

Adenoviral-bone morphogenetic protein-7 and/or doxazosin therapies promote the reversion of fibrosis/cirrhosis in a cirrhotic hamster model

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Received February 28, 2017; Accepted September 15, 2017

DOI: 10.3892/mmr.2017.7785

Abstract. Liver fibrosis occurs in the presence of continuous insults, including toxic or biological agents. Novel treatments must focus on ceasing the progression of cellular damage, promoting the regeneration of the parenchyma and inhibition of the fibrotic process. The present study analyzed the effect of bone morphogenetic protein (BMP)-7 gene therapy with or without co-treatment with doxazosin in a model of liver cirrhosis in hamsters. The serum alanine aminotransferase, aspartate aminotransferase and albumin levels were analyzed spectrophotometrically. Tissue hepatic samples were analyzed by hematoxylin and eosin for parenchymal structure and Sirius red for collagen fiber content. BMP-7 and α -smooth muscle actin (SMA)-positive cells were detected by immunohistochemistry. BMP-7 and collagen type I content in hepatic tissue were analyzed by western blotting, and tissue inhibitor of metalloproteinases (TIMP)-2 and matrix metalloproteinase (MMP)-13 expression levels were detected by reverse transcription-quantitative polymerase chain reaction. The present study detected a significant reduction of collagen type I deposits in the group treated with adenoviral-transduction with BMP-7 and doxazosin. In animals with BMP-7 and doxazosin therapy, α -SMA-positive cells were 31.7 and 29% significantly decreased compared with animals with placebo, respectively. Adenoviral-BMP-7 transduction and/or doxazosin treatments actively induced decrement in type I collagen

deposition via increased MMP-13 and reduced TIMP-2 expression. In conclusion, the adenovirus-BMP-7 gene therapy and the doxazosin therapy are potential candidates for the diminution of fibrosis in the liver, although combination of both therapies does not improve the individual anti-fibrotic effect once cirrhosis is established.

Introduction

Worldwide, liver cirrhosis is considered an important global health burden, with more than one million deaths in 2010 (1). Liver cirrhosis and fibrosis are chronic inflammatory diseases in which excessive wound healing is associated to multiple etiologies such as alcohol abuse, metabolic disorders, immune disease and viral infections (2,3). They are characterized by over deposition of extracellular matrix (ECM), with reduced expression of matrix metalloproteinases (MMPs) and increased expression of tissue inhibitor of metalloproteinases (TIMPs), and the consequent loss of liver functions (4,5). Fibrotic tissue increases the regenerative nodules and the distortion of the vascular function is responsible for most of the complications in this liver disease (6). The activation of hepatic stellate cells (HSCs) to the miofibroblasts phenotype plays a remarkable role during the pathogenesis of fibrosis and the establishment of liver cirrhosis (7). Growing evidence is demonstrating that inhibition of transforming growth factor (TGF)- β , an activator of HSCs, shows promising effects in the treatment of liver fibrosis (7-9). TGF- β is considered as a crucial profibrogenic cytokine involved in the fibroblasts recruitment, differentiation of miofibroblasts, epithelial-mesenchymal transition (EMT) and deposition of ECM (10,11).

Some drugs and pathways are being used as target for the blockage of fibrotic process, like angiogenesis inhibitors, anti-hypertensive drugs as angiotensin inhibitors, downregulation of TGF- β , and cell therapy strategies (12). Recently doxazosin, an α -blocker of adrenergic receptors, has been proposed as a potential antifibrotic drug in an experimental model of

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Key words: fibrosis, cirrhosis, collagen type I, bone morphogenetic protein-7, doxazosin, hepatic stellate cells

cirrhosis induced with carbon tetrachloride (CCl₄). After 6 weeks of treatment with doxazosin, a significant reduction in deposits of collagen type I was observed in cirrhotic livers, which was associated with a decrease in TGF- β synthesis (13). Nevertheless, doxazosin antifibrotic effect at shorter periods of treatment has not been studied on cirrhotic animals. Doxazosin is usually used to treat patients with hypertension by avoiding the norepinephrine binding to α 1-adrenergic receptors (14). Moreover, doxazosin relieves benign prostatic hypertrophy symptoms through the relaxing of the smooth muscles surrounding the prostate, so that it can reduce the urine retention and diminish bladder outlet obstruction (15).

In addition to reducing the complications in cirrhotic patients, new treatments must be focused on ceasing the progression of cellular damage, by promoting the regeneration of parenchyma and the inhibition of fibrotic process. Gene therapy coupled with standard drugs is a promising and viable alternative to alleviate liver chronic diseases in order to recover hepatocellular functions, improve portal pressure and arrest the progression of the liver fibrosis (7,16). Bone morphogenetic proteins (BMPs) are members of related proteins that belong to the superfamily of TGF- β with 47 identified members (17). BMPs have been described as excellent inducers of bone and cartilage, due to their capacity to induce cellular differentiation through cell signaling after receptor binding. BMPs also play pleiotropic roles in various biological processes in digestive tube, heart, brain, gonads, liver and skin (18-21). After binding to their receptors, a downstream Smad signaling is triggered with the consequent activation of target genes, mainly related to cellular proliferation and differentiation (22,23). It has been reported that BMP-7 causes the inhibition of the intracellular signaling initiated by TGF- β , through the blockage of Smad 3/4, and diminishes the effects of TGF- β (24-26). In absence of BMP-7, TGF- β binding to its receptor initiates the phosphorylation of Smad-2 and Smad-3 proteins, which join Smad-4 to form a complex which is translocated to the nucleus and activates the collagen type I synthesis and EMT activation. Otherwise, BMP-7 downstream signaling via activation of Smad-1, Smad-5 and Smad-8 can antagonize the effect of Smad-2/-3 through the heterodimerization with Smad-4, thereby Smad-3 is accumulated outside the nucleus (27,28). Several studies show that recombinant BMP-7 protein administered to animals reduces the fibrosis in kidney, lung and liver, by downregulating fibrotic markers such as collagen, α -smooth muscle actin (SMA) and TGF- β expression (29-32).

