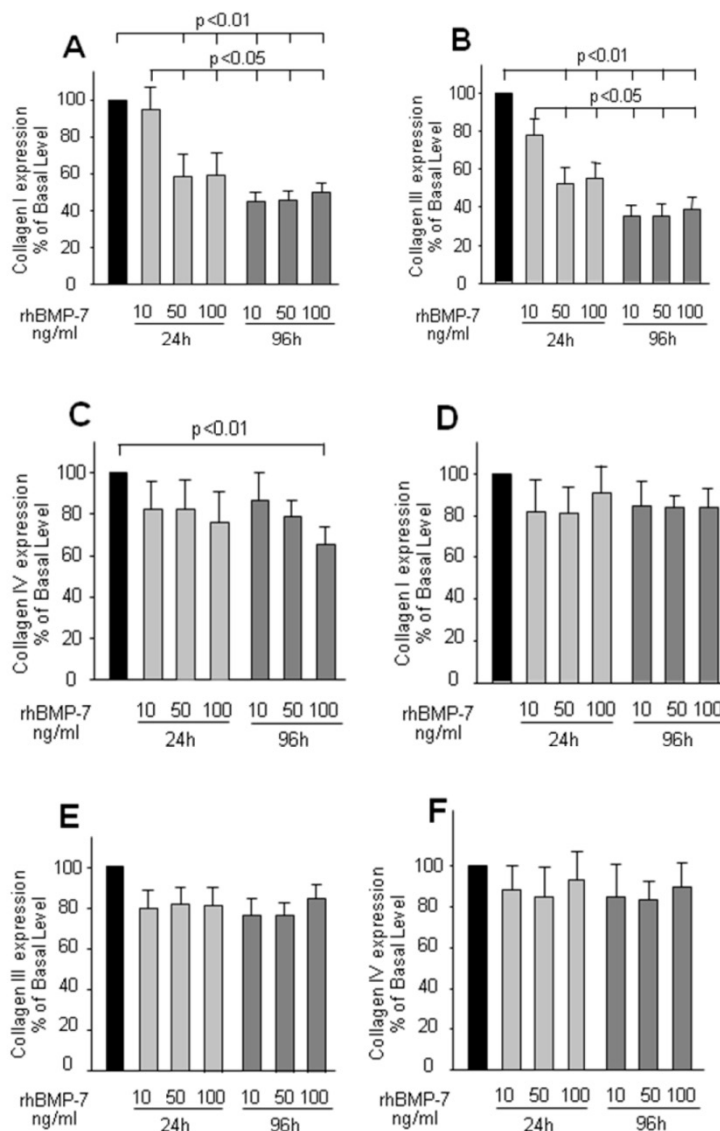


Fig. 2. ELISA results of collagen I, II, IV expression in cell culture supernatants in HSCs and hepatocytes. HSCs and hepatocytes were treated with rhBMP-7 in different concentration (10ng/ml, 50ng/ml, and 100ng/ml) for 24 h and 96 h respectively. The collagen expressed in untreated cells was serviced as control. Black bars, light grey bars, and grey bars indicated no treatment, rhBMP-7 for 24 h and rhBMP-7 for 96 h respectively. Data were means ± SEM of 10 independent experiments (n=10). Chart A, B, C were results from HSCs, and D, E, F were driven in hepatocytes respectively.

Effects of rhBMP-7 on the expression of TGF- β_1 in rat liver

As a potential stimulus of collagen secretion, the expression and location of TGF- β_1 was studied in liver tissue sections by immunohistochemical staining and in liver protein extraction by western-blot. Higher level of cytosolic TGF- β_1 was observed in liver fibrosis (Fig 3A), which was suppressed by rhBMP-7 treatment (Fig 3B). In line with the immunohistochemical pattern, western blots showed a time dependent reduction of TGF- β_1 expression by rhBMP-7 (Fig 4). Compared to the negative control (lane 1) and the positive fibrosis group (lane 5), the expression of TGF- β_1 in HSCs was reduced by rhBMP-7 (lane 2-4) and this inhibitory effect correlated with the length of rhBMP-7 treatment.

