

Cilostazol protects rats against alcohol-induced hepatic fibrosis via suppression of TGF- β 1/CTGF activation and the cAMP/Epac1 pathway

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Abstract. Alcohol abuse and chronic alcohol consumption are major causes of alcoholic liver disease worldwide, particularly alcohol-induced hepatic fibrosis (AHF). Liver fibrosis is an important public health concern because of its high morbidity and mortality. The present study examined the mechanisms and effects of the phosphodiesterase III inhibitor cilostazol on AHF. Rats received alcohol infusions via gavage to induce liver fibrosis and were treated with colchicine (positive control) or cilostazol. The serum alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) activities and the albumin/globulin (A/G), enzymes and hyaluronic acid (HA), type III procollagen (PC III), laminin (LA), and type IV collagen (IV-C) levels were measured using commercially available kits. α -smooth muscle actin (α -SMA), collagen I and III, transforming growth factor- β 1 (TGF- β 1), connective tissue growth factor (CTGF), adenosine 3',5'-cyclic monophosphate (cAMP) and exchange protein directly activated by cAMP (Epac) 1/2 expression in liver tissue were measured using western blotting. The results demonstrated that cilostazol significantly increased the serum ADH and ALDH activities and decreased the liver hydroxyproline levels. Cilostazol increased the serum A/G ratio and inhibited the total serum protein, enzymes, HA, PCIII, LA and IV-C levels. Western blotting revealed that cilostazol effectively decreased liver α -SMA, collagen I and III, TGF- β 1 and CTGF expression. Cilostazol significantly increased the cAMP and Epac1 levels in hepatic tissue. The present study suggests that cilostazol protects rats against AHF via suppression of TGF- β 1/CTGF activation and the cAMP/Epac1 pathway.

Introduction

Liver fibrosis is a wound-healing response to a variety of liver insults, including excessive alcohol intake. Alcohol abuse and chronic alcohol consumption are major causes of alcoholic liver disease (ALD) worldwide (1). Alcohol-induced hepatic fibrosis (AHF) may cause serious hepatic cirrhosis, and is widely accepted as a milestone event in ALD (2). However, recent evidence indicates that liver fibrosis is reversible and that the liver may recover from cirrhosis (3). Therefore, elucidation of the cellular and molecular mechanisms is urgently required to prevent AHF.

The exact mechanism remains unclear, but certain changes produced from excessive alcohol consumption have been clarified. Excessive alcohol damages hepatocytes via the release of cytosolic proteins and enzymes, such as alanine aminotransferase (ALT) and aspartate transaminase (AST), into the circulation (4). Histopathological changes, such as increased inflammatory cell infiltration, fatty changes, accumulation of collagenous fibers and necrotic damage, reinforce toxic liver injury (4). Excessive alcohol also increases serum hyaluronic acid (HA), type III procollagen (PCIII) and laminin (LN) levels (1). Indicators of liver fibrogenesis, including hepatic hydroxyproline collagen I and III levels, are also over expressed (1). Hepatic stellate cells (HSCs) may be activated and transformed into myofibroblast-like cells (5). An increase in α -smooth muscle actin (α -SMA) stimulates the secretion of fibrillar collagens and the deposition of a fibrotic matrix (5). Transforming growth factor- β 1 (TGF- β 1) and connective tissue growth factor (CTGF) are two important cytokines involved in the fibrotic and cirrhotic transformation of the liver and HSCs transformation (6).

Cilostazol is a phosphodiesterase III inhibitor that increases adenosine 3',5'-cyclic monophosphate (cAMP) levels, and is generally used to treat the symptoms of lower extremity peripheral arterial disease (7). Regulation of cAMP may inhibit HSCs activation. Recent studies demonstrated that cilostazol attenuated cholestatic liver injury, improved hepatic functions and decreased portal hypertension and hepatic fibrosis (8). Cilostazol also attenuated HSC activation and protected rats against carbon tetrachloride-induced liver fibrosis (9). Cilostazol protected rats against experimental non-alcoholic fatty liver disease via suppression of mitogen-activated protein kinase activation induced by oxidative stress and

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platelet-derived growth factor (10). However, the effects and mechanisms of cilostazol on AHF are not clear. Therefore, the present study examined the effects of cilostazol on AHF and investigated its potential mechanisms of action.

Materials and methods

Animals and reagents. A total of 212 adult male Sprague-Dawley rats (age, 8-9 weeks; weight, 200-220 g) were purchased from the Experimental Animal Center of Xi'an Central Hospital (Xi'an, China) for use in the present study. Rats were housed at 24±2°C and 50% relative humidity with a 12-h light/dark cycle in the Xi'an Central Hospital Animal Center in accordance with protocols issued by the Xi'an Central Hospital's Institutional Animal Care and Use Committee (approval no. XCH-20170923). All rats received standard chow diet and tap water *ad libitum*.

