Yin-Chen-Hao-Tang alleviates biliary obstructive cirrhosis in rats by inhibiting biliary epithelial cell proliferation and activation

Bing Wang[1,2†], Ming-Yu Sun[1,3†], Ai-Hua Long[1], Hong-Yan Cao[1,3], Shuang Ren[1], Yan-Qin Bian[1,3], Xiong Lu[1], Hong-Tu Gu[1], Cheng-Hai Liu[1,3], Ping Liu[1,3]

[1]Key Laboratory of Liver and Kidney Diseases (Ministry of Education), Institute of Liver Diseases, Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, [3]E-Institute of Shanghai Municipal Education Commission, Shanghai 201203, [2]Shanghai Jiao Tong University Affiliated Sixth People's Hospital, Shanghai 200233, China

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ABSTRACT

Background: Yin-Chen-Hao-Tang (YCHT) consists of three aqueous extracts from *Artemisia capillaris, Gardenia* sp., and prepared *Rheum rhabarbarum* (rhubarb) (3:2:1). YCHT is characterized by its anti-inflammatory properties in liver regulation and relief of jaundice. We aimed to study the effects and mechanisms of action of YCHT on biliary obstructive cirrhosis. **Materials and Methods:** Secondary biliary fibrosis was induced in rats by bile duct ligation (BDL) and scission. One week after BDL, rats were randomly divided into a saline-treated BDL or YCHT-treated BDL group for 4 weeks. Liver function and hepatic hydroxyproline (Hyp) content were assessed. Types I and IV collagen (Col-IV), laminin, fibronectin, alpha smooth muscle actin (α-SMA), and proliferating cell nuclear antigen protein and messenger ribonucleic acid (mRNA) expression were assessed with immunohistochemistry and real-time polymerase chain reaction. **Results:** In the YCHT-treated BDL group, serum total bilirubin, total bile acids, aspartate aminotransferase, alanine aminotransferase, and γ-glutamyl transferase were lower than those in the sham-operated BDL group. The proliferation of bile ducts in hepatic tissues and the Hyp content and Col deposition were also significantly lower than those in control rats. In addition, α-SMA and Col-IV staining was less obvious, and mRNA expression of Procol-α1 (IV), platelet derived growth factor subunit B (PDGF)-B, connective tissue growth factor, and transforming growth factor-beta in proliferative biliary epithelial cells (BECs) in the YCHT-treated BDL group was significantly lower than those in controls. **Conclusions:** YCHT effectively reduces the formation of biliary obstructive cirrhosis mainly via inhibition of BEC proliferation by down-regulation of PDGF-B mRNA expression, inhibition of BEC profibrogenic paracrines, and the epithelial-mesenchymal transition pathological process.

Key words: Biliary epithelium cells, biliary obstructive cirrhosis, platelet derived growth factor subunit B, Yin-Chen-Hao-Tang

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INTRODUCTION

Liver fibrosis is a major histological finding in most chronic liver disease, which is characterized with the overproduction and accumulation of extracellular matrix (ECM). Chronic liver diseases can develop from liver fibrosis into cirrhosis with hepatic insufficiency and portal hypertension.[1] Therefore, prevention of fibrosis is an important strategy for the treatment of liver chronic diseases.[2,3]

Address for correspondence:
Dr. Ping Liu, Institute of Liver Diseases, Shuguang Hospital Affiliated to Shanghai University of Traditional Chinese Medicine, Shanghai, China.
E-mail: liuliver@vip.sina.com.cn.

†These authors contributed equally to this work

The etiology of liver fibrosis is variable, ranging from viral infection to metabolic or genetic factors.[3] Although it is recognized that hepatic stellate cells (HSCs) play a pivotal role in fibrogenesis,[4,5] other liver residential cells like Kupffer cells or cholangiocytes have an important role, particularly in bile salt mediated fibrosis. Cholangiocytes are the epithelial cells that line the biliary tree, and are also called biliary epithelial cells (BECs). BECs have bile absorptive and secretory properties, and are actively involved in cholestasis. Interestingly, it was reported that BECs can undergo an epithelial-mesenchymal transition (EMT),[6] during which BECs proliferate and transform into myofibroblasts under extrahepatic cholestasis, immune injury, or massive hepatic necrosis. This transition produces

pro-fibrogenic cytokines and matrix.[7] Therefore, BECs play an important role in cholestatic fibrosis.

Yin-Chen-Hao-Tang (YCHT) was first described in Shanghan Lun's Treatise on Exogenous Febrile Diseases in 200 AD, and consists of three aqueous extracts from *Artemisia capillaris, Gardenia* sp., and prepared *Rheum rhabarbarum* (rhubarb) in a 3:2:1 ratio. YCHT is characterized by its antiinflammatory properties in the liver and ability to relive jaundice. In recent years, YCHT was reported to have positive effects on liver function in postoperative biliary atresia patients and is thought to promote bile excretion by a cholic acid-independent mechanism.[8,9] In primary biliary cirrhosis (PBC) patients, the combined use of YCHT with ursodesoxycholic was more effective than ursodesoxycholic alone.[10] YCHT could also attenuate the progression of liver fibrosis in CCl_4, choline-deficient, and L-amino-acid-defined diets, and thioacetamide and pig-serum immunity rat models.[11,12] The effects on bile salt mediated fibrosis are not clear, although a recent study showed that YCHT could ameliorate hepatic fibrosis in the bile duct ligation (BDL) rats,[8] mainly via antioxidation. BECs have important roles in both bile salt and fibrogenesis, but YCHT's mechanism of action against bile salt mediated fibrosis by direct regulation of BECs is unclear.