In this study, we analyzed the effect of BMP-7 gene therapy alone or in co-treatment with doxazosin in a model of liver cirrhosis in hamster. Our results suggest that the strategy combining BMP-7 gene therapy and doxazosin is useful for the reversion of liver cirrhosis.

Materials and methods

Animals. Six-to eight-weeks old male golden hamsters (*Mesocricetus auratus*), from 100 to 150 g weight, were maintained in the local Animal Core Facility with 12 h light/dark cycles and fed with Nutricubos (Ralston Purina Company, St. Louis, MO, USA) and water *ad libitum*. All animal experiments were approved by the Animal Welfare and Research Ethics Committee of the Autonomous

University of Aguascalientes (Aguascalientes, Mexico), and were conducted in accordance with institutional guidelines for caring for experimental animals and the national regulatory norm NOM-062-Z00-1999. Thirty animals were randomly distributed as follows: i) intact hamsters (n=5) (Intact group) and ii) experimental hamsters induced with cirrhosis by two intraperitoneal injections of 50 mg/kg of CCl₄ per week for 18 weeks (n=25). Intact animals and five experimental hamsters (Cirrhotic group) were sacrificed at week 18 to demonstrate the fibrosis/cirrhosis grade, collecting blood and liver samples. Photographs in two positions, liver anterior region and portal vein were documented with an Olympus xD Master 2 (SP-550UZ; Olympus Corporation, Tokyo, Japan).

Adenoviral-BMP-7 transduction and doxazosin therapies. Experimental hamsters were divided in four groups with 5 animals per group: i) Placebo, treated orally with water and via portal with a single injection of adenoviral storage buffer as vehicle (10 mM Tris, Ph 8.0, 100 mM NaCl, and 50% glycerol); ii) Doxa, orally treated with doxazosin 1 mg/kg/day; iii) BMP-7: treated via portal with a single injection of 1×10^9 PFU adenoviral vector Ad-BMP-7, as previously reported (33), and iv) BMP-7/Doxa, treated with the same doses and times that BMP-7 and Doxa groups. All treatments were administered for 2 weeks, once cirrhosis was established, and later animals were euthanized for specimen collection of liver and blood. Liver photographs were taken and tissue samples were fixed in neutral formalin. Serum samples were frozen at -80°C until used. The experimental protocol and treatment schedules for each group are illustrated in Fig. 1.

Protein extraction and Western blot analysis. To analyze the expression of BMP-7 and collagen type I in the liver parenchyma, 100 mg of tissue was homogenized in 1 ml of lysis buffer (Tris-HCl 50 mM pH 6.8, N-ethylmaleimide 5 mM, iodoacetamide 3 mM, phenylmethanesulfonyl fluoride 1 mM and tosyl-L-lysine chloromethyl ketone 3 mM) for total protein extraction. The lysate was centrifuged at 40,000 x g for 1 h at 4°C. The supernatant was recovered and proteins quantified with the Bradford method (34).

For western blot, 50 μ g of each protein extract was separated in a 12% SDS-PAGE gel, and proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Blockage was achieved for 1 h at room temperature with TBST (Tris-buffered saline/0.05% Tween-20) and 5% skimmed milk. For immunodetection, membrane was incubated for 1 h at room temperature with: 0.2 μ g/ml goat polyclonal anti BMP-7 (Santacruz Biotechnology, Dallas, Tx, USA), 1 μ g/ml mouse monoclonal anti collagen type I (Sigma-Aldrich, St. Louis, MO, USA) or 1 μ g/ml rabbit polyclonal anti β -actin (Abcam, Cambridge, UK). Then, blots were incubated with the alkaline phosphatase (AP)-conjugated antibodies: 1 μ g/ml rabbit anti-goat (Merk-Millipore, Darmstadt, GE), 0.25 μ g/ml goat anti-mouse (Sigma-Aldrich), or 0.5 μ g/ml goat anti-rabbit (Abcam), respectively. After three washes with TBS, blotting was developed with Sigma Fast BCIP®/NTB (Sigma-Aldrich). Analysis of collagen type I, BMP-7 and β -actin expression was performed with the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Mean of Intensity of collagen type I and BMP-7 bands were normalized respect to that of

Table I. Oligonucleotides sequences.

Gene	Oligonucleotides	Accession number	Amplicon size
MMP-13		XM_005077319.1	246 pb
F	TGTCCTGGCCACTCTCTTCT		
R	GGGTCATCAAGTTTGCCAGT		
TIMP-2		AF260255.1	196 pb
F	TCAAAGGCCCTGACAAAGAC		
R	AGGCTCTTCTTCTGGGTGGT		
β -actin		NM_001281595.1	115 pb
F	CCCCAGAGCAAGAGAGGTAT		
R	CACGCAGCTCGTTGTAGAAG		

MMP-13, matrix metalloproteinase-13; TIMP-2, tissue inhibitor of metalloproteinases; F, forward; R, reverse.

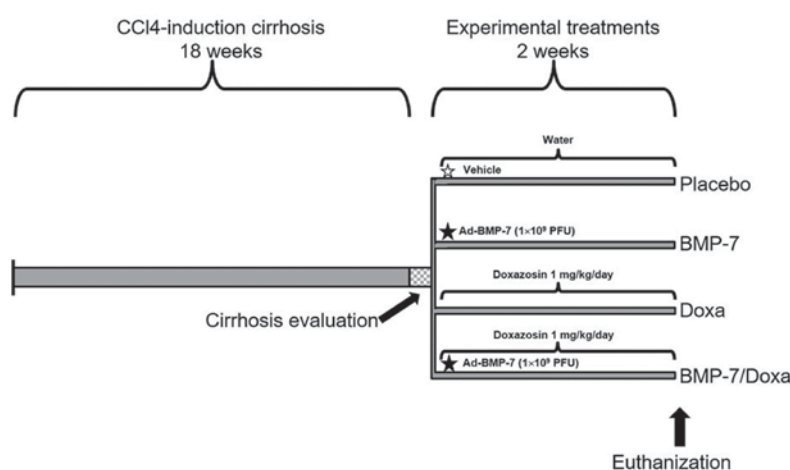


Figure 1. Time schedule of the CCl₄-induced cirrhosis hamster model and experimental treatments. CCl₄, carbon tetrachloride BMP-7; bone morphogenetic protein-7; Doxa, Doxazosin.