The effect of rhBMP-7 on TGF- β_1 receptor expression by HSCs

The expression of TGF- β_1 receptors (T β R-I, T β R-II) was measured by immunofluorescence staining (Fig 5) in isolated primary HSCs. Both T β R-I

(panel A and B) and T β R-II (panel C and D) were mainly located on the membrane of HSCs; however their expression was not affected by the presence of rhBMP-7 within 96 h.

The effect of rhBMP-7 on TGF- β_1 mRNA expression by primary HSCs

RT-PCR analysis of TGF- β_1 mRNA expression in growing HSCs was performed after 96 h rhBMP-7 (50ng/ml) treatment and showed that rhBMP-7 significantly inhibited the expression of TGF- β_1 mRNA by 8.33 fold compared to without rhBMP-7 treatment ($2^{-\Delta\Delta CT}=8.33$, $P<0.05$). (Fig. 6)

The role of rhBMP-7 in the activation of Smad 2 and 3 (Smad2/3) in HSCs and hepatocytes

Immunofluorescence staining in Fig. 7 clearly showed the nuclear accumulation of Smad2/3 in growing HSCs (A) and hepatocytes (C), whereas this signal was eliminated by rhBMP-7 (50ng/ml) in HSCs (B) and in hepatocytes (D) within 96 h.

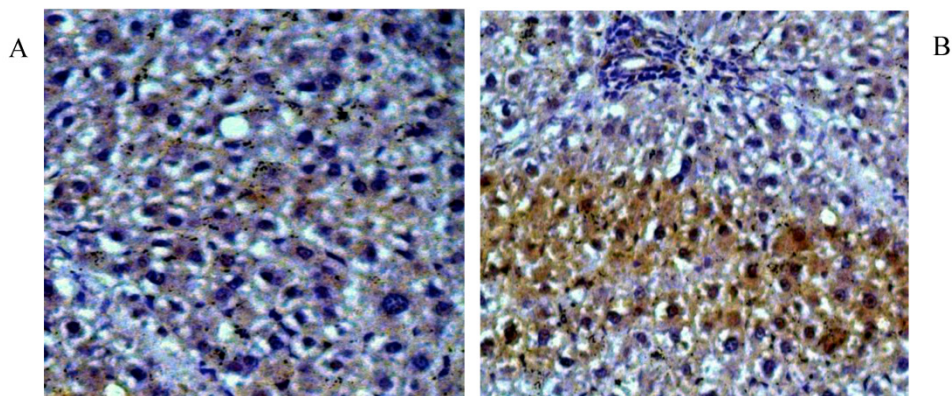


Fig. 3. Immunohistochemical staining for TGF- β_1 in rat liver tissues. Pictures were obtained under 200 X magnification. (A) Liver tissues from positive fibrosis control; (B) Liver section from early-stage treatment. Brown positive staining were the localizations of TGF- β_1 .

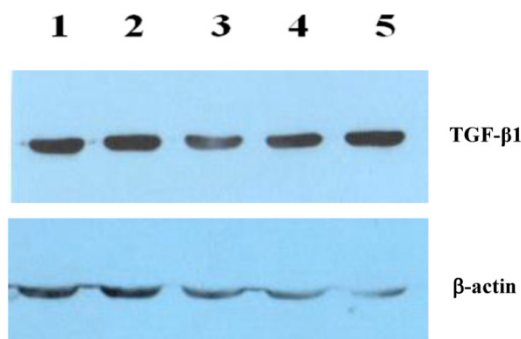


Fig. 4. The expression of TGF- β_1 using the protein extraction in rat liver tissues isolated from different studied group. Lane 1, negative control; lane 2, prophylactic group; lane 3, early-stage treatment; lane 4, late-stage treatment; and lane 5, positive fibrosis control.