In the present study, secondary biliary fibrosis was induced in rats by BDL and scission. We treated BDL rats with YCHT. We aimed to evaluate the mechanism of action of YCHT against bile salt mediated fibrosis by inhibition of BEC proliferation.

MATERIALS AND METHODS

Animals

Inbred male Sprague Dawley rats (200 ± 20 g) were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences. The rats were housed in an air-conditioned room at 25°C with a 12-h darkness/light cycle. The rats received humane care with unlimited accesses to food and water during the study. All of the study's protocols comply with the current ethical considerations of Shanghai University of Traditional Chinese Medicine's (TCMs) Animal Ethic Committee.

Yin-Chen-Hao-Tang preparation

The raw herbs of YCHT, which includes *A. capillaris*, *Gardenia* sp., and prepared rhubarb (3:2:1), were purchased from Shanghai Huayu Pharmaceutical Company (Shanghai, China). Herbs were identified by pharmacognosist, Prof. Zhengtao Wang, from the Institute of Materia Medica, Shanghai University of TCM. YCHT was prepared by Shuguang Hospital Shanghai University of TCM as follows: *A. capillaris* was soaked in 10 times its volume of water for 1 h, followed by decocting for 40 min. Mashed *Gardenia* and rhubarb were then added and further decocted for 15 min. The supernatant was filtered and set aside as solution one. The residue was added to seven times its volume of water for further decocting for 20 min. The supernatant was then filtered and set aside as the second solution. The aqueous extracts were vacuum-dried (60°C) to obtain a powder.

Reagents

A two-step™ anti-rabbit detection reagent horseradish peroxidase (HRP), two-step™ anti-mouse detection reagent (HRP), and liquid diaminobenzidine (DAB) kit were purchased from American Diagnostica Inc. (Stamford, CT). All primary antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Bile duct ligation

Rats were anesthetized with pentobarbital and BDL was performed as described previously.[13] Briefly, under 2% sodium pentobarbital anesthesia, the common bile duct was double-ligated with 3-0 silk thread after a midline abdominal incision. The choledoch was cut after proximal and distal ligation. The common bile duct was exposed and manipulated but not ligated in the sham control group.

Drug treatment groups

One week after BDL, animals were divided into three groups: sham-operated BDL group (n = 12), YCHT-treated BDL group (n = 17), and saline-treated BDL group (n = 15). YCHD was administered once a day for 4 weeks intragastrically at a dose of 0.418/100 g body weight for the YCHD group, which is equivalent to clinical human doses. The sham-operated BDL and saline-treated BDL groups were orally administered an equivalent volume of saline. All animals were sacrificed after 4 weeks of treatment. Blood was obtained for biochemical assay, and liver tissue was either frozen in liquid nitrogen for RNA extraction or fixed with 10% neutral formalin for electron microscopy assay and histology.

Serum parameters of liver function assay

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, total bilirubin (TBil), total bile acid (TBA), and albumin (Alb) content were assayed using kits from Shanghai Institute of Biological Products (Shanghai, China). Levels of alkaline phosphatase (ALP) and γ-glutamyl transferase (GGT) were assessed using kits from Nanjing Jianchen Biotech Institute (Nanjing, China). TBA was analyzed with an Abbott Aeroset Autoanalyzer (Abbott Park, IL).

Hydroxyproline determination

Hepatic hydroxyproline (Hyp) content was used as an indirect measure of tissue collagen (Col) content. Hyp from

liver tissues (100 mg) was determined according to Jamall *et al.*[14] Hyp content is expressed as mg/g of liver wet weight.

Pathological examination

Liver specimens were preserved in 4% paraformaldehyde and dehydrated in a graded alcohol series. Then, specimens were embedded in paraffin blocks, cut into 5-μm-thick sections and placed on glass slides. Sections were then stained with hematoxylin and eosin (H and E) and Picro-Sirius red.

Immunostaining

Immunohistochemistry was performed using a two-step staining method. After deparaffinization and dehydration, microwave antigen retrieval was performed for 5 min before peroxidase quenching with 3% H_2O_2 in phosphate buffered saline (PBS) for 15 min. Then, the sections were blocked with 5% bovine serum Alb for 30 min. Slides were incubated at 4°C with an anti-alpha smooth muscle actin (α-SMA) antibody (A2547, 1:400, Sigma, St. Louis, USA) overnight, then with biotinylated secondary antibodies for 45 min. After that, they were developed with DAB for 3 min and finally counterstained with hematoxylin. For the negative controls, the primary antibody was replaced with PBS. The results were analyzed with an image system IPP6.1 (Olympus, Japan). The immunostained sections were examined with a microscope (×200) coupled to a video camera, connected to a computer-aided color video image analysis system. After capture, images were semi-quantitatively analyzed using an image analysis software IPP6.1. We selected three sections from each group, observed a random visual field, and calculated the ratio between the positive area and the standard measurement window.

