

Basic Study

Improved prescription of taohechengqi-tang alleviates D-galactosamine acute liver failure in rats

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Author contributions: Zhang Y and Luo JX contributed equally to this work; Hu XY and Zhong S designed the study; Hu XY, Zhang Y, Luo JX, Yang F and Lin W performed study; Zhang Y and Yang F analyzed data; and Zhang Y wrote the paper.

Supported by National Key Technology R and D Program, No. 2008ZX10005 and No. 2009ZX10005.

Institutional review board statement: This study was approved by the Institutional Review Board of the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, China.

Institutional animal care and use committee statement: All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, Chengdu, China.

Conflict-of-interest statement: The authors declare that there is no conflict of interest related to this study.

Data sharing statement: There are no additional data available.

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Received: July 8, 2015

Peer-review started: July 9, 2015

First decision: September 29, 2015

Revised: October 13, 2015

Accepted: November 24, 2015

Article in press: November 24, 2015

Published online: February 28, 2016

Abstract

AIM: To investigate the hepatoprotective effect of improved prescription of Taohechengqi-tang (IPTT) against acute liver failure (ALF) in rats.

METHODS: Seventy specific pathogen free male Wistar rats were randomly divided into four groups: control group (normal rats, $n = 10$), ALF group (ALF model, $n = 20$), Stronger Neo-Minophagen C (SNMC) group (ALF model + SNMC, $n = 20$), and IPTT group (ALF model + IPTT, $n = 20$). The ALF model group was administered an intraperitoneal injection of D-galactosamine (1.4 g/kg), and the control group received normal saline intraperitoneally. The SNMC and IPTT groups were treated with SMMC (15.6 mg/kg) or IPTT (28.6 g/kg) by gavage at 24 h intervals, and the ALF and control groups were treated with normal saline. At 36 h after injection, serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, albumin, and cholinesterase and prothrombin

time were determined, and liver histopathological scores were observed by microscopy after hematoxylin and eosin staining. mRNA expression of high mobility group box (HMGB) 1, toll-like receptor (TLR) 4, nuclear factor kappa B (NF- κ B) and caspase-3 were analyzed *via* fluorescence quantitative reverse transcriptase polymerase chain reaction. Proliferating cell nuclear antigen (PCNA) immunohistochemistry in liver tissue was also performed.

RESULTS: D-galactosamine notably decreased the biochemical and coagulation profiles in serum. IPTT not only improved liver function and histopathology but also normalized the gene expression levels in liver tissue. Compared with the model group, in the IPTT and SNMC groups, HMGB1 mRNA/ β -actin (0.06 ± 0.03 , 0.11 ± 0.04 *vs* 0.25 ± 0.04 , $P < 0.05$); TLR4 mRNA/ β -actin (0.07 ± 0.02 , 0.22 ± 0.08 *vs* 0.41 ± 0.22 , $P < 0.05$); NF- κ B mRNA/ β -actin (0.74 ± 0.41 , 1.78 ± 0.64 *vs* 2.68 ± 1.35 , $P < 0.05$); and caspase-3 mRNA/ β -actin levels were all significantly reduced (1.61 ± 0.45 , 2.57 ± 1.04 *vs* 3.41 ± 0.85 , $P < 0.05$). The gene expression levels were significantly lower in the IPTT group than in the SNMC group ($P < 0.05$). Compared with the model group, the PCNA expression in liver tissue was significantly enhanced in the IPTT and SNMC groups (36.34 ± 4.91 , 25.57 ± 2.94 *vs* 17.55 ± 2.40 , $P < 0.05$).

CONCLUSION: IPTT attenuates inflammation in ALF *via* inhibition of HMGB1 production, which may contribute to limited liver regeneration.