β -actin, as internal control and ratios BMP-7/collagen type I were calculated.

Liver function tests. The serum markers of liver damage: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and albumin were quantified in an Biosystems analyzer (bts-350; Biosystems, Quezon City, Philippines).

Histochemical and Immunohistochemical analysis. Tissues were paraffin-embedded and 5 μ m sections were obtained and stained with hematoxylin and eosin (H&E) and Sirius red techniques to evaluate parenchymal architecture and deposits of collagen type I or III as result of ECM rearrangement. Percent of fibrotic area was determined with the measure of fibrotic area in relation to the total area of the tissue. Morphometric analysis was performed with Image Pro Plus Software 4.5.1 (Media Cybernetics, Bethesda, MD, USA). Immunohistochemistry was performed to analyze BMP-7- and α -SMA-positive cells in liver tissues. Briefly, liver tissue slides were incubated with 2 μ g/ml goat polyclonal anti-BMP-7 (Santa Cruz Biotechnology) or 10 μ g/ml rabbit polyclonal anti- α -SMA (Abcam) for 12 h at 4°C. As a secondary antibody,

4 μ g/ml rabbit anti-goat (Merk-Millipore) conjugated with AP or EnVision+Dual Link System-HRP anti Rabbit/Mouse (Dako, Carpinteria, CA, USA), was respectively used. The AP activity for BMP-7 detection was developed with the Sigma Fast BCIP[®]/NTB (Sigma-Aldrich), and the HRP activity for α -SMA detection with Sigma Fast Diaminobenzidine (Sigma-Aldrich). Number of α -SMA-positive cells were counted in all slide and reported as cells/mm². Data was documented in a Zeiss Axioscope 40/40FL microscope (Zeiss, Oberkochen, Germany) and analyzed with the Image Pro Plus Software 4.5.1 (Media Cybernetics).

Isolation of total RNA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from 100 μ g of liver of the control and experimental animals with the SV Total RNA Isolation System (Promega, Madison, WI, USA) following the manufacturer's protocol. Total RNA was stored at -80°C until use. Reverse transcription for cDNA synthesis was performed with 1 μ g of total RNA using the GoScript Reverse Transcription System (Promega) and real time qPCR with the buffer GreenMaster with UNG-clear (Jena Bioscience, Jena, Germany) in a

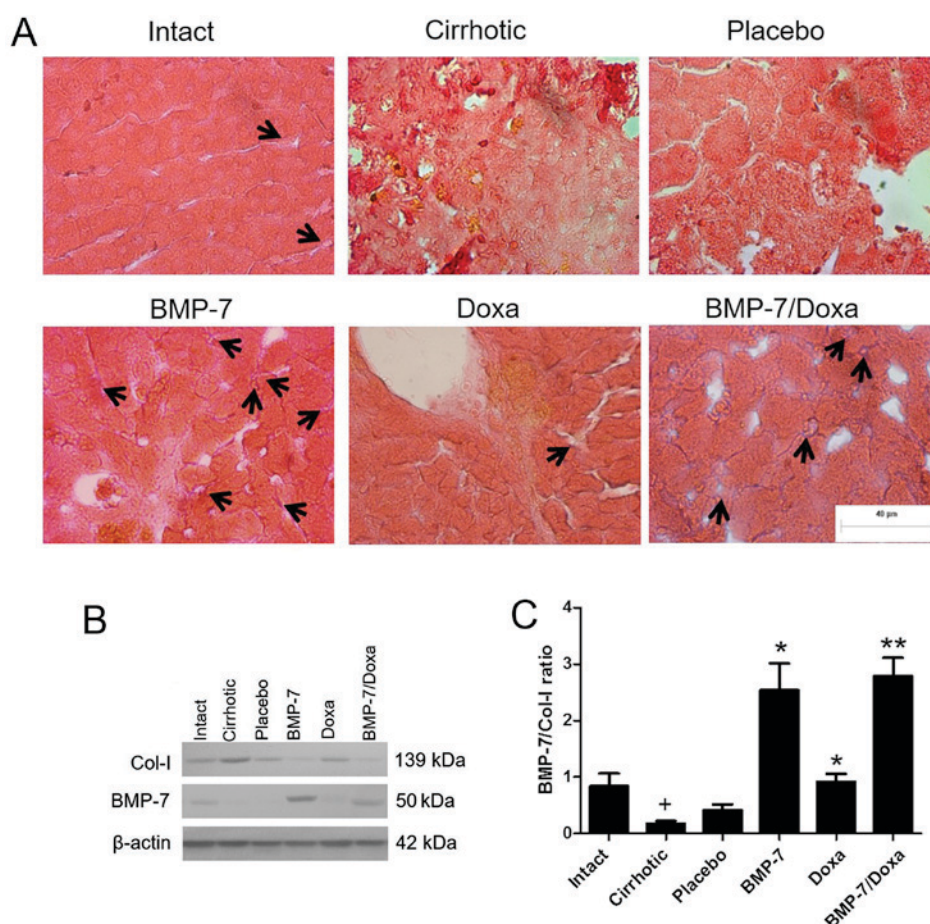


Figure 2. Evaluation of BMP-7 and collagen type I expression in cirrhotic hamsters. (A) Immunohistochemistry for identification of BMP-7-positive secreting hepatocytes (black arrows) after two weeks of adenoviral administration. (B) Western blot for collagen type I (Col-I) and BMP-7 expression in liver tissue. (C) Densitometry determination is represented as BMP-7/collagen type I ratios. Data is presented as mean \pm SEM. * $P < 0.001$ vs. Intact; * $P < 0.05$ and ** $P < 0.005$ vs. Placebo. BMP-7, bone morphogenetic protein; Doxa, Doxazosin, Col-I, collagen type I; SEM, standard error of the mean.