Proliferating cell nuclear antigen positive cell counting

We selected three sections and observed them under a microscope (×100) to determine visual fields of proliferating cell nuclear antigen (PCNA) positive cells. Then, we counted the positive BECs and hepatocytes under a high power lens (×400) in the same fields. Five fields were observed, and their average counts were used for statistical analysis. The discrimination of BECs from hepatocytes depends on the following criteria: (1) The BEC is a single layered columnar epithelial cell, with the longitudinal axis always oriented vertically to the tissue surface, and the hepatocyte has a polyhedral shape, with a diameter of 20-30 μm and 1-2 nucleoli. (2) Arranged BECs form a circular or tubular structure, while hepatocytes form hepatic plates. (3) Outside cells, there is obvious deposited matrix surrounding BECs instead of hepatocytes.[15]

Biliary epithelial cells cut with laser capture microdissection

The liver tissue was embedded in optimal cutting temperature at -20°C and cut into 8-μm pieces onto sterile glass slides. Then, the slides were fixed in 95% ethanol for 5 min and stained with H and E for 30 s. Slides were dehydrated by immersion into 70%, 95%, and 100% ethanol, for 30 s each. The slides were air dried at room temperature for 15 min. Samples were either used immediately for microdissection or stored in air-tight plastic wrapping at -70°C. Frozen tissue sections from saline-treated BDL rats (n = 3), YCHT-treated BDL rats (n = 3), and sham-operated BDL subjects (n = 3) were used for laser capture microdissection. The PALM® MicroLaser Microdissection System (PALM MicroLaser Technologies AG, Burnried, Germany) was use. Staining of frozen sections with H and E enabled the precise selection of BDEs, which have characteristic circular or tubular structures. Laser dissected BECs were then ejected off the slide with a single defocused laser pulse and catapulted directly into the cap of a microfuge tube containing 10 μl of RLT (RNeasy® mini kit; Qiagen Limited, Crawley, UK). For each section, about 1000 bile duct epithelia were laser-captured.

Quantitative real-time polymerase chain reaction analysis of procollagen IV, platelet-derived growth factor subunit B, and connective tissue growth factor

Quantitative real-time polymerase chain reaction (RT-PCR) analysis of rat livers was performed as described previously[16] and expression levels were normalized to β-actin. Primers used in RT-PCR are given in Table 1. In experiments, RNA was isolated from liver and used to direct complementary deoxyribonucleic acid synthesis (RT for PCR, Clontech, Kyoto, Japan). Quantitative RT-PCR

Table 1: Primers for procollagen IV, PDGF-B, CTGF, and β-actin

Name	Primer sequences (5'-3')	Cat gene bank	Product size (bp)
CTGF			
Sense	CTAAGACCTGTGGAATGGGC	NM_010217	383
Antisense	CTCAAAGATGTCATTGTCCCC		
PDGF			
Sense	GAGTGCAAGACGCGTACAGA	NM_033016	135
Antisense	ACTGCACATTGCG GTTATTG		
TGF-β			
Sense	AGGCGGTGCTCGCTTTGT	AJ009862	299
Antisense	CCCGAATGTCT GACGTATTGAA		
αI (IV)			
Sense	TTTCAACGTTTCAGTGGCAG	NM_001845	205
Antisense	TCACTATGCAAGTGGCG		
β-actin			
Sense	TGACGAGGCCCA GAGCAAGA	DQ237887	331 bp
Antisense	ATGGGCACAGTG TGGGTGAC		

CTGF: Connective tissue growth factor; PDGF: Platelet-derived growth factor subunit B; TGF-β: Transforming growth factor-beta

was performed in a fluorescent temperature cycler (ABI) with SYBR Green PCR kit (Takara Bio, Inc., Kyoto, Japan). We normalized expression values to the level of input RNA.

Statistical analysis

Data are expressed as the mean ± standard deviation Statistical analyses were performed with a one-way ANOVA, least significant difference, or Tambane test in SPSS 16.0 software (SPSS Inc., Chicago, Illinois, USA). The count data were compared using a multiple independent samples nonparametric test.

RESULTS

Survival rate, ascites incidence, body weight, liver weight, and spleen weight

Five weeks after the BDL surgery, 7 out of 17 rats in the saline-treated BDL group died (41%), while 5 out of 15 died in the YCHT-treated BDL group (33%). Rats in the saline-treated BDL and YCHT-treated BDL groups had ascites incidences of 90% and 50%, respectively [Figure 1]. Body weight in the saline-treated BDL group was significantly lower, while the liver and spleen weights were significantly higher than those in the sham-operated BDL group. There were no significant differences between the saline-treated BDL and YCHT-treated BDL groups for liver and spleen weight (data not shown).

Changes in serum liver function

The serum TBil level in the saline-treated BDL group was 22 times that of the sham-operated BDL group, and TBA content and activities of ALP, GGT, AST, and ALT were also significantly higher. Alb content was significantly lower than that of the sham-operated BDL group. In the YCHT-treated BDL group, there was significantly lower serum TBil and TBA content and lower GGT, AST, and ALT activity compared with the sham-operated BDL group [Table 2].