Key words: Acute liver failure; Traditional Chinese medicine; Taohechengqi-tang; High mobility group box 1; Inflammation

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Core tip: Acute liver failure (ALF) is a life-threatening condition. Our prospective cohort study demonstrated that high doses of herbs for clearing heat and resolving stasis have a protective effect on LF. However, the curative mechanism is unclear. Our evidence showed that improved prescription of Taohechengqi-tang attenuated the inflammatory reaction of ALF in rats *via* inhibition of high mobility group box 1 production, which may contribute to recovery of limited liver regeneration. We provide evidence for the clinical application of Chinese herbs for clearing heat and resolving stasis.

Zhang Y, Luo JX, Hu XY, Yang F, Zhong S, Lin W. Improved prescription of taohechengqi-tang alleviates D-galactosamine acute liver failure in rats. *World J Gastroenterol* 2016; 22(8): 2558-2565 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v22/i8/2558.htm> DOI: <http://dx.doi.org/10.3748/wjg.v22.i8.2558>

INTRODUCTION

Acute liver failure (ALF) is a life-threatening condition. It is accompanied by an intense inflammatory response, resulting in massive hepatocyte necrosis and fatal multiorgan failure^[1]. Hospital mortality from ALF was as high as 83% in 1973-1978, dropping to 38% in 2004-2008^[2]. The only treatment option shown to improve radically the outcome of LF is emergency liver transplantation (LT)^[3]. Nevertheless, due to a lack of donated livers, nontransplanted mortality in ALF was > 20% with a waiting time of up to 6 wk^[4]. In addition, the complex pathophysiological mechanisms of LT need further study.

According to traditional Chinese medicine (TCR), the syndrome of heat toxin stagnation is a major pattern of LF^[5]. The incidence of the pattern was reported to be 58.6% in an epidemiological investigation from China^[6]. Taohechengqi-tang (TT) is a well-known traditional Chinese herbal formulation, documented in *Shang Han Za Bing Lun* (a book of TCR on febrile and miscellaneous diseases in the Han Dynasty, AD 200-210). Based on TCR theory, TT is a classic formulation for clearing heat and resolving stasis and is used for treating not only diabetes mellitus^[7] but also liver diseases. A recent study indicated that TT protected against chemically induced liver injury, at least partially through an antioxidant-like mechanism^[8].

In our prospective cohort study, high doses of herbs for clearing heat and resolving stasis had a significant protective effect against LF^[9]. However, the curative mechanism is still unclear. The present study was performed to investigate the effect of improved prescription of Taohechengqi-tang (IPTT) in ALF rats and its underlying mechanisms.

MATERIALS AND METHODS

Chemicals

D-galactosamine (D-GalN) was obtained from Hongbang Medical Technology Co. Ltd. (Shanghai, China). Stronger Neo-Minophagen C (SNMC) was purchased from Minophagen Pharmaceutical Co. Ltd. (Tokyo, Japan) and prepared at a concentration of 1.56 mg/mL with distilled water.

Preparation of IPTT

IPTT consists of seven types of TCM ingredients (herbal plants and animal-based materials) (Table 1). All TCMs were purchased from Sichuan Chinese Herbs Co. Ltd. (Chengdu, China) and validated by Prof. Zhu-Yun Yan (Chengdu University of Traditional Chinese Medicine, China). All indicators of the above-mentioned TCMs were in line with the standards of the Chinese Pharmacopoeia (2010). Voucher specimens were deposited at the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine (Chengdu,

Table 1 Herbal prescription of improved prescription of Taohechengqi-tang

Chinese name	Latin name	Origin	Amount in preparation (g)
Chi-shao	<i>Radix paeoniae rubra</i>	Sichuan	100
Tao-ren	<i>Semen persicae</i>	Hebei	50
Hong-hua	<i>Flos carthami</i>	Sichuan	50
Shui-zhi	<i>Hirudo</i>	Sichuan	15
Quan-xie	<i>Scorpio</i>	Henan	10
Mang-xiao	<i>Natrii sulfas</i>	Sichuan	10
Da-huang	<i>Radix et rhizoma rhei palmati</i>	Sichuan	30

China).