Rats were randomly divided into seven groups (20 rats per group) as follows: Control group, Model group, Colchicine group (positive control), Cilostazol (5 mg/kg) group, Cilostazol (10 mg/kg) group, Cilostazol (20 mg/kg) group and Cilostazol-only group (10 mg/kg). Rats in the Model group were exposed to alcohol as described below. Rats in the Colchicine, Cilostazol (5 mg/kg), Cilostazol (10 mg/kg), and Cilostazol (20 mg/kg) groups were exposed to the same dose of alcohol as the Model group and given colchicine or cilostazol (both Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Colchicine (0.1 mg/kg/day) was administered to rats via gavage for 2 weeks. Cilostazol (5, 10 or 20 mg/kg/day) was administered to rats via gavage for 2 weeks. Rats received alcohol for 24 weeks following the completion of colchicine and cilostazol administration. Rats in the Cilostazol-only group received 10 mg/kg cilostazol without alcohol for 2 weeks.

Induction of AHF. Rats received alcohol infusions via gavage to induce liver fibrosis, similar to the procedure used by Zhang *et al* (11). Alcohol was administered at 5.0 g/kg/day from 1-4 weeks, 7.0 g/kg/day from 5-8 weeks, 9.0 g/kg/day from 9-12 weeks and 9.5 g/kg/day from 13-24 weeks. Rats were sacrificed at the end of 24 weeks for the following assays.

Determination of serum alcohol dehydrogenase (ADH) and acetaldehyde dehydrogenase (ALDH) activities. Blood samples were immediately taken from sacrificed rats and centrifuged at 3,000 x g for 10 min at 4°C to obtain serum. ADH and ALDH activities in serum were measured using the Alcohol Dehydrogenase Activity Assay kit (cat. no. A083-2; Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and Aldehyde Dehydrogenase Activity Assay kit (cat. no. ALDH-2-G; Suzhou Comin Biotechnology Co., Ltd., Suzhou, China) via a colorimetric method.

Determination of liver hydroxyproline. Liver hydroxyproline was examined as previously described by Wang *et al* (12). Briefly, rats were sacrificed and livers were harvested and cut into slices. Liver slices were homogenized in 10% (w/v) phosphate buffer (0.5 mol/l potassium phosphate; cat. no. P3786; Sigma-Aldrich, Merck KGaA) and hydrolyzed in 12 M HCl at 100°C for 12 h. Following hydrolysis, the pH was adjusted to pH 7.0 and the samples were centrifuged

at 1,000 x g for 10 min at 4°C. The hydroxyproline content in each tissue sample was examined using the spectrophotometric method, previously described by Bergman and Loxley (13). Briefly, hydroxyproline was oxidised by chloramine T (cat no. 402869; Sigma-Aldrich, Merck KGaA) at room temperature for 5 min. Following oxidation, chloramine T was removed using perchloric acid (cat no. 311421, Sigma-Aldrich, Merck KGaA) and Ehrlich's reagent was added to each sample and heated at 60°C for 25 min. Finally, they samples were cooled to room temperature and the absorbance was measured at a wavelength of 558 nm to calculate the hydroxyproline levels.

Determination of serum levels of albumin/globulin, enzymes and HA, LN, IV-C and PCIII. Serum levels of albumin, globulin, enzymes [total protein (TP), total bilirubin (TBIL), ALT, AST, alkaline phosphatase (AKP) and glutamyltransferase (γ -GT)], HA, LN, type IV collagen (IV-C) and PCIII were determined using radioimmunoassay (RIA) kits. Albumin (cat. no. 452106), globulin (cat. no. 325214), TP (cat. no. 320175), TBIL (cat. no. 235109), ALT (cat. no. 635921), AST (cat. no. 102307), AKP (cat. no. 471256) and γ -GT (cat. no. 120523) kits were from Shanghai Institute of Biological Products Co., Ltd. (Shanghai, China). HA (cat. no. HY-10088), LN (cat. no. HY-10087), IV-C (cat. no. bs-0806P) and PCIII (cat. no. HY-E0007) RIA kits were purchased from Beijing Sino-uk Institute of Biological Technology (Beijing, China). Albumin (A) and globulin (G) levels were used to calculate the A/G value. Enzyme levels (TP, TBIL, ALT, AST, AKP, γ -GT) were used to evaluate the degree of hepatic injury. HA, LN, IV-C and PCIII levels were used to evaluate the degree of AHF.

Western blot analysis. A liver sample of ~10 g was collected from the left lobe of the liver and rinsed thoroughly with ice-cold PBS (pH=7.4). Liver samples were homogenized, and total protein was extracted using HEPES extraction buffer (Santa Cruz Biotechnology, Inc.). Total protein was quantified using a bicinchoninic acid assay kit (Santa Cruz Biotechnology, Inc.) and 40 μ g protein/lane were separated via SDS-PAGE on a 12% gel. The separated proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and blocked for 1 h at room temperature with 5% milk. The membranes were incubated with primary antibodies against α -SMA (1:1,000; cat. no. 19245), TGF- β 1 (1:1,000; cat. no. 3709), CTGF (1:1,000; cat. no. 86641), exchange protein directly activated by cAMP (Epac)-1 (1:1,000; cat. no. 4155), Epac2 (1:1,000; cat. no. 43239) and β -actin (1:1,000; cat. no. 4970; all Cell Signaling Technology, Inc., Danvers, MA, USA), and collagen III (1:1,000; cat. no. sc-271249) and I (1:1,000; cat. no. sc-376350; Santa Cruz Biotechnology, Inc.) overnight at 4°C. Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (1:3,000; cat. no. sc-2354; Santa Cruz Biotechnology, Inc.) for 1 h at 4°C. Protein bands were visualized using the ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Little Chalfont, UK) and Bio-Rad ChemiDoc™ Imaging System (Bio-Rad Laboratories, Inc.). Protein expression was quantified using Image-Quant TL software (GE Healthcare Life Sciences).