StepOne Real-Time PCR Machine (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Oligonucleotides were designed to target on MMP-13, TIMP-2, and actin as a reference control (Table I). The RT-PCR program used for all targets was: 50°C for 2 min, 95°C for 45 sec, 40 cycles of 95°C for 45 sec and 60°C for 45 sec, and extension at 72°C for 12 sec. Relative quantification of target mRNA expression was calculated by the $\Delta\Delta C_t$ method using β -actin for normalization (35).

Statistical analysis. GraphPad Prism V5 software (GraphPad Software, Inc., La Jolla, CA, USA) was employed for statistical analysis. Data are expressed as the mean \pm standard error of the mean (SEM) of five animals. Significant differences between mean values were evaluated by using the two tailed Student's *t* test. Statistical significance was considered at $P < 0.05$.

Results

Expression of BMP-7 and its relationship with collagen type I levels in hepatic parenchyma of transduced CCl₄-induced cirrhosis hamsters. The administration of 1×10^9 PFU of the vector via porta was efficiently tolerated by animals. To confirm protein expression of BMP-7 in the hepatic

parenchyma of transduced animals, immunohistochemistry and Western blot was developed in liver tissue (Fig. 2). Immunohistochemical examination showed BMP-7 secreting hepatocytes in liver from BMP-7 and BMP-7/Doxa animals (Fig. 2A, black arrows). BMP-7 native production was also detected in Intact and Doxa groups, and remains almost undetectable in Placebo and Cirrhotic animals. Similar results were obtained by western blot analysis, as seen in Fig. 2B. Although basal expression of BMP-7 was present in Intact, Cirrhotic, Placebo and Doxa groups, the expression of the protein was much higher in the groups BMP-7 and BMP-7/Doxa after 2 weeks of adenoviral delivery. Also, BMP-7 expression was correlated with collagen type I production, since an increase in BMP-7 levels in BMP-7 and BMP-7/Doxa groups was associated with a decrease in the levels of collagen type I (Fig. 2B). The densitometric analysis revealed that cirrhotic animals decreased BMP-7/Col-I ratio, but vector administration significantly increased 6.1- and 6.7-fold BMP-7/Col-I ratio in BMP-7 and BMP-7/Doxa groups, respect to Placebo animals (Fig. 2C). When doxazosin was administered alone, although collagen type I expression was similar to Placebo group, there was also an increase in BMP-7/Col-I ratio in these animals that was 2-fold higher than in Placebo group.