Hepatic inflammation and collagen deposition changes

The liver lobules exhibited a clear structure in the sham-operated BDL group. In the saline-treated BDL group, there were extensive proliferative bile ducts and bile canaliculi. The aggregation of myofibroblasts and ECM deposition was seen around BECs. Neither inflammation nor necrosis was observed, but lymphocyte infiltration was sometimes observed in the portal area. The proportion of liver parenchymal cells decreased significantly. However, the morphology of hepatocytes appeared to be normal. Compared with the saline-treated BDL group, the YCHT-treated BDL group exhibited significantly less proliferation of the small bile ducts and more proportion of liver parenchymal cells [Figure 2a].

Only a small quantity of Col was seen in the portal area and on central venous walls in the sham-operated BDL group. In contrast, abundant ECM was deposited around the proliferated bile ducts with less deposition in the liver parenchyma in the saline-treated BDL group. Compartments

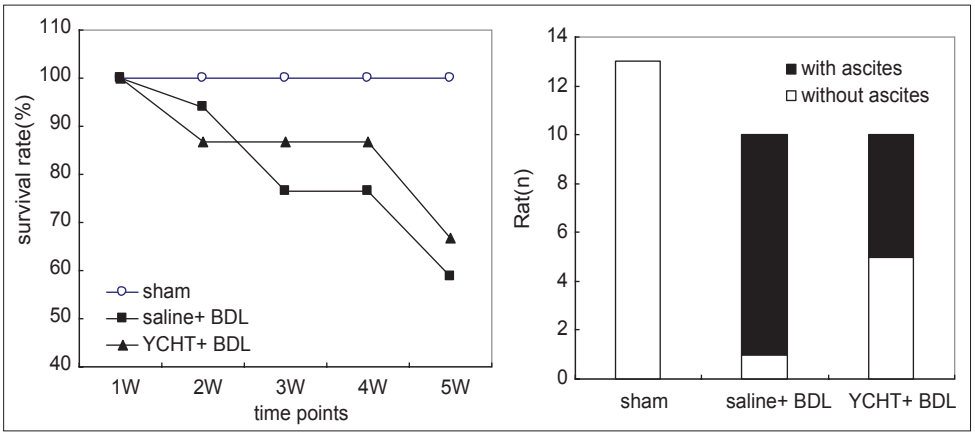

Figure 1: Effect of Yin-Chen-Hao-Tang (YCHT) on the survival rate and ascites incidence in cirrhotic rats. Sham group, *n* = 10; saline + bile duct ligation (BDL) group, *n* = 17; YCHT + BDL group, *n* = 15. The YCHT group had significantly lower ascites incidence than control rats

Table 2: Effect of YCHT on serum liver function parameters in cirrhotic rats

Group	*n*	ALT (U/L)	AST (U/L)	T. Bil (μmol/L)	TBA (μmol/L)	GGT (U/L)	ALP (U/L)	Alb (g/L)
Sham	12	128.24±14.08**	38.55±4.26**	0.26±0.14**	26.40±10.79**	3.01±1.82**	326.58±94.48**	26.68±2.61**
Saline+BDL	10	276.77±53.60	106.49±31.69	5.78±3.00	76.33±24.93	115.94±63.15	595.90±144.11	14.21±3.02
YCHT+BDL	10	212.96±60.00*	70.46±27.50*	3.21±2.35*	45.60±13.79**	3.01±1.82**	517.80±165.43	17.53±6.76

YCHT: Yin-Chen-Hao-Tang; BDL: Bile duct ligation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TBil: Total bilirubin; TBA: Total bile acid; GGT: γ-glutamyl transferase; ALP: Alkaline phosphatase; Alb: Albumin. *P<0.05; **P<0.01 versus saline+BDL

were formed between proliferative bile ducts and Col, which then connected with adjacent compartments to surround and separate the liver lobules, thus forming tubercles or false lobules. The area where the bile canaliculi excessively proliferated was free from large hepatocyte aggregation. The fibrosis level of hepatic tissues in the YCHT-treated BDL group was significantly less extensive than that in the saline-treated BDL group [Figure 2].

Ultrastructure changes under transmission electron microscope

Cholestasis was not found in hepatocytes or BECs in the sham-operated BDL group. There were many microvilli in the bile duct cavity, and no bile thrombi were observed. In the saline-treated BDL group, cholestasis was found in both hepatocytes and in the cytoplasm of proliferative BECs. Additionally there were bile thrombi in the bile duct cavity, and fewer microvilli. Abundant deposition of Col and aggregation of fibroblasts was observerd around the BECs. In the YCHT-treated BDL group, fibroblast aggregation around the BECs, cholestasis extent in both hepatocytes and epidermal cells, and Col deposition were all less extensive than those in the saline-treated BDL group [Figure 3].