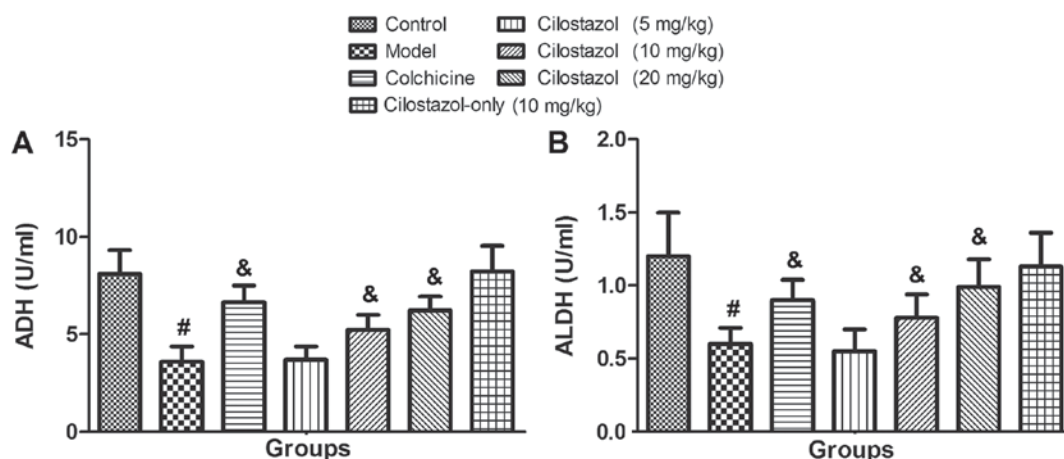


Figure 1. Effects of cilostazol on serum (A) ADH and (B) ALDH activities. Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Colchicine, rats received oral alcohol and colchicine (0.1 mg/kg/day); Cilostazol (5, 10 or 20 mg/kg), rats received oral alcohol and cilostazol at 5, 10 or 20 mg/kg/day; Cilostazol-only (10 mg/kg), rats received oral cilostazol at 10 mg/kg/day. Data are presented as the mean \pm standard deviation (n=20/group). [#]P<0.05 vs. Control; [&]P<0.05 vs. Model. ADH, alcohol dehydrogenase; ALDH, acetaldehyde dehydrogenase.

Measurement of hepatic cAMP using ELISA. Hepatic cAMP was measured using an ELISA kit (cat. no. RPN225; GE Healthcare, Chicago, IL, USA) and the method described by Miller *et al* (14). Hepatic cAMP levels were normalized to total liver weight, and the results are expressed as pmol/mg tissue.

Statistical analysis. All data are expressed as the mean \pm standard error of the mean. Statistical significance was determined using one-way analysis of variance followed by a Dunnett's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of cilostazol on serum ADH and ALDH activities. Fig. 1 presents the serum ADH and ALDH levels. After rats were received treatment with alcohol for 24 weeks, significantly decreased serum ADH and ALDH activities were observed in the Model group compared with the Control group (P<0.05). Rats that received colchicine and alcohol exhibited a significant recovery of serum ADH and ALDH activities compared with the Model group (P<0.05). Cilostazol treatment (5 mg/kg/day) did not significantly alter serum ADH and ALDH activities, but 10 and 20 mg/kg/day cilostazol significantly increased these activities compared with the Model group (P<0.05). Cilostazol treatment (10 mg/kg/day) without alcohol did not significantly affect serum ADH or ALDH activity compared with the Control group.

Effects of cilostazol on liver hydroxyproline levels. Fig. 2 presents the liver hydroxyproline levels. Liver hydroxyproline levels in the Model group were significantly increased compared with the Control group (P<0.05). Colchicine treatment significantly recovered liver hydroxyproline levels compared with the Model group (P<0.05). Cilostazol treatment (5 mg/kg/day) did not significantly alter liver hydroxyproline levels, but 10 and 20 mg/kg/day cilostazol significantly decreased these levels compared with the Model group (P<0.05). Cilostazol treatment (10 mg/kg/day) without

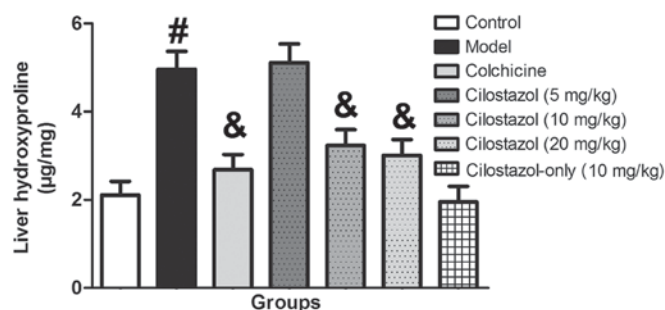


Figure 2. Effects of cilostazol on liver hydroxyproline levels. Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Colchicine, rats received oral alcohol and colchicine (0.1 mg/kg/day); Cilostazol (5, 10 or 20 mg/kg), rats received oral alcohol and cilostazol at 5, 10 or 20 mg/kg/day; Cilostazol-only (10 mg/kg), rats received oral cilostazol at 10 mg/kg/day. Data are presented as the mean \pm standard deviation (n=20/group). [#]P<0.05 vs. Control; [&]P<0.05 vs. Model.

alcohol did not significantly alter liver hydroxyproline levels compared with the control group.