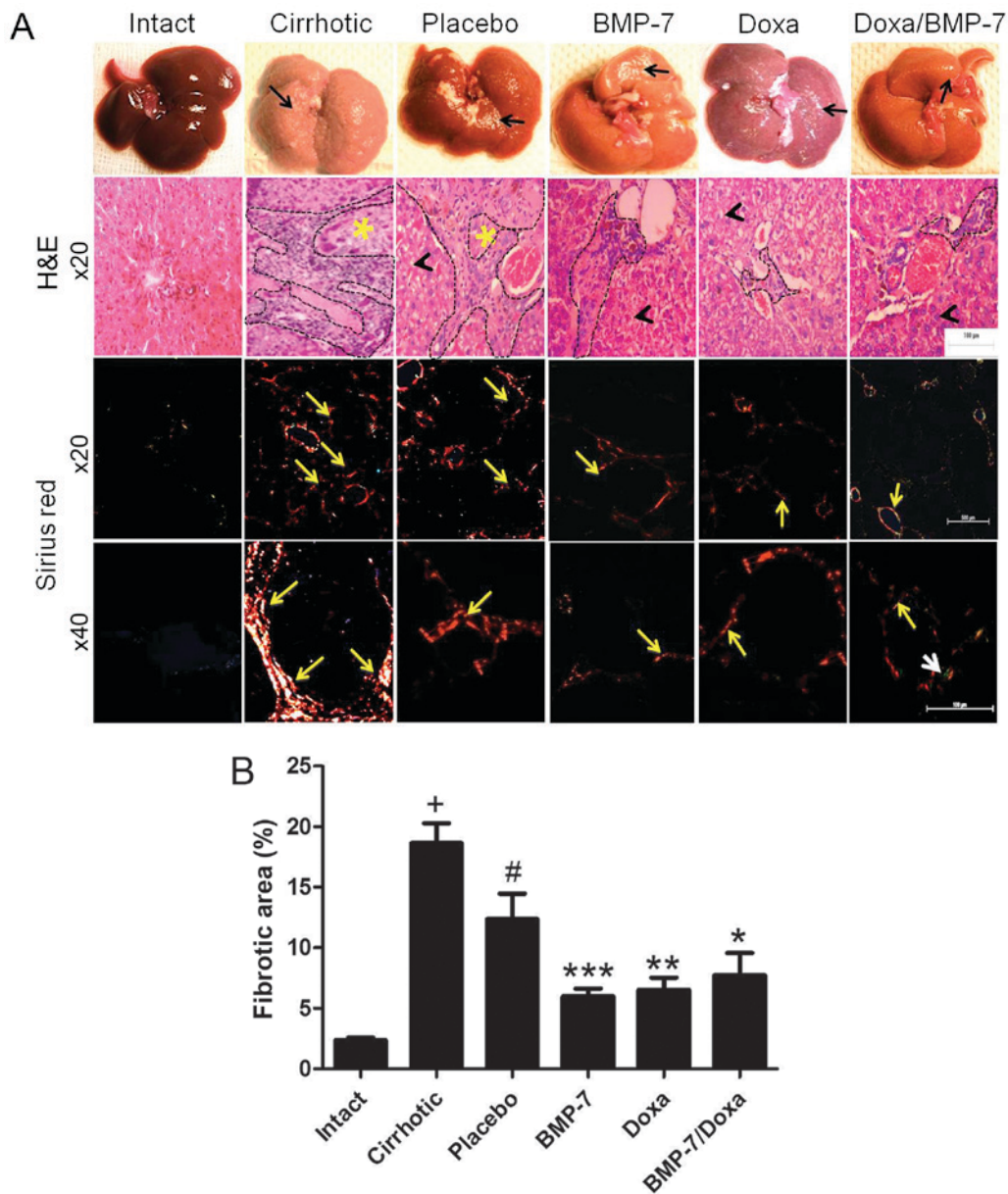


Figure 3. Liver macroscopic observation and histological analysis for the acinus architecture remodeling and collagen deposition in experimental groups. (A) Representative pictures of livers from experimental groups and the corresponding microphotographs of slides stained with H&E (magnification, x20) or Sirius red (magnification, x20 and x40) techniques. Black arrows show granulated aspect of the livers; black arrowheads indicate histological aspect of hepatocytes; black dashed lines demarcate inflammatory infiltrates; yellow asterisks mark regenerative nodules; yellow arrows point to collagen type I fiber; and white arrow to collagen type III fibers. (B) Percentage of fibrotic area was determined by measuring the amount of fibrotic area in respect to whole area. Data is presented as mean \pm SEM. * $P < 0.001$ vs. Intact; # $P < 0.01$ vs. Cirrhotic; + $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$ vs. Placebo. BMP-7, bone morphogenetic protein; Doxa, Doxazosin; SEM, standard error of the mean.

Regression of cirrhosis with α -adrenergic receptor antagonist and therapy with BMP-7. The histological changes and accumulation of collagen type I were evaluated by studying liver architecture and collagen type I deposition by H&E and Sirius red staining under light microscopy and polarized-light microscopy (Fig. 3A). The macroscopic observation of Intact group livers evidenced normal aspect, shape and dark-browned color. The H&E staining showed the liver parenchyma well organized in acinus with a central vein. Moreover, Sirius red staining did not reveal collagen type I accumulation at magnification, x20 and x40. In the Cirrhotic group, livers had granulated surface (black arrow); the H&E images showed a wide area of inflammatory infiltrate and possible fibrosis

(black dashed line) that delimited the regenerative nodules (yellow asterisk). Sirius red staining evidenced several circles of collagen type I surrounding regenerative nodules (x20, yellow arrow), in which collagen type I accumulated in the periphery (x40, yellow arrow). The livers in the Placebo group were similar to the Cirrhotic group, with granulated surface (black arrow). We observed an inflammatory infiltrate (black dashed outline) in the tissue architecture, and hepatocytes appeared edematous (black arrowhead). After observation at magnification, x20 and x40 with Sirius red staining, liver slides had lesser aggregation of collagen type I fibers (yellow arrows) when compared to the Cirrhotic group. Livers from BMP-7 group had reduced granulation on their surface (black arrow),

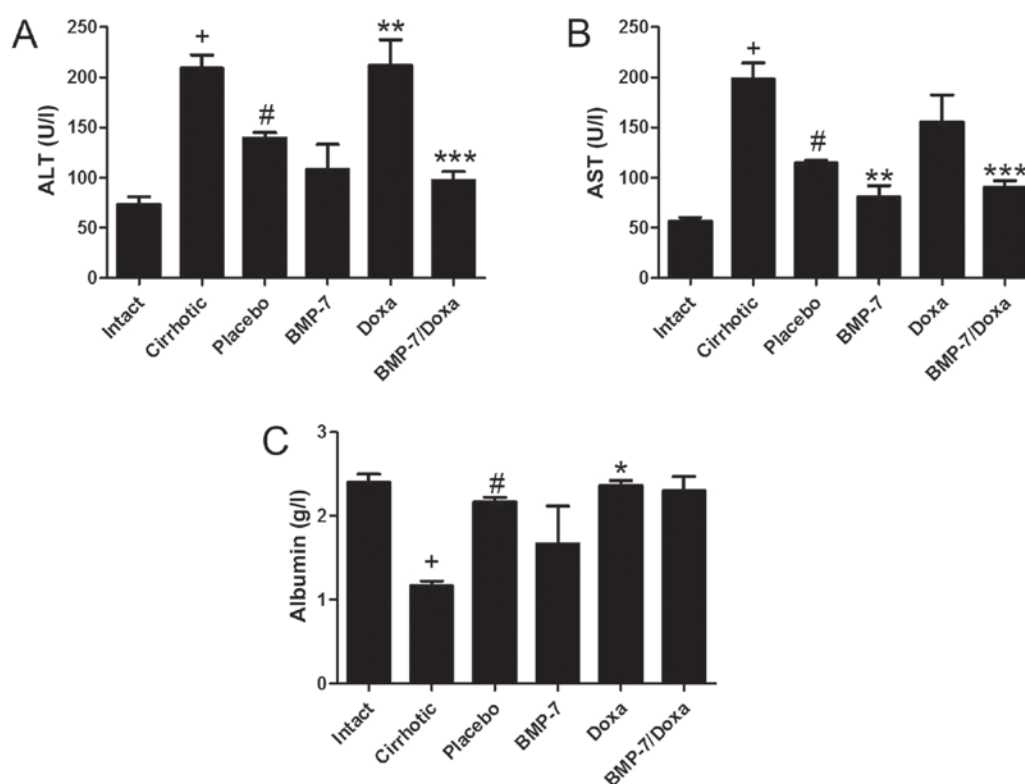


Figure 4. Liver function assessment by quantification of serum levels of (A) ALT, (B) AST activities and (C) albumin concentration. Data is presented as mean \pm SEM. ⁺P<0.001 vs. Intact; [#]P<0.005 vs. Cirrhotic; ^{*}P<0.05, ^{**}P<0.01 and ^{***}P<0.005 vs. Placebo. ALT, alanine aminotransferase; BMP-7, bone morpho-genetic protein; AST, aspartate aminotransferase; Doxa, Doxazosin; SEM, standard error of the mean.