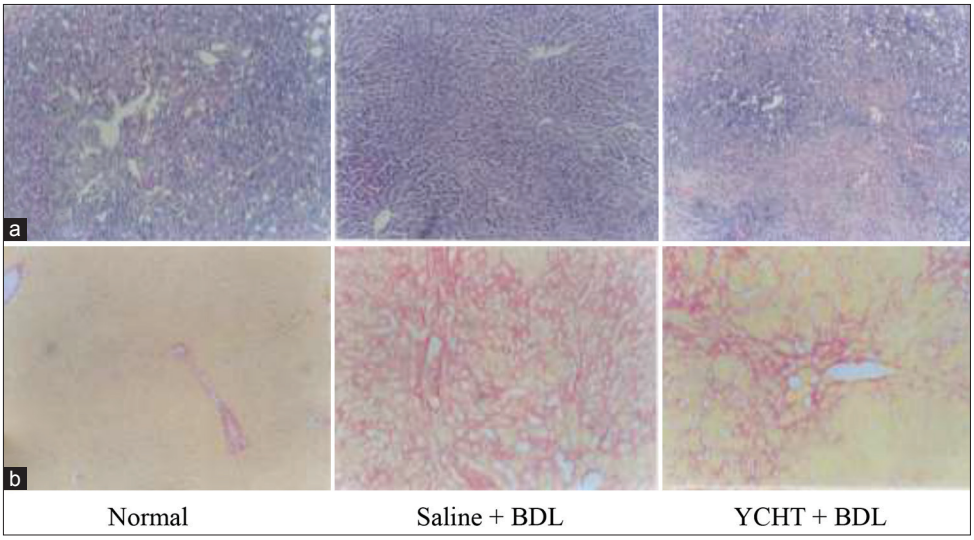

Figure 2: Effect of Yin-Chen-Hao-Tang (YCHT) on liver inflammation and collagen (Col) deposition in cirrhotic rats. (a) H and E staining for inflammation. (b) Picro-Sirius red staining for Col deposition. YCHT remarkably attenuated hepatic inflammation and Col deposition in bile duct ligation-induced cirrhotic rats

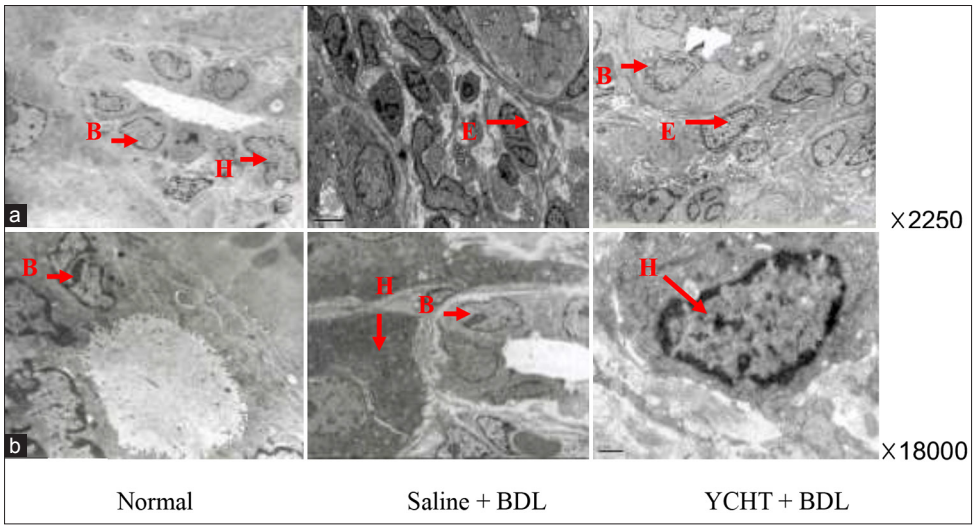

Figure 3: Effect of Yin-Chen-Hao-Tang (YCHT) on the liver microstructure in cirrhotic rats. (a) ×2250. (b) ×18000, observed by transmission electron microscope. In the sham group, biliary epithelial cells (BECs) formed the bile duct cavity, and there were many microvilli, but little collagen deposition around the BECs. In the saline + bile duct ligation (BDL) group, there was cholestasis in both hepatocytes and BECs, fewer microvilli, and bile thrombi appeared. Extracellular matrix (ECM) deposition and desmocyte aggregation could be seen around the bile duct, and there was cholestasis in hepatocytes. In the YCHT + BDL group, there was less cholestasis in hepatocytes and BECs, no bile thrombus, and less ECM deposition around bile duct compared with the saline + BDL group (H: Hepatocyte; B: Biliary epithelial cell; E: Extracellular matrix)

Changes in hydroxyproline content in hepatic tissues

Hepatic Hyp content in the saline-treated BDL group was four-fold that of the sham-operated BDL group. Hyp content was significantly lower in the YCHT-treated BDL group compared with the saline-treated BDL group [Table 3].