The extraction process of IPTT was as follows: *Radix paeoniae rubra* 100 g, *Semen persicae* 50 g, *Flos carthami* 50 g, *Hirudo* 15 g, *Scorpio* 10 g, *Natrii sulfas* 10 g, and *Radix et rhizoma rhei palmate* 30 g were added in 1.5 volumes of water, soaked for 30 min, and decocted three times for 20, 25, and 30 min, respectively. The decoction was mixed and concentrated to 2.86 g/mL (1 mL extract contained 2.86 g herbal mixture).

Animals and treatments

Male specific pathogen free Wistar rats weighing 150 ± 20 g were obtained from Shanghai Experimental Animal Co. Ltd. (Shanghai, China) and housed in the laboratory animal center at our university. Seventy Sprague-Dawley rats were randomly divided into four groups: control group ($n = 10$ rats), model group ($n = 20$), SNMC group ($n = 20$), and IPTT group ($n = 20$). For the SNMC group, rats were treated with SNMC 15.6 mg/kg daily by gavage. For the IPTT group, rats were treated with IPTT 28.6 g/kg per day by gavage. The dosages used in rats were calculated from the formula: $\text{Dose}_{\text{rat}} = \text{Dose}_{\text{human}} \times (\text{habeas index}_{\text{rat}} / \text{habeas index}_{\text{human}}) \times (\text{body weight}_{\text{human}} / \text{body weight}_{\text{rat}}) \times 2/3^{[10]}$. First, the translational coefficient 6.25 was produced based on the formula. Then, the dose used in humans was converted to that used in rats through the translational coefficient. For the control and model groups, rats were treated with saline 10 mL/kg per day by gavage. All rats were treated by gavage for 3 d before induction of ALF, once daily, for a total of 5 d.

For the preparation of rats with D-GalN-induced ALF, rats were administered an intraperitoneal injection of D-GalN (1.4 g/kg). The control group was given intraperitoneally the same dose of saline. Thirty-six hours later, all rats were sacrificed. Blood samples (6 mL) were collected *via* the femoral artery, centrifuged at 3000 rpm for 10 min, and the sera were kept at -70°C for detection of biochemical parameters. The livers were rapidly removed, and tissues were removed from the left lobes, fixed in 10% neutral formalin, and retained for further analyses, including hematoxylin and eosin (HE) staining, fluorescence quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and proliferating cell nuclear antigen (PCNA)

immunohistochemistry assay.

Biochemistry

Serum biochemical parameters^[11] closely associated with liver function, including mainly alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), albumin (ALB), cholinesterase (CHE), and prothrombin time (PT), were determined according to the manufacturers' instructions.

Histopathological scores

Liver tissues obtained from each rat were fixed, embedded in paraffin, and cut into 3–5 mm sections. HE staining was used to evaluate the degree of hepatocyte necrosis. Microscopic injuries were graded according to a semiquantitative scoring system on a scale of 0 to 4 (0 = no discernable necrosis, 1 = bridging necrosis, 2 = confluent necrosis, 3 = sub-massive necrosis, and 4 = massive necrosis)^[11]. In order to minimize bias, each slide was read by three pathologists in a blinded manner and observed three times.

Fluorescence qRT-PCR

mRNA expression of high mobility group box (HMGB) 1, toll-like receptor (TLR) 4, nuclear factor kappa B (NF- κ B) and caspase-3 were analyzed *via* qRT-PCR and the $2^{-\Delta\Delta\text{Ct}}$ method. In accordance with the manufacturer's protocol (Invitrogen, Carlsbad, CA, United States), total RNAs were extracted from pulverized liver tissues with TRIzol reagent, then reverse-transcribed into cDNA by the ABI Step-One Plus Real Time PCR System (Applied Biosystems, Foster City, CA, United States). For HMGB1, the 5' primer sequence was 5' TGTTCTGAGTACCGCCCAA3', and the 3' primer sequence was 5' TTTCGCTGCATCAGGTTTTC3'. For TLR4, the 5' primer sequence was 5' CCAGGAAGGCTTCCACAAGA3', and the 3' primer sequence was 5' AATTCGACCTGCTGCCTCAG3'. For NF- κ B, the 5' primer sequence was 5' GCACGAGGCTCCTTTTCTCAA3', and the 3' primer sequence was 5' CGTTTTTCTTCAATCCGGTGG3'. For caspase-3, the 5' primer sequence was 5' ACCGATGTCGATGCAGCTAA3', and the 3' primer sequence was 5' AGGTCCGTTCGTTCCAAAAA3'. For the housekeeping gene (β -actin), the 5' primer sequence was 5' AAGGAGGCAAAGGACACCA3', and the 3' primer sequence was 5' AATGGCCCCCTTCACAGTTA3'.