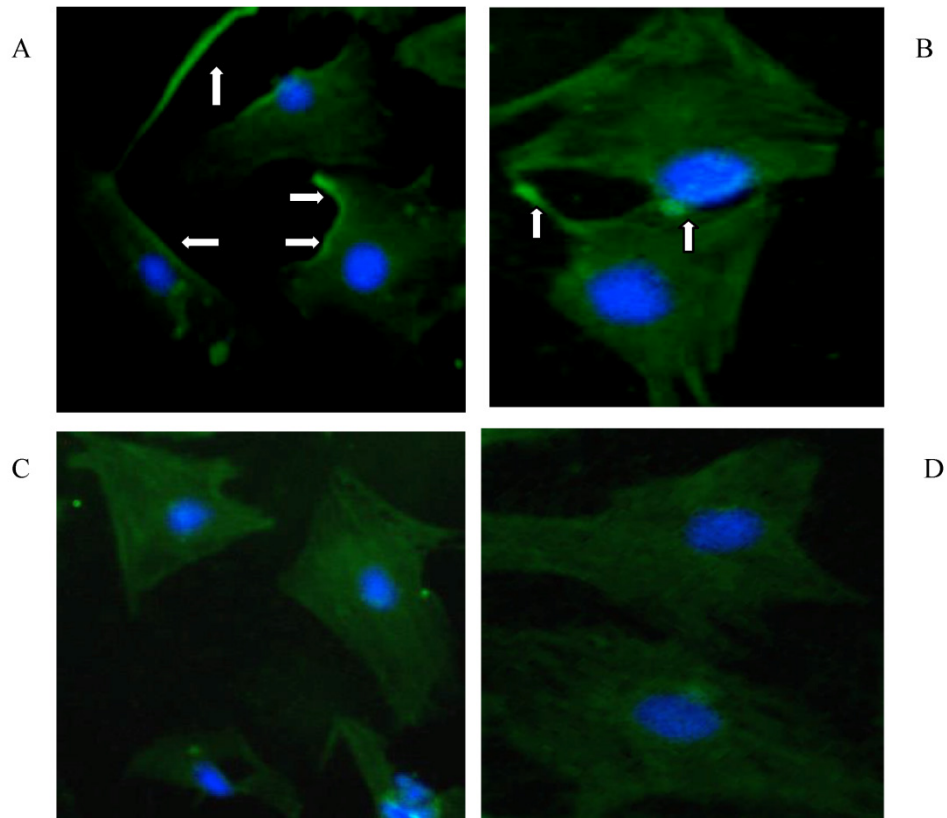


Fig. 5. Immunofluorescence staining for TGF- β_1 binding receptors (T β R-I, T β R-II) in HSCs cultured for 96 h with (B and D) and without (A and C) 50 ng/ml rhBMP-7 treatment. The arrows indicated the expression of T β R-I, T β R-II on the cell membrane of HSCs.

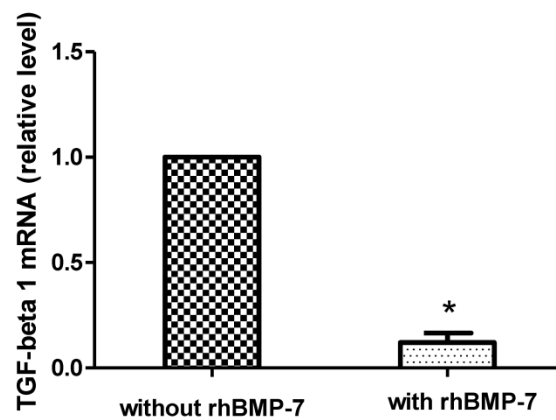


Fig. 6. rhBMP-7 affects the relative level of mRNA TGF- β_1 . TGF- β_1 mRNA expression was reduced treatment with rhBMP-7 compared without rhBMP-7 as detected by real-time RT-PCR. * $P < 0.05$.