liver parenchyma showed less inflammatory infiltrate (black dashed line) surrounding hepatocytes with notable reduction of edema (black arrowhead), and reduced fibers of collagen type I (yellow arrows). Doxazosin-treated cirrhotic hamsters had little granulation on the liver surface (black arrow); like in the BMP-7 group, scarce inflammatory infiltrate (black dashed line) and some edematous hepatocytes were observed (black arrowhead), and collagen type I fibers (yellow arrows) were markedly reduced when compared to Placebo group. In the group that received the double treatment (BMP-7/Doxa), the surfaces of the livers were barely granulated (black arrow), liver parenchyma showed few inflammatory infiltrate (black dashed line) surrounding morphologically healthy hepatocytes (black arrowhead). Outstandingly, the observation of the Sirius red staining showed scarce deposits of collagen type I (yellow arrows) and merging fibers of collagen type III (white arrow).

The measurement of the fibrotic area clearly demonstrated a significant 7.8-fold increment of collagen type I deposits in liver of cirrhotic animals. Placebo group showed a spontaneous 33.6% decrease of these collagen deposits. However, the treatment with adenoviral BMP-7-transduction induced a reduction of 51.6% of collagen type I deposits, and Doxa group had 47.1% reduced collagen type I accumulation, when compared to Placebo group. The percent of the fibrotic area was reduced 37.4% in cirrhotic hamster treated with doxazosin and BMP-7 in comparison to the Placebo group (Fig. 3B). BMP-7 did not improve the antifibrogenic effect of doxazosin, as there were not differences between Doxa and BMP-7/Doxa groups. These data demonstrated that both adenoviral-BMP-7 gene and doxazosin treatment are potential candidates for decreasing

liver fibrosis, although combination of both therapies do not improve their individual antifibrotic effect once cirrhosis is established.

Recovery of liver function in BMP-7 transduced and/or Doxa treated animals. The plasma levels of liver enzymes ALT and AST, and albumin were measured to determine the liver function in experimental groups (Fig. 4). Liver function tests supported the histological findings in 50 mg/kg CCl₄-induced hamsters treated with BMP-7 and BMP-7/Doxa. As expected, the cirrhotic group had significantly increased levels of ALT and AST, and diminished concentration of albumin when compared to Intact group. Animals from Placebo group showed a spontaneous recovery of these biochemical markers of hepatic damage, as they decrease in 33.2 and 42.3% the levels of ALT and AST and increase in 86.2% albumin concentration in blood. In the BMP-7 group, adenoviral-BMP-7 transduction significantly lowered the levels of AST (29.6%) as compared to the Placebo group, although ALT and albumin remained without significant changes. However, after two weeks of doxazosin treatment (Doxa group), ALT levels and albumin were 34.1 and 6% higher than in the Placebo group, respectively, which presumably indicates hepatotoxic activity of doxazosin. Moreover, adenoviral-BMP-7 transduction and doxazosin treatment induced significant diminution of ALT and AST levels, while concentration of albumin was unchanged, as compared to Placebo group. These results suggest that BMP-7 over-expression combined with doxazosin may reduce the biochemical abnormalities after hepatic damage induced

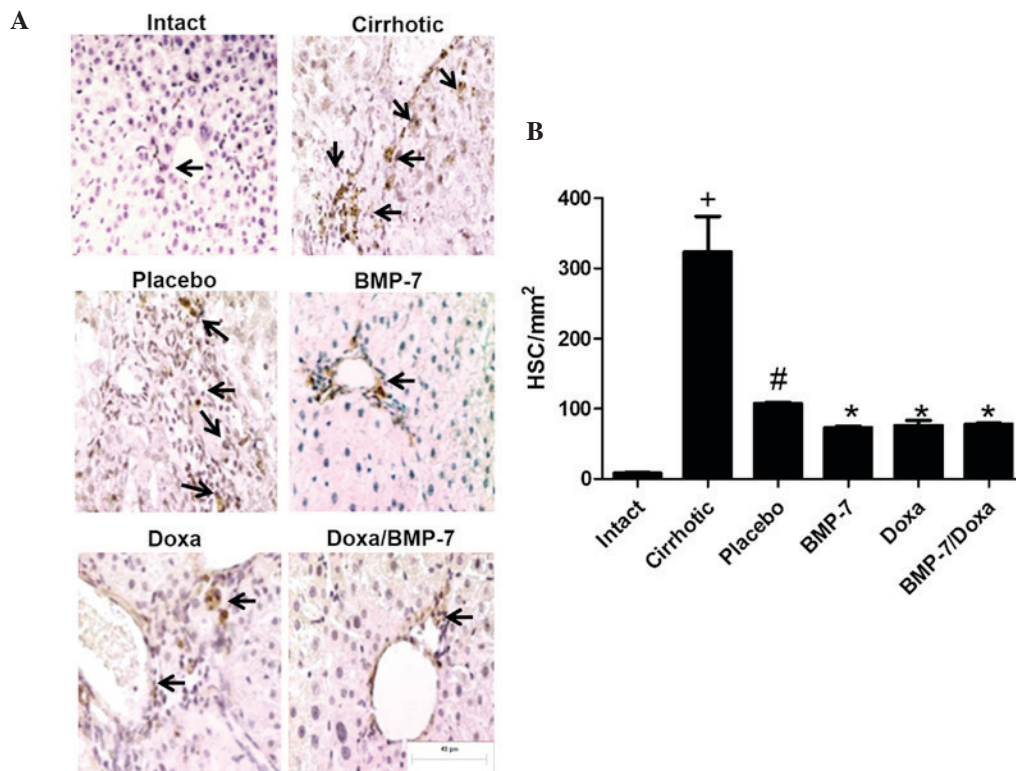


Figure 5. Immunohistochemical quantification of α -SMA-positive cells in cirrhotic livers after adenoviral-BMP-7 transduction and doxazosin treatments. (A) Representative microphotographs observed at 20x, black arrows indicate HSCs. (B) Morphometric analysis of α -SMA-positive cells per unit of area. Data is presented as mean \pm SEM. ^{*}P<0.001 vs. Intact; [#]P<0.005 vs. Cirrhotic; ^{*}P<0.005 vs. Placebo. HSC, hepatic stellate cells; BMP-7, bone morphogenetic protein; Doxa, Doxazosin; SEM, standard error of the mean.