PCNA immunohistochemistry

PCNA staining of the formalin-fixed and paraffin-embedded liver tissues was performed with an immunohistochemistry kit purchased from Boster Bioengineering Co. Ltd. (Wuhan, China). The number of PCNA-positive cells was counted in five random fields at $40\times$ magnification for each section. An average of the percentage of PCNA-positive cells was taken over these fields^[12]. Sections were examined

Table 2 Biochemistry and histopathology

Group	<i>n</i>	ALT (U/L)	AST (U/L)	TBiL (μmol/L)	ALB (g/L)	CHE (U/L)	PT (s)	Histopathological scores
Control	10	35.15 ± 6.01	151.61 ± 20.87	1.55 ± 0.43	35.25 ± 4.19	557.40 ± 43.23	13.60 ± 1.73	0
Model	20	441.10 ± 60.36 ^a	887.80 ± 128.47 ^a	38.04 ± 6.84 ^a	23.67 ± 3.21 ^a	343.92 ± 68.93 ^a	31.80 ± 5.02 ^a	3.69 ± 0.38 ^a
SNMC	20	267.18 ± 41.45 ^b	380.49 ± 55.38 ^b	24.37 ± 4.03 ^b	26.65 ± 4.50	378.50 ± 83.53	29.93 ± 3.83	2.78 ± 0.31 ^b
IPTT	20	127.45 ± 25.33 ^{b,c}	264.04 ± 63.43 ^{b,c}	19.37 ± 3.26 ^{b,c}	30.22 ± 4.30 ^{b,c}	488.61 ± 76.41 ^{b,c}	23.78 ± 4.34 ^{b,c}	2.06 ± 0.40 ^{b,c}

^a*P* < 0.05 *vs* control group; ^b*P* < 0.05 *vs* group; and ^c*P* < 0.05 *vs* SNMC group. Control group: Negative control group; Model group: Injected with D-GalN; SNMC group: Injected with D-GalN and treated with SNMC; IPTT group: Injected with D-GalN and treated with modified Taohechengqi-tang.