Changes in expressions of collagen-I, collagen-IV, fibronectin, laminin, and alpha smooth muscle actin in hepatic tissues

In the sham-operated BDL group, little Col-I or α-SMA positive staining was observed on the intrahepatic vascular walls, Col-IV and laminin (LM) was observed mainly on the vascular walls and bile cells walls, and fibronectin (FN) was continuously expressed in hepatic sinusoid. In the saline-treated BDL group, Col-I staining obvious in the liver parenchyma, but not around the BECs. There were no obvious changes in Col-IV staining on the hepatic sinusoid, but it was strongly expressed around the proliferative BECs. FN and LM were also positively expressed around the proliferative bile duct. α-SMA positive staining was observed on the myofibroblasts around the proliferative bile duct. The areas of positive staining of Col-I, Col-IV, LM, FN, and α-SMA from the YCHT-treated BDL group were much less extensive than those in the saline-treated BDL group [Figure 4].

Changes in proliferating cell nuclear antigen-positive biliary epithelial cells and hepatocytes

Compared with the sham-operated BDL group, the saline-treated BDL group exhibited a significantly greater number of PCNA-positive BECs, and a significantly lower number of PCNA-positive hepatocytes [Figure 5]. Compared with the saline-treated BDL group, the YCHT-treated BDL group exhibited significantly fewer PCNA-positive BECs and significantly more PCNA hepatocytes.

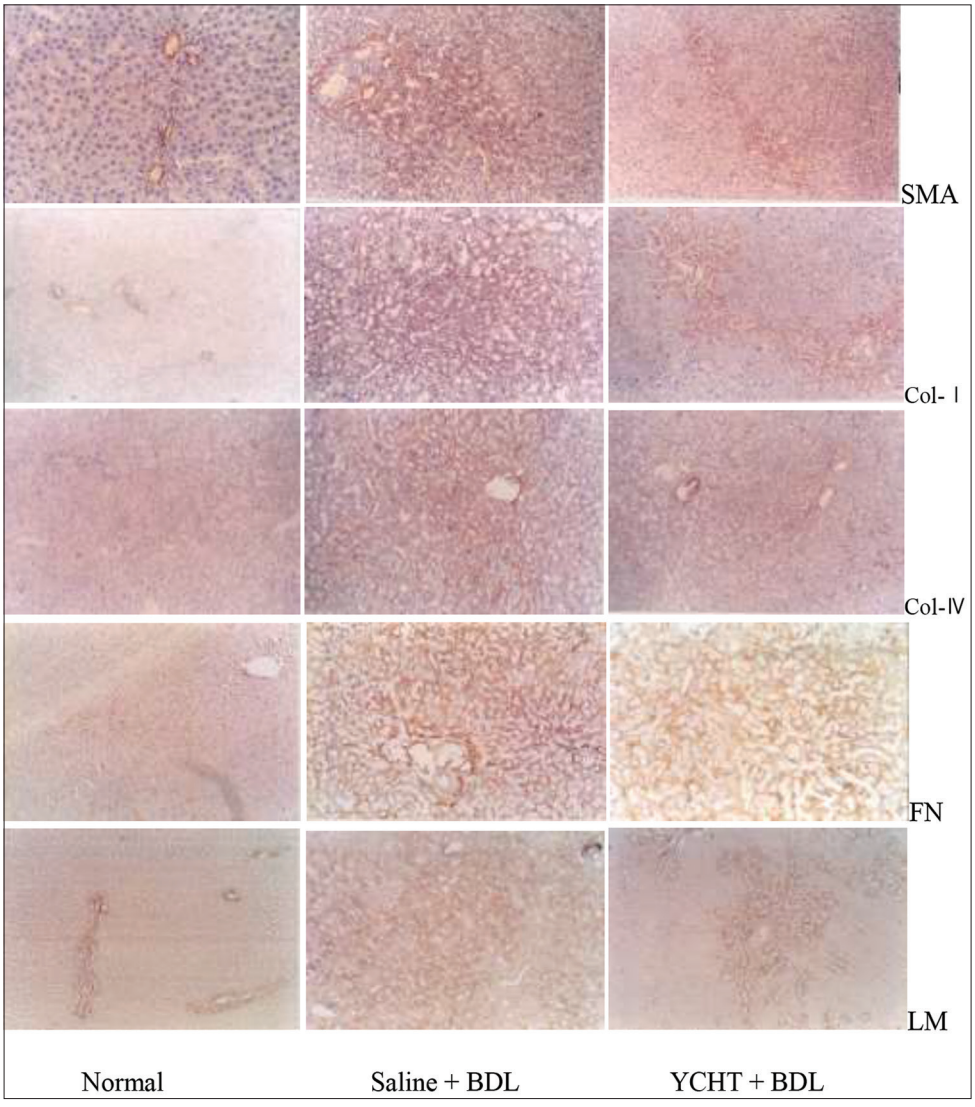

Figure 4: Effect of Yin-Chen-Hao-Tang on liver extracellular matrix expression in cirrhotic rats. The liver tissues were immunohistologically stained with specific antibodies, and their expression levels were semi-quantitatively analyzed. **P* < 0.05, ***P* < 0.01, versus saline + bile duct ligation

Table 3: Effect of YCHT on hepatic Hyp contents and proliferation of BECs in cirrhotic rats

Group	*n*	Hyp content (μg/g liver)	Proliferation of BECs grade			
			–	+	++	+++
Sham	12	130.7±25.5**	12	0	0	0
Saline+BDL	10	538.2±105.6	0	1	1	8
YCHT+BDL	10	391.9±135.8*	0	6	3	1