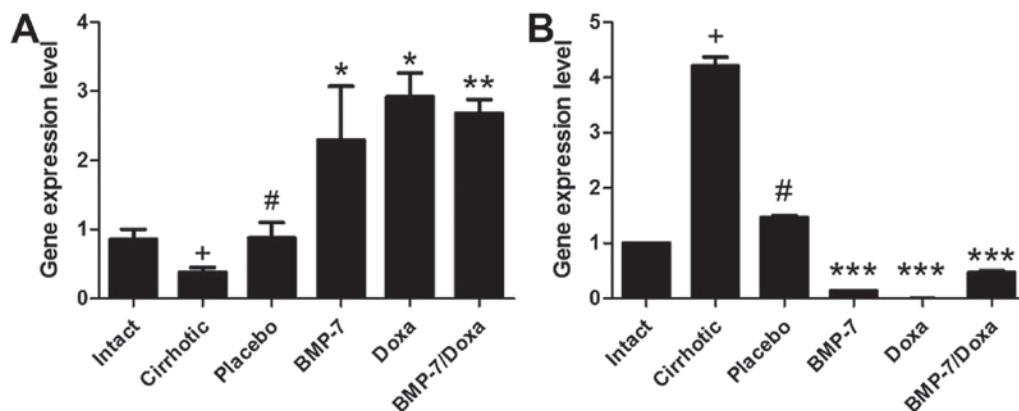


Figure 6. Gene expression levels of (A) MMP-13 and (B) TIMP-2. Data is presented as mean \pm SEM. ^{*}P<0.005 vs. Intact; ^{*}P<0.05 vs. Cirrhotic; ^{*}P<0.05, ^{**}P<0.01 and ^{***}P<0.001 vs. Placebo. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; BMP-7, bone morphogenetic protein; Doxa, Doxazosin; SEM, standard error of the mean.

with CCl₄, even when two weeks of doxazosin treatment appear to induce toxic effects in the cirrhotic liver.

Analyses of hepatic fibrosis by immunohistochemistry for HSCs. To correlate changes in number of activated HSCs to the fibrosis reduction, we analyzed the number of α -SMA-positive cells in hepatic tissue from 50 mg/kg CCl₄-induced hamsters treated with adenoviral-BMP-7 transduction and/or doxazosin (Fig. 5A). As expected, the amount of activated HSCs in liver of cirrhotic animals was 40.4-fold higher than in Intact hamsters. When cirrhotic animals were

placebo administered there was a spontaneous decrease of 66.9% in the number of liver activated HSCs. In the BMP-7 and Doxa groups, α -SMA-positive cells were 31.7 and 29% significantly lower than in the Placebo. Combination of BMP-7-adenoviral transduction and Doxa did not improve reduction of α -SMA-positive cells in liver parenchyma once cirrhosis was established (Fig. 5B). Taken together, these data indicated that Doxa and BMP-7 exert the antifibrotic effect, at least in part, by reduction of HSCs; although the combination of both treatments does not increase the reduction of this activated cell population.

Changes in MMP-13 and TIMP-2 expression are associated to antifibrotic effect of experimental treatments. We further examined MMP-13 (Fig. 6A) and its inhibitor TIMP-2 (Fig. 6B) mRNA expression, as both molecules are involved in ECM remodeling. As it is known that occurs in cirrhotic damage, MMP-13 expression was significantly decreased in liver of Cirrhotic animals while that of TIMP-2 was increased. Placebo administration increased 2.3-fold MMP-13 expression and diminished in a 65.2% TIMP-2 mRNA levels. Consistent with the histological data, the experimental treatments were associated with upregulated MMP-13 mRNA expression, as well as downregulated TIMP-2 mRNA. In the BMP-7 group, MMP-13 and TIMP-2 were inversely regulated with increment of 2.6-fold and reduction of 11.2-fold, respectively, when compared to Placebo group. Also, Doxa treatment promoted a 3.3-fold increased expression of MMP-13 and almost undetectable expression of TIMP-2. Nevertheless, BMP-7/Doxa group had 3.0-fold increased expression of MMP-13 mRNA and TIMP-2 mRNA was 3.1-fold lowered than Placebo group. These data suggest that BMP-7-adenoviral transduction and/or doxazosin treatments induce decrement in the collagen type I deposition by increasing MMP-13 expression and reducing TIMP-2 expression. These findings are consistent with collagen type I accumulation in the cirrhotic animals. However, the combined BMP-7/Doxa therapy does not exceed the benefits observed in the separate treatments.

Discussion

Cirrhosis represents the major pathological outcome of continuous insult to the liver and it also precludes the development of hepatocellular carcinoma in some patients. Currently, drugs targeting fibrosis in clinical practice are attracting the interest of researchers, who have generated innovative therapeutic strategies addressed to target fibrogenic cytokines, such as TGF- β , with blocking peptides (36), or through the inhibition of HSCs activation (37). Thus, in the present study, we evaluated whether the drug therapy with the α -adrenergic blocker doxazosin or gene therapy through adenoviral transduction of BMP-7, could have antifibrotic effects as individual or combined treatments in a hamster model of CCl₄-induced cirrhosis.

