

Sophocarpine attenuates liver fibrosis by inhibiting the TLR4 signaling pathway in rats

Hui Qian, Jian Shi, Ting-Ting Fan, Jiao Lv, Si-Wen Chen, Chun-Yan Song, Zhi-Wu Zheng, Wei-Fen Xie, Yue-Xiang Chen

Hui Qian, Jian Shi, Ting-Ting Fan, Si-Wen Chen, Chun-Yan Song, Wei-Fen Xie, Yue-Xiang Chen, Department of Gastroenterology, Shanghai Changzheng Hospital, Second Military Medical University, Shanghai 200003, China

Hui Qian, Jiao Lv, Zhi-Wu Zheng, Department of Gastroenterology, the 411th Hospital of PLA, Shanghai 200081, China

Author contributions: Qian H, Shi J and Fan TT contributed equally to this study who performed the majority of the experiments and prepared the manuscript with assistance from Chen SW and Song CY; Lv J and Zheng ZW performed the immunohistochemical staining and data analysis; Xie WF designed the experiments and supervised the project; Chen YX designed the experiments, analyzed the data and wrote the manuscript.

Supported by The National Natural Science Foundation of China, Nos. 30971343, 81270486, 81000167 and 81370009

Correspondence to: Yue-Xiang Chen, Professor, Department of Gastroenterology, Shanghai Changzheng Hospital, Second Military Medical University, 415 Fengyang Rd, Shanghai 200003, China. yuxchen1807@163.com

Telephone: +86-21-81885343 Fax: +86-21-81886824

Received: September 7, 2013 Revised: November 13, 2013

Accepted: November 28, 2013

Published online: February 21, 2014

Abstract

AIM: To explore the effect of sophocarpine on experimental liver fibrosis and the potential mechanism involved.

METHODS: Sophocarpine was injected intraperitoneally in two distinct rat hepatic fibrosis models induced either by dimethylnitrosamine or bile duct ligation. Masson's trichrome staining, Sirius red staining and hepatic hydroxyproline level were used for collagen determination. Primary hepatic stellate cells (HSCs) were isolated and treated with different concentrations of sophocarpine. Real-time reverse transcription-polymerase chain reaction was used to detect the mRNA levels of fibrotic markers and cytokines. The expression of pathway proteins was measured by Western blot. The Cell Counting

Kit-8 test was used to detect the proliferation rate of activated HSCs treated with a gradient concentration of sophocarpine.

RESULTS: Sophocarpine decreased serum levels of aminotransferases and total bilirubin in rats under chronic insult. Moreover, administration of sophocarpine suppressed extracellular matrix deposition and prevented the development of hepatic fibrosis. Furthermore, sophocarpine inhibited the expression of α -smooth muscle actin (SMA), interleukin (IL)-6, transforming growth factor- β 1 (TGF- β 1), Toll-like receptor 4 (TLR4), and extracellular-related kinase (ERK) in rats. Sophocarpine also down-regulated the mRNA expression of α -SMA, collagen I, collagen III, TGF- β 1, IL-6, tumor necrosis factor- α and monocyte chemoattractant protein-1, and decreased protein levels of TLR4, p-ERK, p-JNK, p-P38 and p-IKK *in vitro* after Lipopolysaccharide induction. In addition, sophocarpine inhibited the proliferation of HSCs accompanied by a decrease in the expression of Cyclin D1. The protein level of proliferating cell nuclear antigen was decreased in activated HSCs following a gradient concentration of sophocarpine.

CONCLUSION: Sophocarpine can alleviate liver fibrosis mainly by inhibiting the TLR4 pathway. Sophocarpine may be a potential chemotherapeutic agent for chronic liver diseases.

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Key words: Liver fibrosis; Sophocarpine; Toll-like receptor 4; Hepatic stellate cells; Cytokines

Core tip: Sophocarpine significantly ameliorated liver function and hepatic fibrosis in both the dimethylnitrosamine and bile duct ligation models. In addition, sophocarpine inhibited the activation and proliferation of hepatic stellate cells *in vitro*, which contributed to

the anti-fibrotic effect of sophocarpine *in vivo*. Toll-like receptor 4 signaling was blocked by sophocarpine *in vivo* and *in vitro*, accompanied by a reduction in pro-inflammatory and fibrotic cytokines, as well a decrease in the expression of Cyclin D1 and proliferating cell nuclear antigen.