*P<0.05; **P<0.01 versus saline+BDL. BDL: Bile duct ligation; BECs: Biliary epithelial cells cells; YCHT: Yin-Chen-Hao-Tang; Hyp: Hydroxyproline

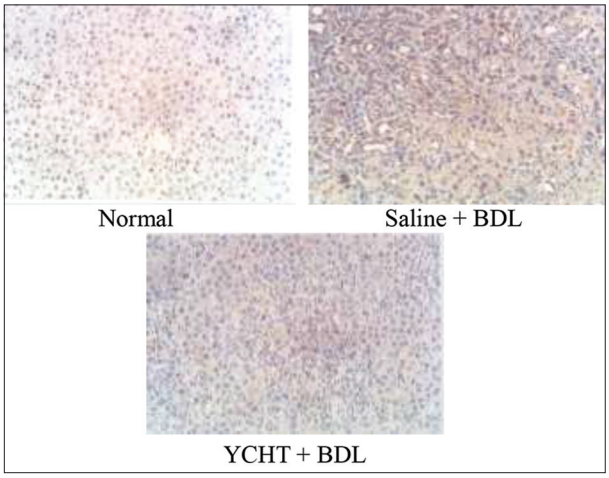

Figure 5: Proliferating cell nuclear antigen positive cells in hepatic tissue. Yin-Chen-Hao-Tang (YCHT) inhibited biliary epithelial cell proliferation significantly and improved hepatocytes proliferation. Sham operation group, $n = 3$; saline + bile duct ligation (BDL) group, $n = 3$; YCHT + BDL group, $n = 3$. YCHT. *$P < 0.05$, **$P < 0.01$, versus saline + BDL

Changes in messenger ribonucleic acid expression of procollagen α1 (IV), platelet derived growth factor-B, connective tissue growth factor, and transforming growth factor-beta in biliary epithelial cells

Real-time-PCR revealed that messenger ribonucleic acid (mRNA) expression of procol α1(IV), platelet derived growth factor subunit B (PDGF-B), and connective tissue growth factor (CTGF) mRNA was 16-, 12-, and 9-fold greater in the saline-treated BDL group compared with the sham-treated BDL group. Procol α1(IV), PDGF-B, CTGF, and transforming growth factor-beta (TGF-β1) mRNA expression in the YCHT-treated BDL group was significantly lower at 43.9%, 56.3%, 41%, and 48% that of their respective values in the saline-treated BDL group [Figure 6].

DISCUSSION

Yin-Chen-Hao-Tang is a classic TCM recipe for jaundice and is frequently used for chronic liver diseases.[11,12] In our previous study, we found that YCHT could attenuate rat liver fibrosis induced by dimethylnitrosamine, and the mechanism is associated with the suppression of Kupffer cells and HSC activation.[17,18] In the study, we induced a fibrotic model in rats by BDL, and found that YCHT can reduce the ascites incidence in model rats, and decrease ECM component deposition including LM, FN, Col-I, and Col-IV. It can also reduce hepatic Hyp content, and inhibit HSC activation in hepatic tissue. These results indicate that YCHT could have therapeutic action against cholestatic liver fibrosis.

Bile duct ligation causes obvious periductal liver fibrosis and pathological lesions appear around the bile duct epithelium. After BDL, bile acids accumulate in the canaliculi and lead to extrahepatic cholestasis, which triggers the compensatory proliferation and expansion of BECs.[19] Following the BECs' mitogenic phase, chronic obstruction of the bile duct causes myofibroblast activation and ultimately results in biliary fibrosis and cirrhosis. Biliary cirrhosis often occurs in human diseases such PBC and primary sclerosing cholangitis. Moreover, bile acids from cholestasis can damage the hepatocytes inducing cell apoptosis and peroxidative injury, which may result in HSC activation. It was reported[20] that YCHT can protect against apoptosis *in vivo* and *in vitro*, exerts choleretic effects, and increases canaliculum bile flow in a bile acid-independent manner. In this study, we found that YCHT could improve serum liver function, particularly by decreasing TBil level, and inhibiting BEC proliferation. Therefore, YCHT can protect hepatocytes against injury and inhibit BEC proliferation by reducing cholestasis, and these actions may contribute to YCHT-mediated inhibition of fibrosis.

In chronic liver injury, HSCs become activated and transdifferentiate into myofibroblasts which promote ECM synthesis and play a role in liver fibrogenesis. BECs not only play an important role in bile canaliculi proliferation, but also in fibrosis formation. We found that BECs proliferated in the cirrhotic liver, as observed by PCNA staining and increased PDGF-B mRNA expression. These proliferative BECs may extend into the liver parenchyma from the portal area and destroy the normal hepatic structure. However, YCHT decreased the PDGF-B mRNA, PCNA positive cells, and bile canaliculi formation. PDGF-B is one of the strongest mitogenic factors, YCHT could inhibit BEC proliferation by down-regulating PDGF-B gene expression and restoring normal liver structure.