Effects of cilostazol on serum A/G ratio, protein, enzyme, HA, LN, IV-C and PCIII levels. Table I presents the effects of cilostazol on the serum A/G ratio. Alcohol treatment reduced the serum A/G ratio from 1.08 ± 0.11 to 0.63 ± 0.08 (P<0.05). Colchicine and 10 and 20 mg/kg/day cilostazol significantly increased the serum A/G ratio to 0.94 ± 0.12 , 0.86 ± 0.08 and 0.91 ± 0.07 , respectively (P<0.05 vs. Model group). Cilostazol treatment (10 mg/kg/day) without alcohol did not significantly alter serum A, G or A/G levels. Table II presents the levels of serum protein and enzymes (TP, TBIL, ALT, AST, AKP and γ -GT). Alcohol treatment significantly increased serum levels of TP, TBIL, ALT, AST, AKP and γ -GT compared with the Control group (P<0.05). Colchicine and 10 and 20 mg/kg/day cilostazol significantly inhibited the increase in these proteins and enzymes compared with the Model group (all P<0.05). Treatment with 10 mg/kg/day cilostazol without alcohol did not significantly alter serum protein or enzymes compared with the Control group. Table III presents the serum levels of HA,

Table I. Effects of Cilostazol on serum albumin, globulin and A/G in rats.

Measurement	Control	Model	Colchicine	Cilostazol (5 mg/kg)	Cilostazol (10 mg/kg)	Cilostazol (20 mg/kg)	Cilostazol-only (10 mg/kg)
Albumin (g/l)	41.2±6.5	40.3±5.5	40.6±5.4	41.6±4.3	40.7±4.7	41.1±3.9	40.3±5.4
Globulin (g/l)	38.2±4.2	63.5±4.8	43.1±4.9	60.3±5.1	47.6±3.9	45.1±4.3	39.9±4.9
A/G	1.08±0.11	0.63±0.08 ^a	0.94±0.12 ^b	0.69±0.07	0.86±0.08 ^b	0.91±0.07 ^b	1.12±0.15

Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Colchicine, rats received oral alcohol and colchicine (0.1 mg/kg/day); Cilostazol (5, 10 or 20 mg/kg), rats received oral alcohol and cilostazol at 5, 10 or 20 mg/kg/day; Cilostazol-only (10 mg/kg), rats received oral cilostazol at 10 mg/kg/day. Data are presented as the mean ± standard deviation (n=20/group).

^aP<0.05 vs. Control; ^bP<0.05 vs. Model. A/G, albumin/globulin.

Table II. Serum levels of protein and enzymes in rats.

Measurement	Control	Model	Colchicine	Cilostazol (5 mg/kg)	Cilostazol (10 mg/kg)	Cilostazol (20 mg/kg)	Cilostazol-only (10 mg/kg)
TP (g/l)	70.6±6.9	88.3±7.1 ^a	72.1±6.8 ^b	85.4±7.5	75.2±5.9 ^b	71.0±6.6 ^b	66.7±5.7
TBIL (μmol/l)	1.7±0.4	7.8±1.3 ^a	3.2±0.5 ^b	7.6±1.1	3.8±0.3 ^b	3.5±0.6 ^b	1.6±0.5
ALT (U/l)	50.3±10.5	154.5±20.6 ^a	80.9±21.3 ^b	139.6±22.6	92.3±23.4 ^b	87.8±21.8 ^b	55.2±8.9
AST (U/l)	132.5±23.3	254.6±36.6 ^a	166.8±35.9 ^b	241.6±30.8	195.7±34.2 ^b	184.2±31.3 ^b	125.5±21.4
AKP (U/l)	135.4±36.5	251.2±35.2 ^a	157.4±26.5 ^b	239.2±23.5	188.1±25.5 ^b	167.4±34.2 ^b	132.9±33.7
γ-GT (U/l)	3.5±1.2	21.2±2.4 ^a	6.5±2.1 ^b	19.9±2.7	10.3±3.1 ^b	8.2±2.8 ^b	3.4±1.3

Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Colchicine, rats received oral alcohol and colchicine (0.1 mg/kg/day); Cilostazol (5, 10 or 20 mg/kg), rats received oral alcohol and cilostazol at 5, 10 or 20 mg/kg/day; Cilostazol-only (10 mg/kg), rats received oral cilostazol at 10 mg/kg/day. Data are presented as the mean ± standard deviation (n=20/group).

^aP<0.05 vs. Control; ^bP<0.05 vs. Model. TP, total protein; TBIL, total bilirubin; ALT, alanine aminotransferase; AST, aspartate transaminase; AKP, alkaline phosphatase; GT, glutamyltransferase.

LN, IV-C and PCIII in rats. Alcohol treatment significantly increased these indicators of AHF compared with the Control group (all P<0.05). However, co-treatment with colchicine or 10 mg/kg/day and 20 mg/kg/day cilostazol significantly decreased these indicators compared with the Model group (all P<0.05). Treatment with cilostazol (10 mg/kg/day) without alcohol did not significantly alter serum levels of HA, LN, IV-C or PC III compared with the Control group.