Qian H, Shi J, Fan TT, Lv J, Chen SW, Song CY, Zheng ZW, Xie WF, Chen YX. Sophocarpine attenuates liver fibrosis by inhibiting the TLR4 signaling pathway in rats. *World J Gastroenterol* 2014; 20(7): 1822-1832 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i7/1822.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i7.1822>

INTRODUCTION

Liver fibrosis is a wound-healing response to chronic liver injury and is characterized by the accumulation of extracellular matrix (ECM), which depends on the balance between ECM synthesis and degradation. Following liver injury, hepatic stellate cells (HSCs) are the predominant ECM-producing cell type in the liver^[1,2]. The activation and proliferation of HSCs, characterized by the morphological transition to myofibroblast-like cells, have been well established as the central events in the pathogenesis of hepatic fibrosis^[3]. Previous studies have suggested that inhibition of the activation, proliferation and migration of HSCs may be an attractive anti-fibrotic therapy^[4].

In cirrhotic rats and patients, intestinal bacterial overgrowth can occur, including Gram-negative and -positive bacteria^[5]. Lipopolysaccharide (LPS), a major cellular component of Gram-negative bacteria, aggravates liver cirrhosis with increased permeability of the intestinal mucosal barrier. LPS-induced HSC activation has been proved to be an important mechanism in liver injury^[6]. Quiescent murine HSCs responsive to LPS can express Toll-like receptor 4 (TLR4) similar to *in vivo*-activated HSCs, even at the low LPS dose of 1 ng/mL^[7]. In addition, activated murine HSCs express TLR4 and respond to LPS with up-regulation of extracellular-related kinase (ERK) phosphorylation, interleukin (IL)-6 and transforming growth factor- β 1 (TGF- β 1)^[7,8]. TLR4-mutant mice displayed a profound reduction in hepatic fibrogenesis in three different experimental models of biliary or toxic fibrosis^[7]. These results confirm the critical role of TLR4 signaling in regulating HSC activation which affects the risk of hepatic fibrosis progression.

Sophocarpine is a matrine-type quinolizidine alkaloid widespread in the genus *Sophora*. Basic and clinical studies have shown that sophocarpine possesses a variety of pharmacological effects, such as anti-inflammatory, immuno-regulatory, anti-virus and anti-tumor^[9-11]. Moreover, our previous research proved that sophocarpine alleviated the progression of non-alcohol steatohepatitis through the down-regulation of inflammatory cytokines *in vivo*^[12]. More importantly, sophocarpine has minor toxic side effects and significant potential for clinical application.

In this study, we demonstrated that sophocarpine ameliorated liver fibrosis by inhibiting the activation and proliferation of HSCs in rats. In addition, negative regulation of the TLR4 signaling pathway contributed to the effects of sophocarpine which decreased the expression of profibrotic and inflammatory cytokines such as tumor necrosis factor (TNF)- α , TGF- β 1 and IL-6, and reduced Cyclin D1 and proliferating cell nuclear antigen (PCNA).

MATERIALS AND METHODS

Sophocarpine

Sophocarpine (High Performance Liquid Chromatography purity > 98%) was obtained from Winherb Medical Science Co., Ltd (Shanghai, China). The sophocarpine used was endotoxin-free, as determined by the limulus lysate assay, with a minimum detectable level of 8 pg/L.