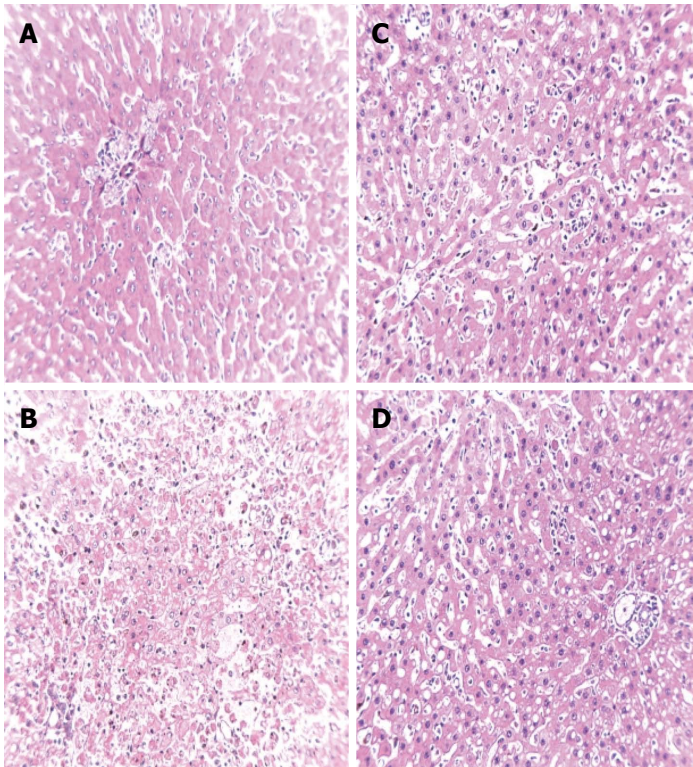


Figure 1 Hematoxylin and eosin staining. A: Control, negative control group; B: Model group, injected with D-GalN; C: SNMC group, injected with D-GalN and treated with SNMC; D: IPTT group, injected with D-GalN and treated with modified Taohechengqi-tang. D-GalN: D-galactosamine; SNMC: Stronger Neo-Minophagen C; IPTT: Improved prescription of Taohechengqi-tang.

microscopically, and photographs were collected using a digital image-capture system (CX40; Olympus, Tokyo, Japan).

Statistical analysis

Continuous variables were summarized as mean ± SD. The histopathological scores, biochemical parameters, mRNA, and PCNA expression were analyzed by one-way analysis of variance (ANOVA), followed by the Student's *t* test. All analyses were performed with SPSS version 17.0 (Chicago, IL, United States). All *P* values were two-sided, and *P* < 0.05 was considered statistically significant.

RESULTS

Biochemical profile in serum

The serum parameters are summarized in Table 2. D-GalN significantly increased serum ALT, AST, TBiL, and PT, while it decreased serum ALB and CHE. The effects were significantly reversed in the IPTT and

SNMC groups, especially the IPTT group.

Histopathological scores

The histopathological results are shown in Table 2 and Figure 1. Light microscopy revealed no liver necrosis in the control group, but massive hepatocyte necrosis was observed in rats treated with D-GalN. Compared to the model group, necrosis was markedly reduced in the IPTT and SNMC groups, and IPTT had a more significant impact.

mRNA expression

mRNA expression was observed as shown in Table 3. Compared to the model group, mRNA expression of HMGB1, TLR4, NF-κB, and caspase-3 were markedly reduced in the control, IPTT, and SNMC groups. Moreover, IPTT was found to have a more significant impact than SNMC.

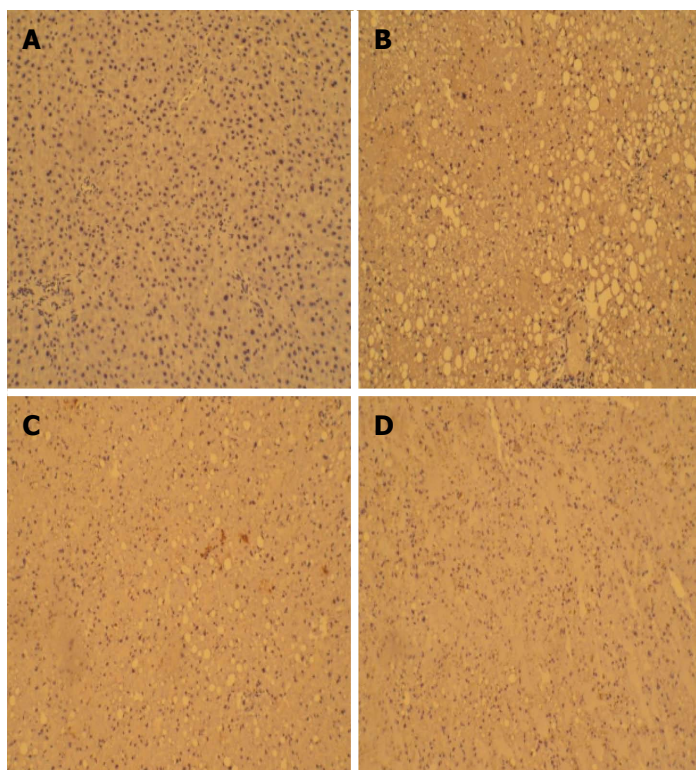
PCNA immunohistochemistry

Table 3 and Figure 2 show that the mean PCNA-

Table 3 mRNA expression and proliferating cell nuclear antigen immunohistochemistry