Effects of cilostazol on liver α-SMA and collagen III and I expression. α-SMA and collagen III and I expression were measured in liver tissue using western blotting to confirm the effects of cilostazol on AHF. Protein levels of α-SMA and collagen III and I increased significantly in the Model group compared with the Control group (P<0.05; Fig. 3). Colchicine (0.1 mg/kg/day) and cilostazol (10 mg/kg/day) significantly decreased the levels of these proteins compared with the Model group (P<0.05). Treatment with cilostazol (10 mg/kg/day) without alcohol did not significantly alter liver α-SMA or collagen III and I expression compared with controls.

Effects of cilostazol on liver TGF-β1, CTGF, Epac1/2 and cAMP levels. TGF-β1, CTGF and Epac1/2 expression were measured in liver tissue using western blotting, and cAMP

levels were measured using ELISA to investigate the mechanisms of cilostazol on AHF. TGF-β1 expression was significantly increased in the Model group compared with the Control group (P<0.05), and colchicine (0.1 mg/kg/day) and cilostazol (10 mg/kg/day) significantly inhibited this increase compared with the Model group (P<0.05; Fig. 4A). CTGF expression was significantly increased in the Model group compared with the Control group (P<0.05), and 10 mg/kg/day cilostazol significantly inhibited this increase (P<0.05 vs. Model group; Fig. 4B). Colchicine (0.1 mg/kg/day) did not significantly alter CTGF expression compared with the Model group. Treatment with cilostazol (10 mg/kg/day) without alcohol did not significantly affect liver TGF-β1 or CTGF expression compared with the Control group. Epac1 expression was decreased significantly in the Model group compared with the Control group (P<0.05), and cilostazol greatly enhanced Epac1 expression (P<0.05 compared with the Model group; Fig. 5A and B). Cilostazol treatment without alcohol also significantly increased Epac1 expression compared with the Control group (P<0.05). Epac2 expression was significantly decreased in the Model and Cilostazol groups compared with the Control group (P<0.05; Fig. 5A and C). There was no significant difference between the Model and Cilostazol groups. Cilostazol treatment without alcohol did not significantly alter Epac2 expression compared with the

Table III. Serum levels of HA, LN, IV-C and PCIII in rats.

Measurement	Control	Model	Colchicine	Cilostazol (5 mg/kg)	Cilostazol (10 mg/kg)	Cilostazol (20 mg/kg)	Cilostazol-only (10 mg/kg)
HA	201.6±32.3	451.3±34.5 ^a	233.4±36.1 ^b	457.8±39.1	286.4±32.2 ^b	259.3±28.7 ^b	195.6±23.4
LN	33.1±2.8	56.9±3.8 ^a	38.9±4.1 ^b	58.7±4.5	44.6±5.1 ^b	40.5±3.4 ^b	32.6±3.1
IV-C	18.4±3.1	39.1±3.5 ^a	21.3±2.7 ^b	38.7±3.4	25.2±4.4 ^b	23.3±3.7 ^b	17.9±2.6
PCIII	47.6±10.3	210.7±32.4 ^a	103.8±10.9 ^b	203.9±24.5	169.8±13.6 ^b	150.6±16.8 ^b	45.8±9.5

Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Colchicine, rats received oral alcohol and colchicine (0.1 mg/kg/day); Cilostazol (5, 10 or 20 mg/kg), rats received oral alcohol and cilostazol at 5, 10 or 20 mg/kg/day; Cilostazol-only (10 mg/kg), rats received oral cilostazol at 10 mg/kg/day. Data are presented in ng/ml and as the mean ± standard deviation (n=20/group).

^aP<0.05 vs. Control; ^bP<0.05 vs. Model. HA, hyaluronic acid; LN, laminin; IV-C, type IV collagen; PCIII, type III procollagen.