Animal fibrosis models and sophocarpine administration *in vivo*

Male Sprague-Dawley rats (190 \pm 15 g) were housed in cages with stainless-steel wire tops under standard animal laboratory conditions in the specific pathogen-free-grade animal room of the Experimental Animal Center of the Second Military Medical University. The rats had free access to standard rat chow and water. This study was approved by the Ethics Committee of the Second Military Medical University, Shanghai, China. Two distinct models of hepatic fibrosis were induced in rats using dimethylnitrosamine (DMN) injection (10 mg/kg, three injections per week for 4 wk) or bile duct ligation (BDL) as described previously^[13]. For the BDL model, seven rats served as controls and underwent sham surgery. Three days after surgery, 24 BDL rats were randomly divided into two groups and treated as follows: the model group ($n = 12$, Ringer's solution) and sophocarpine treatment group ($n = 12$, 20 mg/kg sophocarpine dissolved in Ringer's solution). For the DMN model, seven rats were included as controls. Fourteen days after DMN injection, the 24 DMN rats were divided as described above (12 in the model group and 12 in the sophocarpine treatment group). Sophocarpine and placebo solution were injected intraperitoneally once a day. The animals were sacrificed 3 wk after BDL or 4 wk after DMN administration.

Serum biochemical analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total bilirubin (TB) were determined in all experimental rats using the appropriate kits from Sigma-Aldrich.

Histological examination and immunohistological staining

All paraffin-embedded liver samples were stained with hematoxylin and eosin for histopathological examination. Masson's trichrome staining and Sirius red staining were used for collagen determination. For the semiquantitative analysis, connective tissues stained with Sirius red were

Table 1 Primers used for detection of gene transcription

Gene	Forward	Reverse
α -SMA	5'-ACTGGGACGACATGGAAAAG-3'	5'-CATCTCCAGAGTCCAGCACA-3'
Pro-collagen I	5'-AGGCATAAAGGGTCATCGTG-3'	5'-ACCGTTGAGTCCATCTTTGC-3'
Collagen III	5'-GTCCACGAGGTGACAAAAGGT-3'	5'-CATCTTTCCAGGAGTCCA-3'
TNF α	5'-AGATGTGGAACCTGGCAGAGG-3'	5'-CCCATTGGGAACCTTCCT-3'
IL-6	5'-CCGGAGAGGAGACTTCACAG-3'	5'-ACAGTGCATCATCGCTGTT-3'
TGF β 1	5'-ATACGCTGAGTGGCTGCT-3'	5'-TGGGACTGATCCCATGATT-3'
MCP-1	5'-ATGCAGTTAATGCCCACTC-3'	5'-TTCCTTATGGGGTCAGCAC-3'
TLR4	5'-TGCTCAGACATGGCAGTTTC-3'	5'-TCAAGGCTTTCCATCCAAC-3'
Myd88	5'-GAGATCCCGAGTTTGAGAC-3'	5'-CTGTTTCGCTGGTTGCGTA-3'
TRAF6	5'-AGGGTACAATACGCCCTCAG-3'	5'-GCGGGTAGAGACTTCACAGC-3'
ERK1	5'-TCCAAGGGCTACACCAAATC-3'	5'-AGGTAGTTTCGGGCCTTCAT-3'
JNK1	5'-GCCACAAAATCCTCTTCCA-3'	5'-CACATCGGGGAACAGTTTCT-3'
Cyclin D1	5'-GCGTACCCTGACACCAATCT-3'	5'-GGCTCCAGAGACAAGAAACG-3'
β -actin	5'-GCCAACACAGTGTGCTGG-3'	5'-TGATCCACATCTGCTGGAAGG-3'

measured on an image analyzer (Image-Pro Plus, Media Cybernetics) by a technician blinded to the samples. The intensity of collagen deposition or protein expression was calculated as the percentage of the positive area in the corresponding field of liver tissue. Immunohistochemical examinations were carried out to determine the expression of α -smooth muscle actin (SMA) (BM0002, Boster, Wuhan, China), TGF- β 1 (sc-146, Santa Cruz), IL-6 (ab6672, Abcam), TLR4 (ab13556, Abcam) and ERK1/2 (4695, Cell Signaling).

Measurement of hepatic hydroxyproline content

Total hepatic hydroxyproline levels in all experimental rats were determined in the hydrolysates of liver samples as previously described^[13]. One hundred mg of wet liver samples were subjected to acid hydrolysis to determine the amount of hydroxyproline according to the protocol outlined in the Hydroxyproline Testing Kit (A030-2, Jiancheng, Nanjing, China).