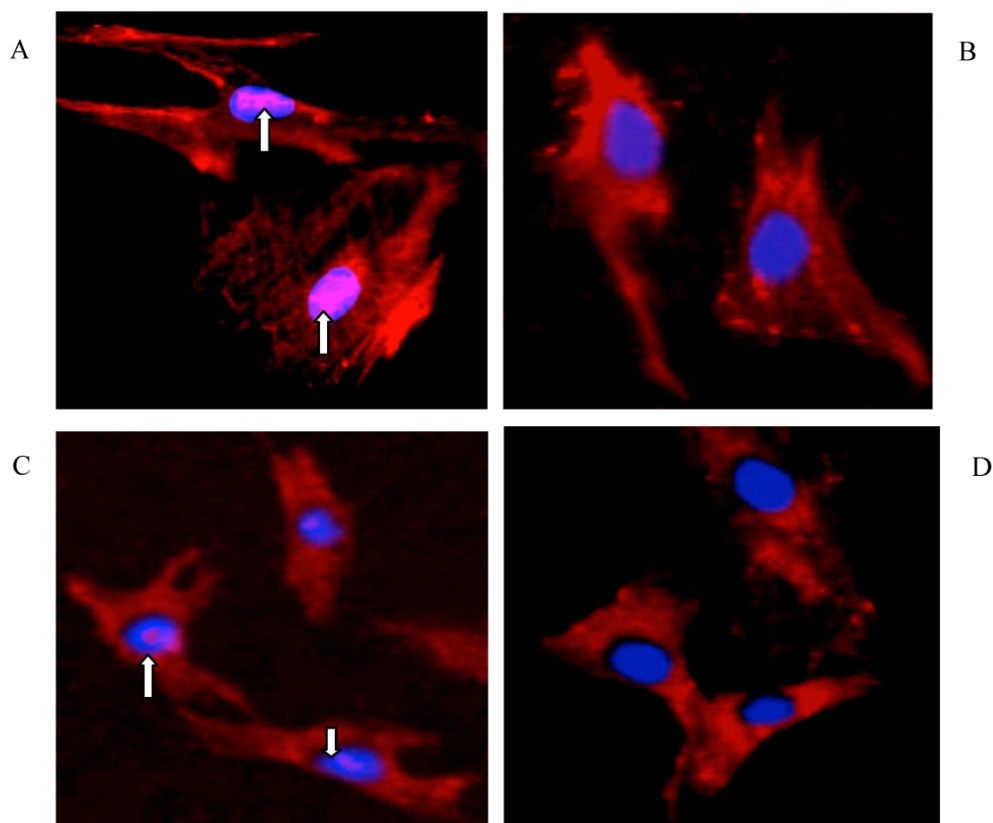


Fig. 7. Immunofluorescence staining for Smad2/3 in HSCs and hepatocytes. Primary rat HSCs and hepatocytes were cultured for 96 h with (B and D) and without (A and C) 50ng/ml of rhBMP-7 for 96 h. Smad2/3 expression was represented as pink staining and was indicated by arrows. The nuclear was shown as blue staining.

Discussion

In this study, a stable animal model of liver fibrosis has been established by repeated intraperitoneal injection of porcine serum. Compared to adenoviral vectors or other viral transduction systems induced liver fibrosis (37), our method avoided problems due to the immune responses against to viruses. Additionally the combination of in vivo and in vitro models has been for the first time used to investigate mechanism of liver fibrosis. Evidence claimed HSC as the major extracellular matrix producer in liver and its dysfunction resulted in a deregulated deposition of ECM (38-40). Activity of HSC by cytokines, such as TGF- β_1 , was demonstrated playing an important role in the progression of liver fibrosis. Hence the investigation in the function of HSC and the underlying mechanism are reasonable in liver fibrosis (1).