Biliary epithelial cells can secrete cytokines such as TGF-α, PDGF-B, and TGF-β1, which are pro-fibrogenic and can activate quiescent HSCs through paracrine signaling.[21] We found that α-SMA, which is a marker of HSC activation, was higher after BDL. However, YCHT lowered α-SMA expression in the cirrhotic liver. Gene expression in BECs were examined, and YCHT was found to down-regulate

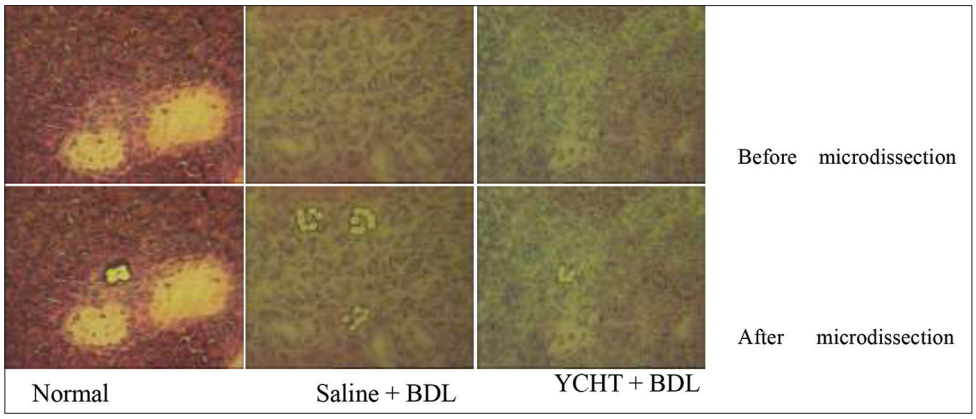

Figure 6: Changes in messenger ribonucleic acid (mRNA) expressions of transforming growth factor-beta (TGF-β1), Procollagen (Col) α1 (IV), platelet derived growth factor subunit-B (PDGF-B), and connective tissue growth factor (CTGF) in BECs. (a) Biliary epithelial cells (BECs) were removed. (b) TGFβ1, Col-IV, PDGF-B, and CTGF mRNA expressions in BECs detected by real-time polymerase chain reaction. (c) Yin-Chen-Hao-Tang inhibited BECs proCol α1 (IV), PDGF-B, CTGF, and TGF-β1 mRNA expression. **P* < 0.05, ***P* < 0.01, versus saline + bile duct ligation

TGF-α, PDGF-B, and TGF-β1 mRNA levels, which suggested that YCHT can exert its antifibrotic action by inhibiting BECs paracrine effects.

The origin of myofibroblasts in the liver remains unclear, aside from residential HSCs and fibroblasts. However, it was reported that hepatocytes and BECs can transdifferentiate into myofibroblasts.[9] Some researchers found that BECs in cholangiocarcinoma and CCl_4 models may generate basilar membrane proteins and Col.[22,23] Xia *et al*.[24] found that most BECs had α-SMA in the epithelium after BDL *in vivo* and in cultured BECs after TGF-β1 incubation *in vitro*. BECs were observed to migrate into the periductal region via the impaired basement membrane and transform into myofibroblasts, which was called the EMT and is thought to play a pivotal role in organ fibrosis. Our immunohistochemistry indicated that ECM including Col-IV, LM, and FN were strongly expressed around the proliferative BECs in the cirrhotic liver. Transmission electron microscope revealed that ECM components were mainly deposited on basolateral BECs, and RT-PCR revealed that BECs had significantly higher procol αI (IV) mRNA expression. These data suggest that BECs in the BDL model have potential fibrogenic ability. Although we did not check epithelial markers such as CK-19, α-SMA positive staining as observed around the proliferative BECs, and CTGF and TGF-β1 mRNA expression was higher in BECs, which are the features of myofibroblasts. Therefore, BECs in the BDL model might undergo EMT, and obtain the capability to synthesize ECM. This EMT generation plays a significant role in biliary fibrosis and cirrhosis. YCHT inhibited ECM deposition and gene expression of CTGF and TGF-β1, and decreased α-SMA expression in the BDL model. Therefore, the action of YCHT against biliary cirrhosis is probably associated with the inhibition of BEC EMT, in addition to inhibition of HSC activation.

Previous studies have demonstrated that YCHT has various active compounds for liver diseases, like 6,7-dimethylesculetin. This compound from *A. capillaris* can accelerate bilirubin clearance in blood by activating CAR (constitutive androstane receptor) in primary hepatocytes and inducing CYP2B10 expression.[25] Genipin, a metabolite of *Gardenia*, exerts potent choleretic effects, and markedly inhibited activated HSC α-SMA expression and HSC activation.[9] Emodin from rhubarb is effective for reducing DNA synthesis and migration of PDGF-B-stimulated HSCs through inhibition of phosphorylation of PDGF receptor-beta and downstream signaling pathways.[26]

A recipe such as YCHT can exert better comprehensive advantages with multiple components. Based on our results, we confirmed the beneficial action of YCHT against biliary cirrhosis induced by BDL, and conclude that its mechanism is closely associated with the inhibition of BEC proliferation and activation. This inhibition also inhibits the profibrogenic paracrine effects BECs, which indirectly stimulate HSC production of ECM, thus inhibiting the EMT through which BECs directly produce ECM.

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