Cell culture and treatment

Primary HSCs were freshly isolated as previously described^[14]. The primary HSCs were cultured in plastic cell culture dishes. Forty-eight hours later, sophocarpine was added at the concentrations of 25, 50 or 200 mg/mL for 48 or 72 h, and the cells were then treated with LPS (Sigma, 2 ng/mL) for 30 min (Western blotting) or 12 h [Real-time reverse transcription-polymerase chain reaction (RT-PCR)]. Activated HSCs were derived from the primary HSCs which were cultured for approximately 14 d and passaged for 2-3 passages, the cells were then treated with sophocarpine (100-1000 μ g/mL) for 0-5 d. These cells were cultured in Dulbecco's modified medium containing 10% fetal bovine serum.

RT-PCR

Total RNA was extracted from the cells and liver tissues (3 animals representative of each group) with Trizol reagent (Invitrogen, Carlsbad, CA, United States), and cDNA was synthesized according to the manufacturer's instructions (TAKARA, Japan). Transcription levels were detected by

real-time RT-PCR with a SYBR Green PCR Kit (Applied Biosystems, Foster City, CA, United States). Primer sequences are listed in Table 1.

Western blot analysis

Western blot analyses of α -SMA (ab5694, Abcam), Collagen I (ab6308, Abcam), TLR4 (ab13556, Abcam), PCNA (ab29, Abcam), GAPDH (BSAP0063, Bioworld), p-JNK (4668, Cell Signaling), JNK (9258, Cell Signaling), p-ERK1/2 (4370, Cell Signaling), ERK1/2 (4695, Cell Signaling), p-P38 (4511, Cell Signaling), P38 (8690, Cell Signaling), p-IKK α/β (2697, Cell Signaling) and IKK α/β (sc-7607, Santa Cruz) were performed according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, United States).

Measurement of HSC proliferation

Activated HSCs were plated in triplicate wells on a 96-well plate at 3×10^3 cells/well and cultured for 24 h. These cells were then treated with sophocarpine at the concentrations of 0, 100, 200, 400, 600 and 1000 μ g/mL. The number of metabolically active and viable cells was detected colorimetrically at 450 nm using the Cell Counting Kit-8 (DOJINDO, Tokyo, Japan) assays.

Statistical analysis

Results are presented as the mean of three independent experiments (mean \pm SD). The two-sided independent Student's *t* test was performed to analyze the differences in gene expression levels, hydroxyproline content and histology data. *P* < 0.05 was considered statistically significant.

RESULTS

Sophocarpine ameliorates liver function in fibrotic rats

To assess the effect of sophocarpine on liver function, serum aminotransferases and total bilirubin were determined. Liver function tests showed that sophocarpine significantly down-regulated the concentrations of aminotransferases and total bilirubin in both the DMN and BDL models (Table 2, Figure 1A and B). TB concentra-

Table 2 Effect of sophocarpine on the improvement in serum alanine aminotransferase, aspartate aminotransferase and total bilirubin levels in both models of liver fibrosis

Group	ALT (U/L)	AST (U/L)	TB (mmol/L)
Normal	29.29 ± 1.76	123.67 ± 29.06	1.29 ± 0.28
DMN model	68.27 ± 3.43	176.9 ± 8.99	8.09 ± 1.35
DMN + sophocarpine	48.58 ± 4.52 ¹	108.42 ± 15.46 ¹	3.00 ± 0.48 ¹
Sham operation	28.83 ± 2.22	121.00 ± 36.72	2.00 ± 0.63
BDL model	150.50 ± 23.6	959.50 ± 255	139.67 ± 16.23
BDL + sophocarpine	91.86 ± 6.71 ¹	464.14 ± 182.6 ¹	123.86 ± 9.69

¹Compared with the model group, $P < 0.05$. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; DMN: Dimethylnitrosamine; BDL: Bile duct ligation.