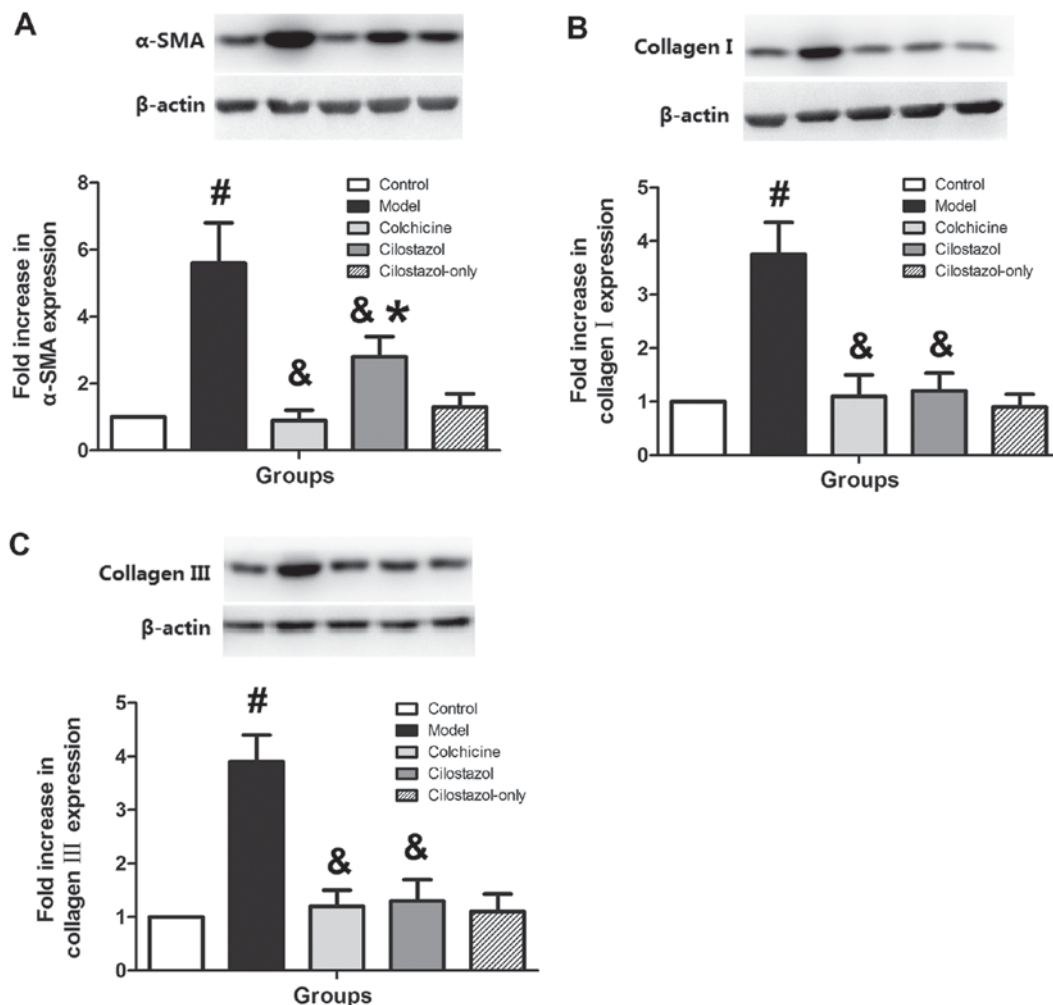


Figure 3. Effects of cilostazol on liver (A) α-SMA and (B) collagen I and (C) III expression. Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Colchicine, rats received oral alcohol and colchicine (0.1 mg/kg/day); Cilostazol, rats received oral alcohol and cilostazol at 10 mg/kg/day; Cilostazol only, rats received oral cilostazol at 10 mg/kg/day. Data are presented as the mean ± standard deviation (n=8/group).

[#]P<0.05 vs. Control; [&]P<0.05 vs. Model; ^{*}P<0.05 vs. Colchicine. α-SMA, alpha smooth muscle actin.

Control group. cAMP levels were significantly decreased in the Model group compared with the Control group (P<0.05; Fig. 5D), and cilostazol significantly enhanced these levels (P<0.05 vs. Model group). Cilostazol treatment without alcohol also significantly increased cAMP levels compared with the Control group (P<0.05; Fig. 5D).

Discussion

Chronic alcohol consumption produces many harmful consequences, and liver failure is one of the most serious effects. Chronic alcohol consumption may lead to fatty liver disease, alcoholic hepatitis and alcoholic liver fibrosis (15). Liver

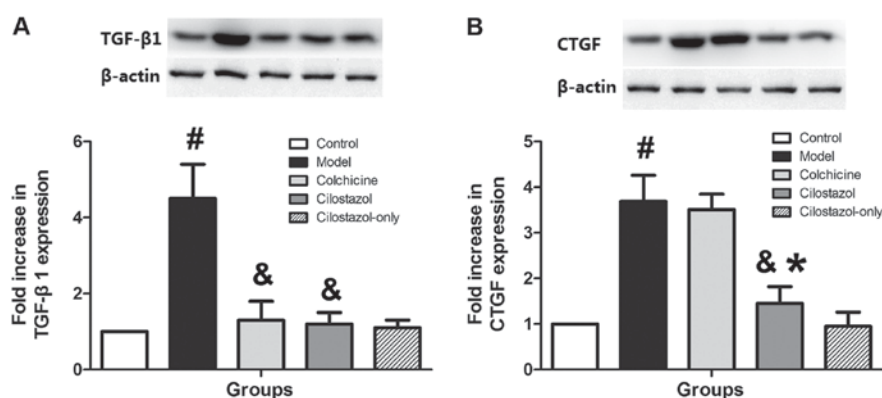


Figure 4. Effects of cilostazol on liver (A) TGF- β 1 and (B) CTGF expression. Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Colchicine, rats received oral alcohol and colchicine (0.1 mg/kg/day); Cilostazol, rats received oral alcohol and cilostazol at 10 mg/kg/day; Cilostazol only, rats received oral cilostazol at 10 mg/kg/day. Data are presented as the mean \pm standard deviation ($n=8$ /group). [#] $P<0.05$ vs. Control; [&] $P<0.05$ vs. Model; ^{*} $P<0.05$ vs. Colchicine. TGF- β 1, transforming growth factor- β 1; CTGF, connective tissue growth factor.