Spontaneous reversion of the liver cirrhosis after cessation of the CCl₄-induction has been well documented (38-40). We observed spontaneous improvement in the fibrotic process and liver function in animals from Placebo group. Chávez *et al* demonstrated spontaneous resolution of liver fibrosis in vehicle treated CCl₄-induced rats. The placebo treatment also diminished the levels of ALT in CCl₄-induced cirrhosis even after 4 weeks of discontinued induction (41). Besides, other authors reported that the spontaneous reversal of cirrhosis after 6 weeks of vehicle daily administration was accompanied by diminution of α -SMA positive cells and down-expression of TIMP-1, as well by up-expression of MMP-13 (42). In our study, drug therapy with doxazosin and BMP-7 showed greater recovery than spontaneous reversion, outstanding their promissory role in the treatment of fibrotic liver.

BMP-7 has inhibitory downstream activity over TGF- β signaling, as Smad-1, -5 and -8, avoiding the activation of

Smad-3/4 mediated by TGF- β (24-26,43). Thus, when BMP-7 was administered, fibrosis deposition, some liver functions, HSCs hyperplasia, and expression changes in MMP and TIMP were favored to retrieve the functionality of the liver. Studies have also demonstrated how oral administration of a recombinant adeno-associated virus (AAV)-mediated BMP-7 transduction reduces the hepatic fibrosis and suppresses the activation of HSCs (44). Moreover, gene delivery of BMP-7 via polyethylenimine-conjugated gold nanoparticles in corneal tissue demonstrated diminution of α -SMA positive cells and fibronectin, in model of corneal fibrosis after photorefractive keratectomy in rabbits (45). Besides, administration of recombinant human BMP-7 reduced tubular damage and tubulointerstitial fibrosis in streptozotocin-induced diabetic mice with renal disease (46). Recently, natural compounds have shown to promote the activation of BMP-7/Smad signaling, which in turn modified the serum, biochemical and histological markers of hepatic damage, indicating a diminution of liver fibrosis in a model of bile duct ligation-induced liver fibrosis (47).

Previous studies have reported a significant reduction in deposits of collagen type I in cirrhotic hamsters treated with Doxazosin, when it was administered during 6 weeks after liver damage development (13). The effect was related with a decrease in TGF- β synthesis. We showed that when Doxazosin is only administered for 2 weeks, the beneficial effect on cirrhosis is maintained, as demonstrated by the decrease in the fibrotic area and collagen type I deposit in the liver of cirrhotic CCl₄-induced hamsters. The antifibrotic effect of doxazosin has also been reported in renal fibrosis and myocardial interstitial fibrosis (48-50). As HSCs, the main fibrinogenic cells in liver, express α_1 -AR (51,52) we argue that is the selective inhibition of this receptor in HSCs which is causing the decrease in number of activated cells and therefore in collagen synthesis. Some differences are reported between 2 or 6 weeks of treatment with Doxazosin in relation to liver enzymes. When it was administered through 6 weeks ALT was significantly decreased (13), but no significant change in liver enzymes is observed after 2 weeks of treatment. However, the increase in plasma albumin after 2 weeks of treatment is indicative of liver function recovery.

As both experimental therapies are targeted to blockade (BMP-7) or inhibit (doxazosin) TGF- β secretion, we hypothesized that co-administration of both therapies could have an improved effect on ECM deposition in the treated hamsters. In the present study, adenoviral-BMP-7 transduction as well as doxazosin therapy, demonstrated to reduce, significantly, the deposition of ECM, particularly of collagen type I, and to diminish the expression of α -SMA in activated HSCs. We found that combination of both treatments do have neither synergic nor an additive effect on liver cirrhosis. Because of both treatments are targeted to blockade the same fibrogenic mechanisms (13,31), probably the total capacity of inhibition has been achieved by individual therapies and then the combination is not able to improve the antifibrotic effect.

In addition, ALT levels were significantly reduced when combined therapies were administered, but AST and albumin productions were unchanged by comparison to BMP-7. It is not surprising that doxazosin treatment maintains increased levels of AST or ALT after two weeks of treatment, but apparent liver toxicity to the liver is reduced when BMP-7 is also administered.

Previously, doxazosin was reported to cause liver toxicity at doses of 1 mg/kg/day at six weeks in the same model (13), moreover in the present study this toxicity is maintained at 2 weeks of treatment; outstanding BMP-7 gene therapy reduced toxic effect of doxazosin. These data are consistent with other study that have shown the protective effect of BMP-7 to hepatocytes, with improved liver function and regeneration (53).

Hepatocyte proliferation is also related to changes in the expression of regulatory genes involved in ECM remodeling (53,54). In this regard, Arendt *et al* demonstrated that MMP-13 and TIMP-1 are differentially expressed after TGF- β deprivation (55). Besides, previous studies had shown that the N-Myc downstream-regulated gene 2 abrogates the profibrogenic TGF- β activity by diminution of the expression of TIMP-2 mRNA (56). We demonstrated that down-expression of TIMP-2 mRNA correlate with reduction of collagen type I deposition in BMP-7 and doxazosin treated groups, however combinatory therapies were not as efficient as doxazosin alone.

In conclusion, the current study indicates that BMP-7 diminish the deposition of collagen type I by hampering the activation-proliferation of HSCs, and likely increasing hepatocyte proliferation. Although BMP-7 gene therapy did not improve the antifibrotic effect of doxazosin, it reduced its toxic effect in the liver. The combination of gene and drug therapies may not favor the increment of therapeutic effects, but its application can reduce adverse effects of conventional drugs.

Acknowledgements

The present study was supported by the grants (nos. 241312 and 134487) awarded by the National Council of Science and Technology (CONACYT), and PIBB15-10N from the Autonomous University of Aguascalientes. LRAM is a Postdoctoral Fellow awarded by CONACYT (grant no. 176507). Authors are grateful to Beatriz Orozco for excellent technical assistance and animal care.

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