Group	<i>n</i>	HMGB1	TLR4	NF-κB	Caspase-3	PCNA (%)
Control	10	0.01 ± 0.01	0.04 ± 0.02	0.49 ± 0.25	1.36 ± 0.26	7.48 ± 0.90
Model	20	0.25 ± 0.04 ^a	0.41 ± 0.22 ^a	2.68 ± 1.35 ^a	3.41 ± 0.85 ^a	17.55 ± 2.40
SNMC	20	0.11 ± 0.04 ^b	0.22 ± 0.08 ^b	1.78 ± 0.64 ^b	2.57 ± 1.04 ^b	25.57 ± 2.94 ^b
IPTT	20	0.06 ± 0.03 ^{b,c}	0.07 ± 0.02 ^{b,c}	0.74 ± 0.41 ^{b,c}	1.61 ± 0.45 ^{b,c}	36.34 ± 4.91 ^{b,c}

^a*P* < 0.05 *vs* control group; ^b*P* < 0.05 *vs* model group; and ^c*P* < 0.05 *vs* SNMC group. HMGB: High mobility group box; TLR: Toll-like receptor; NF: Nuclear factor; PCNA: Proliferating cell nuclear antigen; Control group: Negative control group; Model group: Injected with D-GalN; SNMC group: Injected with D-GalN and treated with SNMC.

**Figure 2** Proliferating cell nuclear antigen immunohistochemistry.

A: Control, negative control group; B: Model group, injected with D-GalN; C: SNMC group, injected with D-GalN and treated with SNMC; D: IPTT group, injected with D-GalN and treated with modified Taohechengqi-tang. D-GalN: D-galactosamine; SNMC: Stronger Neo-Minophagen C; IPTT: Improved prescription of Taohechengqi-tang.

positive rates were significantly higher in the model, IPTT, and SNMC groups than in the control group. The PCNA-positive rate in the IPTT group was highest among the three groups.

DISCUSSION

In our study, IPTT not only remarkably improved the biochemical and coagulation profiles in serum, but it also reduced histopathological scores in ALF induced by D-GalN.

Inflammation plays a central role in the development of ALF. The net loss of hepatocytes mainly contributes to overwhelming injury with subsequent cell death rather than a lack of regeneration^[13,14]. Similar to severe acute pancreatitis and sepsis, ALF initially starts with a systemic inflammatory response syndrome (SIRS phase) and is followed by a compensatory anti-inflammatory response syndrome^[15,16]. There is an imbalance in both the pro- and anti-inflammatory systems, leading to immune-inflammatory dysregulation^[17]. The SIRS in ALF, whether or not precipitated

by infection, is characterized by prolonged hypercytokinemia, which is associated with the systemic complications (e.g., encephalopathy, infection, and renal failure) and poor outcome^[18-20].

Overactivation of the TLR4/NF-κB pathway is correlated with these pathological events in ALF. TLR4 is a member of the TLR family recognizing pathogen-associated molecular patterns^[21]. One of its ligands is bacterial lipopolysaccharide (LPS)^[22]. Once TLR4 recognizes LPS or LPS-CD14 complex, macrophages and dendritic cells are activated to present antigen to helper T cells. Furthermore, upregulated TLR4 signaling activates NF-κB via a MyD88-dependent pathway and/or MyD88-independent pathway. NF-κB is a downstream intracellular molecule of different receptors and induces the release of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, and IL-6^[23-25]. TNF-α, the core of the cytokine network, still plays an important role in the development of hepatocyte apoptosis. The binding of TNF-α to the TNF receptor 1 triggers the apoptotic cascade mediated by proapoptotic Bcl-2 family

proteins^[26,27].