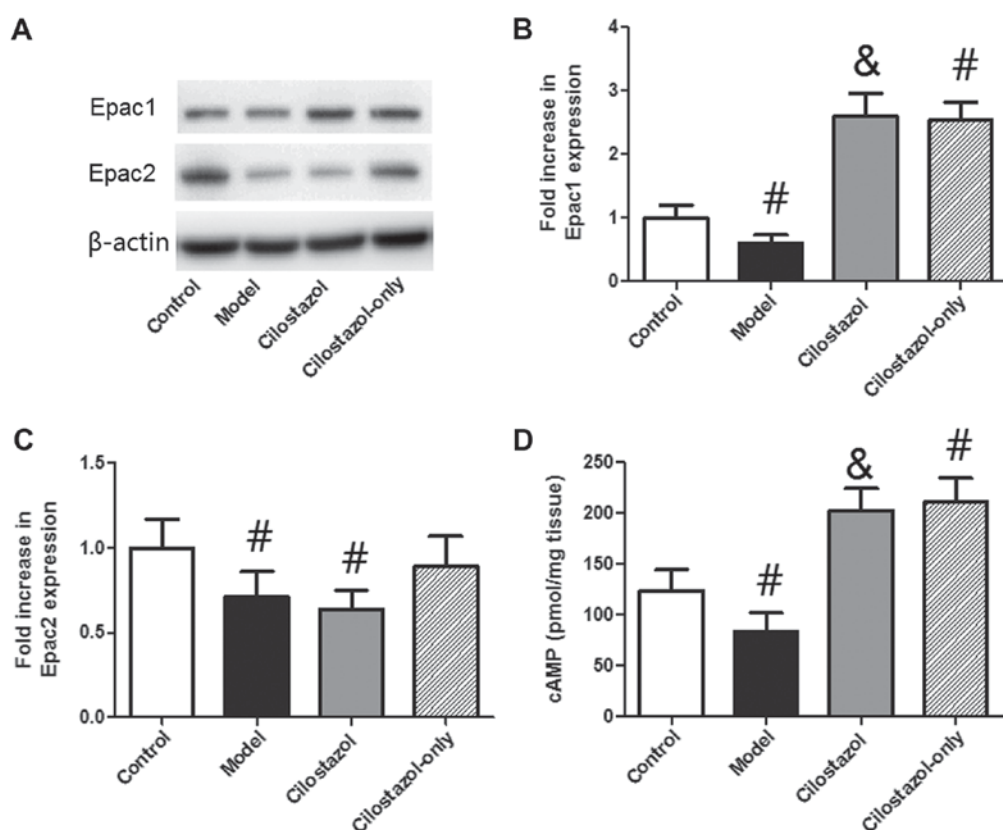


Figure 5. Effects of cilostazol on liver Epac1/2 expression and cAMP level. (A) Western blotting of (B) Epac1, (C) Epac2 and (D) cAMP. Control, rats received no treatment; Model, rats received oral alcohol infusions to induce liver fibrosis; Cilostazol, rats received oral alcohol and cilostazol at 10 mg/kg/day; Cilostazol only, rats received oral cilostazol at 10 mg/kg/day. Data are presented as the mean \pm standard deviation ($n=8$ /group). [#] $P<0.05$ vs. Control; [&] $P<0.05$ vs. Model. cAMP, adenosine 3',5'-cyclic monophosphate; Epac, exchange protein directly activated by cAMP.

fibrosis is an important public health concern because of its high morbidity and mortality (16). The present study examined the effects and mechanisms of the phosphodiesterase III inhibitor cilostazol, which is clinically used to treat lower extremity peripheral arterial disease, on alcohol-induced liver fibrosis. The results demonstrated that in alcohol-treated rats, the levels of liver hydroxyproline, α -SMA, collagen III and collagen I, and serum levels of HA, LN, IV-C and PCIII in rats were all significantly increased. These data indicated that the model of AHF was successful. Cilostazol significantly increased serum

ADH and ALDH activities and decreased liver hydroxyproline levels. Cilostazol increased the serum A/G ratio and inhibited serum TP, TBIL, ALT, AST, AKP and γ -GT, HA, LN, IV-C and PCIII levels. Western blotting revealed that cilostazol effectively decreased α -SMA, collagen III and I, TGF- β 1 and CTGF expression in the liver. Cilostazol significantly increased Epac1 expression and cAMP level in liver tissue.

The development of AHF is associated with the oxidation of alcohol to acetaldehyde, which stimulates the production of extracellular matrix (ECM) components, such as

type I collagen, via HSC activation (17). The activation of HSCs is a milestone event in the development of AHF as these cells are the primary source of ECM in the response of the liver to alcohol consumption (18). HSCs transform to myofibroblast-like cells, proliferate and eventually become fibrogenic (19). Alcohol is metabolized via various catabolic metabolic pathways. ADH is the primary enzyme that oxidizes alcohol to acetaldehyde (20), which is converted to acetate via ALDH (21). The present study treated rats with alcohol for 24 weeks and demonstrated a significant decrease in serum ADH and ALDH activities in the Model group. This decrease suggests that alcohol was deposited in the liver tissue and induced hepatotoxicity over time. Colchicine or cilostazol administration with alcohol significantly recovered serum ADH and ALDH activities. These results suggest that cilostazol enhances ADH and ALDH activities to accelerate alcohol metabolism and protect the liver from alcohol assault.