Liver fibrosis in rat was triggered by porcine serum in a time and dose dependent manner, in which the earliest lesion was observed after two weeks. Porcine serum induced liver fibrosis in rat was significantly inhibited by rhBMP-7, the effect of which was dependent on the duration of porcine serum

treatment and the length of rhBMP-7 treatment. Correlated with in vivo results, rhBMP-7 significantly reduced the expression of collagen type-I and -III by primary HSCs, which is considered as the major source of fibrillar collagens and of other ECM proteins (7, 38-40).

A strong TGF- β_1 signal was obtained either in rat liver tissue or in primary HSCs derived from fibrotic liver and this was decreased by rhBMP-7. The variation of the TGF- β_1 signal correlated with the level of collagen deposition and the gradient of liver fibrosis in vivo and in vitro and might indicate that TGF- β_1 underlied the porcine serum induced liver fibrosis.

TGF- β_1 plays an important role in initiating and maintaining fibrogenesis in many organs including the liver (41-43). In hepatic fibrogenesis, TGF- β_1 is believed to be involved in the synthesis and deposition of extracellular matrix components like fibronectin, collagens type I, III, and IV via an autocrine or a paracrine mechanism; however the detailed mechanism still remains unclear (44, 45). As a member of the TGF- β superfamily, BMP-7 was assumed to reverse fibrosis in the kidney by decreasing pro-inflammatory cytokines, inhibiting EMT and inducing matrix met-

alloproteinase activity (25-26, 31). In addition, the BMP-7 dependent resistance on the TGF- β_1 induced liver fibrosis in rats was explained by the inhibition of EMT in a cell culture model (32) and by the block of collagen expression in an animal model (27). Moreover, the upregulatory effect of TGF- β_1 on ECM expression together with the transdifferentiation to myofibroblasts in HSCs was considered as important fibrogenic contributions to fibrotic liver diseases (7, 11-12, 46-48). Furthermore, the pathological change of hepatocytes during the fibrogenic process was related to TGF- β_1 driven apoptosis and mesenchymal transition of hepatocytes into fibroblasts or fibroblast like cells (32).

To clarify the antifibrotic mechanism of rhBMP-7, we examined the activity of TGF- β_1 intracellular signal pathway in primary HSCs. The expression of TGF- β_1 binding receptor: T β R-I and T β R-II was not affected by rhBMP-7, while the expression of TGF- β_1 either on protein level or the mRNA transcription was significantly down-regulated by rhBMP-7. It was logic that the nuclear translocation of Smad-2/-3 was also interrupted by rhBMP-7 both in HSCs and in hepatocytes. The transcription of collagen genes was triggered by Smad-2/-3 phosphorylation (49-52). Therefore the inhibitory effect of rhBMP-7 on liver fibrosis depended on the decrease of TGF- β_1 and its signalling cascade, which was triggered by porcine serum.

In liver fibrosis, ECM accumulation was linked to activated fibroblasts which originated from hepatocytes by EMT that was induced by TGF- β_1 and inhibited by BMP-7 (32). Our finding together with other studies suggested that the inhibitory effect of rhBMP-7 on liver fibrosis occurred by decreased collagen deposition and the resistance to EMT. Whether the regulation of rhBMP-7 on TGF- β_1 signalling pathway occurred on the posttranscriptional level remained to be investigated.

In conclusion, we provided new evidence that rhBMP-7 could act as a prophylactic therapy for porcine-serum induced liver fibrosis through the decrease of TGF- β_1 expression, and the inhibition of Smad2/3 in HSCs and hepatocytes, which led to reduce collagens deposition.

Abbreviations

BMP-7, bone morphogenic protein-7; HSC, hepatic stellate cells; TGF- β_1 , transforming growth factor beta1; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-to-mesenchymal transition; RT-PCR, reverse transcriptase-polymerase chain reaction; T β R I and II, TGF- β_1 receptor type I and II; ECM, extracellular matrix.

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Conflict of interest

There is no conflict of interest for any author with the content of this paper.

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