HMGB1 is a nuclear protein with cytokine-type functions that is passively released during necrosis or actively secreted during immune cell activation^[28]. There are many receptors, including the receptor for advanced glycation endproducts, TLR2, TLR4, and TLR9^[29], to which HMGB1 can bind. TLR4 is the critical receptor mediating the inflammatory activity of HMGB1^[30,31]. Once the HMGB1/TLR4/NF- κ B pathway is activated, overexpression of TNF- α induces hepatocyte necrosis and activates the TNF- α -mediated extrinsic apoptotic pathway^[32]. Our results support the anti-inflammatory and antiapoptotic effects of IPTT, as the liver expression of HMGB1, TLR4, NF- κ B, and caspase-3 was significantly reduced in ALF rats. Moreover, IPTT improved hepatocyte regeneration.

Acute liver injury may recover *via* the process of hepatocyte regeneration once the injury is discontinued. After massive liver injury, the capacity of hepatocyte proliferation was not lost, only severely suppressed^[33]. In a recent study, HMGB1 was reported to impair hepatocyte regeneration after acetaminophen overdose, and blockade of HMGB1 enhanced liver recovery^[34,35]. Our results showed that severe liver damage limited hepatocyte regression. However, the hepatoprotective effect of IPTT might contribute to liver regeneration. Furthermore, IPTT may be sensitive to the stimulation of growth factors, such as HGF and transforming growth factor (TGF) α , and progress into the cell cycle for replication^[36].

IPTT has been used for a long time in the Affiliated Hospital of Chengdu University of Traditional Chinese Medicine^[9] and is composed of seven different types of TCM. Among them, *Radix paeoniae rubra* was effective in attenuating hepatocyte apoptosis, and this effect was partly mediated through the activation of the mitochondrial pathway and subsequent regulation of expression of particular proapoptotic genes^[37]. *Semen persicae* protected against cisplatin-induced hepatotoxicity by reducing cisplatin-induced oxidative stress^[38]. *Flos carthami* protected the liver from long-term alcohol injury, which was related to enhanced antioxidant capacity and inhibition of TGF- β 1 expression^[39]. *Radix et rhizoma rhei palmate* promoted bioavailability and liver protective effects and prevented and treated hepatic encephalopathy in rats with thioacetamide-induced ALF^[40,41]. IPTT combined these Chinese herbal medicines following the principle of clearing heat and resolving stasis. Our data suggested that IPTT protected against ALF through its anti-inflammatory, antiapoptotic, and regeneration promoting effects. It provided evidence for clinical application of Chinese herbs for clearing heat and resolving stasis.

In conclusion, IPTT attenuates inflammatory reaction of ALF in rats *via* inhibition of HMGB1 production, which may contribute to recovery of limited liver regeneration.

ACKNOWLEDGMENTS

The authors would like to thank the members of the Department of Infectious Diseases, Affiliated Hospital of Chengdu University of Traditional Chinese Medicine, and the Animal Care and Scientific Committee of Chengdu University of Traditional Chinese Medicine for their technical support.

COMMENTS

Background

Acute liver failure (ALF) is a severe clinical syndrome with high mortality. In previous studies, Chinese herbal medicine has been shown to improve the outcome of LF. Nevertheless, pharmacological research of Chinese medicine is still lacking.

Research frontiers

A systemic inflammatory response syndrome in ALF is characterized by prolonged hypercytokinemia, which is associated with the systemic complications and poor outcome. The high mobility group box (HMGB)1/Toll-like receptor 4/nuclear factor- κ B pathway has been the recent research focus in inflammation.

Innovations and breakthroughs

The present study demonstrates that improved prescription of Taohechengqitang (IPTT) attenuated the inflammatory reaction of ALF. One of the mechanisms is likely related to inhibition of HMGB1 production, which contributes to recovery of liver regeneration.

Applications

The data from this study provide a rational basis for IPTT for treatment of LF in clinical practice. It also provides evidence for clinical application of Chinese herbs for clearing heat and resolving stasis.

Terminology

ALF is a clinical syndrome in which abrupt onset, manifesting as jaundice and coagulopathy, is complicated within 2 wk by grade II + encephalopathy in a patient with undiagnosed chronic liver disease.

Peer-review

The experiments appear to be well conducted and the conclusions drawn are justified. The manuscript has novelty and significance.

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