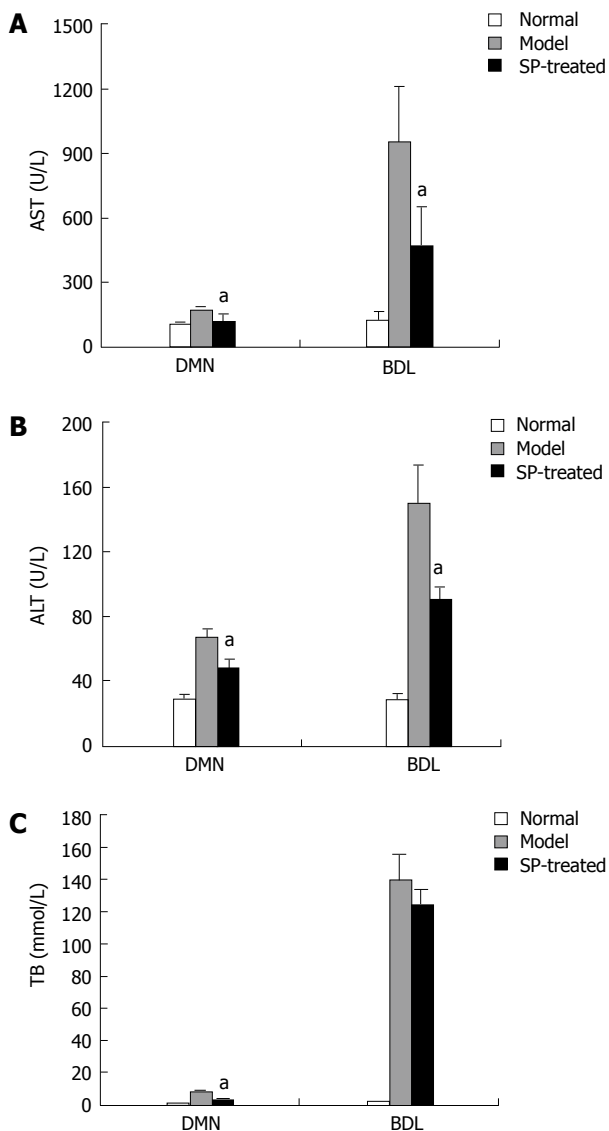


Figure 1 Sophocarpine ameliorates liver function in fibrotic rats. Serum was collected from each group of rats. AST (A), ALT (B) and TB (C) levels were determined to assess liver function in the sophocarpine-treated group compared to each model group ($P < 0.05$, by two-tailed Student's *t* test). ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; TB: Total bilirubin; DMN: Dimethylnitrosamine; BDL: Bile duct ligation.

tion in sophocarpine-treated rats showed a decrease by approximately 70% compared with the DMN rat model,

however, there was no significant decrease in the sophocarpine-treated BDL group (Table 2, Figure 1C).

Sophocarpine attenuates hepatic fibrosis induced by DMN or BDL in rats

We next examined the potential effect of sophocarpine on the two distinct fibrotic models. Liver sections stained with Masson's trichrome stain showed that periportal fibrosis with fibrous septa extended to adjacent portal tracts and the terminal hepatic venue in the DMN model; in the BDL model, livers showed extensive bile duct proliferation, a detrimental collapse of liver parenchyma and overt ECM deposition around the reactive bile ductile (Figure 2A). Following administration of sophocarpine, the ECM area (Masson's staining) was reduced by 55.6% and 39.3% ($P < 0.05$) in DMN and BDL rats, respectively (Figure 2B). The contents of hydroxyproline in the sophocarpine-treated rats were lower than those in the model rats ($261.17 \pm 20.45 \mu\text{g/g}$ in the sophocarpine-treated DMN group *vs* $361.17 \pm 20.55 \mu\text{g/g}$ in the DMN model group, $P < 0.05$; $178 \pm 15.89 \mu\text{g/g}$ in the sophocarpine-treated BDL group *vs* $259.33 \pm 23.18 \mu\text{g/g}$ in the BDL model group, $P < 0.05$) (Figure 2C). The mRNA expression of α -SMA, TGF- β 1 and alpha 1 type I procollagen detected by RT-PCR was reduced significantly in the sophocarpine-treated groups in both the DMN and BDL models ($P < 0.05$) (Figure 2D and E).