Chronic alcohol assault also promotes the release of cytosolic proteins and enzymes, such as TBIL, ALT, AST, AKP and γ -GT, into the circulation (4). The present study demonstrated that alcohol treatment significantly increased serum levels of TP, TBIL, ALT, AST, AKP and γ -GT. However, colchicine and 10 and 20 mg/kg/day cilostazol significantly inhibited the release of these protein and enzymes. Liver hydroxyproline level increased significantly in the Model group, and 10 and 20 mg/kg/day cilostazol treatment significantly decreased these levels. Cilostazol significantly increased the serum A/G ratio. Cilostazol also significantly inhibited the increased levels of serum HA, LN, IV-C and PCIII, which are indicators of liver fibrogenesis. These results suggest that cilostazol effectively inhibited biomarkers of liver fibrogenesis and collagen deposition. This mechanism of action may delay the progression of hepatic fibrosis and alleviate hepatic injury.

α -SMA is a marker of HSC transformation into myofibroblast-like cells and the secretion of fibrillar collagens (collagen III and I) (5). α -SMA and collagen III and I expression were measured in liver tissue using western blotting to confirm the effect of cilostazol on AHF. The protein levels of α -SMA and collagen III and I expression increased significantly in the Model group. Rats treated with 0.1 mg/kg/day colchicine or 10 mg/kg/day cilostazol exhibited significantly decreased levels. These results suggest that cilostazol prevents initiation of the fibrotic process and synthesis of excessive connective tissue components.

CTGF is a cysteine-rich peptide that is synthesized and secreted by fibroblastic cells following TGF- β activation. CTGF is a downstream mediator of TGF- β -induced fibroblast proliferation (22). Previous studies demonstrated an upregulation of CTGF expression in numerous fibrotic diseases, such as atherosclerosis, pancreas, kidney, and liver fibrosis (23,24). Duncan *et al* (22) demonstrated that CTGF mediated TGF- β -induced fibroblast collagen synthesis, and the inhibition of CTGF synthesis prevented granulation tissue formation via the inhibition of collagen synthesis and fibroblast accumulation. TGF- β 1 and CTGF expression were measured in liver tissue to further investigate the mechanism of cilostazol on AHF. TGF- β 1 expression increased significantly following 24 weeks of alcohol administration, and 0.1 mg/kg/day colchicine and 10 mg/kg/day cilostazol significantly inhibited this

increase. CTGF expression was also significantly increased in the Model group, and 10 mg/kg/day cilostazol significantly inhibited this increase. Colchicine (0.1 mg/kg/day) did not alter CTGF expression. These results reveal that the TGF- β 1/CTGF pathway is involved in the protective effects of cilostazol in AHF. Cilostazol inhibited CTGF expression, and the positive control treatment, colchicine, did not affect expression.

The effects of cilostazol were measured on cAMP level and Epac1/2 expression in liver tissues to further examine the association between cilostazol and TGF- β 1/CTGF signaling in the antifibrotic action of cilostazol. Previous studies have reported that the cAMP/Epac1/2 pathway served a key role in the effect of cilostazol in other tissues and cells including, bone, aortic endothelial cells and progenitor cells (25-27). A number of previous studies revealed the importance of Epac1 and Epac2 as mediators of the antifibrotic effects of cAMP (28-30). Epac1 and Epac2 exhibit different cAMP-binding sites. Increased cAMP restrains fibroblast function to exert its anti-fibrotic effects. The mechanisms of these effects include fibroblast proliferation inhibition, fibroblast death and ECM protein synthesis inhibition (31). Activation of Epac1 or Epac2 in different tissues inhibits profibrotic actions in the body, such as collagen and DNA synthesis (31). The present results demonstrated that Epac1 expression and cAMP level decreased significantly in the Model group, and cilostazol greatly enhanced this reduction. However, cilostazol did not significantly alter Epac2 expression. These results indicate that cilostazol effectively activates the cAMP/Epac1 signaling pathway in liver tissue. Multiple studies previously established the association between cAMP and TGF- β 1/CTGF. Huang *et al* (32) demonstrated that liraglutide improved myocardial fibrosis following myocardial infarction via inhibition of CTGF and activation of cAMP in mice. Weng *et al* (33) revealed that high expression of TGF- β 1 and its downstream pathways in MDCK cells produced a significant and negative effect of cAMP-PKA on TGF- β 1-induced p-ERK1/2 and FN expression. Satish *et al* (34) also demonstrated that increasing cAMP levels potentially inhibited myofibroblast formation and the accumulation of ECM components via inhibition of TGF- β 1 stimulation of α -SMA, CTGF, and collagen I and III. Therefore, it is reasonable to hypothesize that cilostazol inhibits TGF- β 1/CTGF expression via activation of the cAMP/Epac1 signaling pathway in liver tissue to suppress hepatic fibrosis development.

The present study demonstrated that cilostazol protected rats against AHF via suppression of TGF- β 1/CTGF activation. Further studies are required to confirm the exact mechanisms, but these results provide a novel potential strategy to prevent AHF and associated liver injury. The present study focused on the preventive effects of cilostazol on AHF. However, whether cilostazol reverses AHF is not clear. The therapeutic effect of cilostazol on AHF requires further study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KH designed the study and prepared the manuscript. YZ performed the experiments. ZY collected and analyzed the data.

Ethics approval and consent to participate

The present study was approved by the Xi'an Central Hospital's Institutional Animal Care and Use Committee (Xi'an, China; approval number: XCH-20170923).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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