Expression of pro-fibrotic cytokines and TLR4 signaling pathway-related proteins is suppressed in sophocarpine-treated rats

We subsequently determined the effects of sophocarpine on the expression of pro-fibrotic cytokines. Immunohistochemical examination revealed that α -SMA, IL-6 and TGF- β 1 protein expression was significantly elevated in both the DMN and BDL models, and was suppressed following sophocarpine administration (Figure 3). Furthermore, from the observed effects of sophocarpine on HSCs *in vitro*, the expression of TLR4 signaling pathway-related proteins, such as TLR4 and ERK1/2, was also increased in the DMN and BDL models. Sophocarpine down-regulated these proteins *in vivo* as shown by immunohistochemistry (Figure 3). These results indicated that sophocarpine blocked the TLR4 signaling pathway which aggravated the progression of liver fibrosis and reduced pro-fibrotic cytokine expression.

Sophocarpine inhibits the activation of hepatic stellate cells

RT-PCR was performed to determine the gene expression of inflammatory and fibrotic markers in HSCs treated with a gradient concentration of sophocarpine at 48 and 72 h to observe the effect of sophocarpine during activation of HSCs. In contrast to the freshly isolated and self-activated HSCs, dose gradient sophocarpine-treated cells showed significantly lower expression of α -SMA, collagen I, collagen III, TGF- β 1 and inflammatory cytokines, including IL-6, TNF- α and monocyte chemoattractant protein-1 (MCP-1) (Figure 4A). Furthermore,

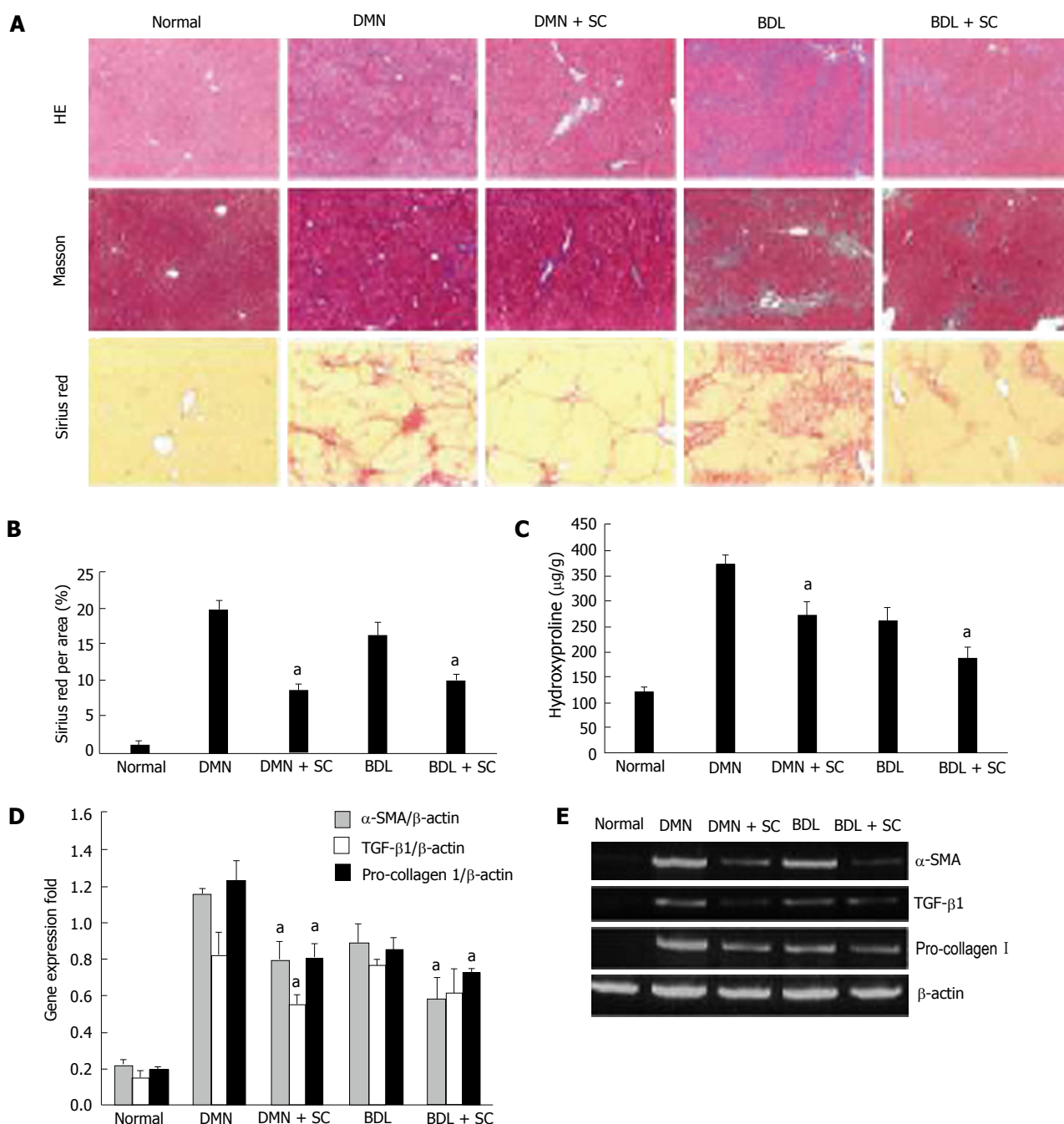


Figure 2 Sophocarpine attenuates hepatic fibrosis induced by dimethylnitrosamine or bile duct ligation in rats. DMN and BDL were used to construct two types of hepatic fibrosis models to evaluate the therapeutic effect of sophocarpine. A: Liver fibrosis in each group was assessed by HE ($\times 40$), Masson's trichrome ($\times 40$) and Sirius red staining ($\times 40$); B: The percentage of Sirius-red in fibrotic livers was quantified using an image analysis system ($^{\ast}P < 0.05$); C: The amount of hydroxyproline in fibrotic livers was detected in the sophocarpine-treated group compared with each model group ($^{\ast}P < 0.05$); D, E: Real-time RT-PCR was employed to examine the expression of α -SMA, TGF- β and pro-collagen I in fibrotic livers following sophocarpine administration compared with the control models ($^{\ast}P < 0.05$ by two-tailed Student's *t* test). DMN: Dimethylnitrosamine; BDL: Bile duct ligation; TGF- β : Transforming growth factor- β ; RT-PCR: Reverse transcription-polymerase chain reaction; SMA: Smooth muscle actin.

the protein expression of α -SMA and collagen I was depressed by sophocarpine in HSCs (Figure 4B). We also measured the above-mentioned gene expression at different culture times. The mRNA expression of α -SMA, collagen III, IL-6, TNF- α and MCP-1 decreased by 38.6%, 43.7%, 59.5%, 45.2% and 55.6% at 72 h compared to 48 h, respectively (Figure 4C). These results confirmed that sophocarpine had a dose- and time-dependent inhibitory effect on the activation of HSCs.

Sophocarpine blocks the LPS-induced TLR4 signaling pathway affecting the activation of HSCs

We then detected mRNA expression of TLR4 pathway-related genes induced by LPS using RT-PCR at 72 h. The gene expression of TLR4 and Myd88 showed a significant dose-dependent decrease in sophocarpine-treated HSCs (Figure 4D). Subsequently, we found that the protein expression of TLR4, p-ERK1, p-JNK1, p-P38 and p-IKK decreased markedly in sophocarpine-treated

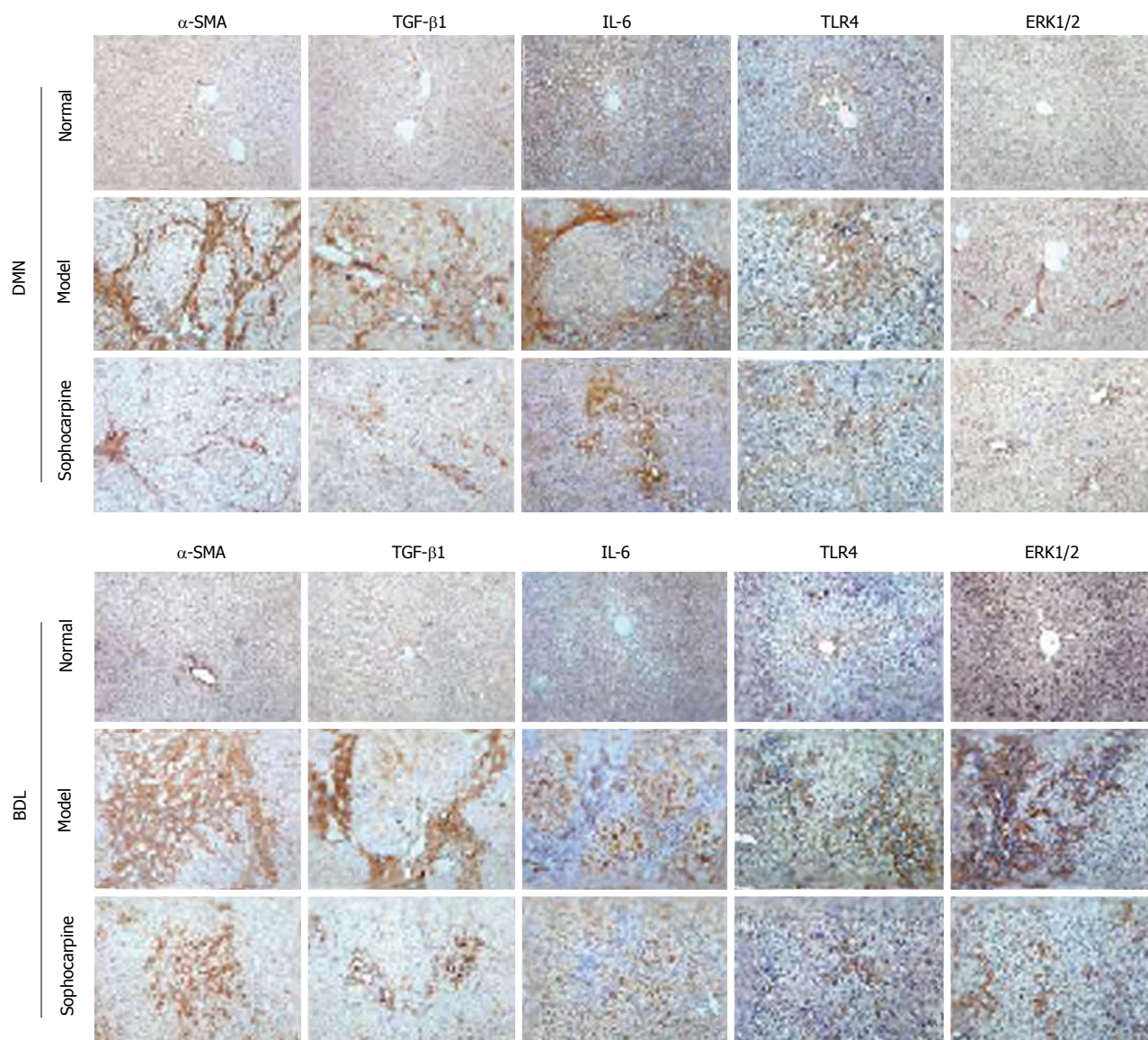


Figure 3 Expression of pro-fibrotic cytokines and toll-like receptor 4 signaling pathway related-proteins is suppressed in sophocarpine-treated rats. Immunohistochemical analysis of the protein expression of α -SMA, TGF- β 1, IL-6, TLR4 and ERK1/2 in the liver tissue of each group was performed as described in Materials and Methods. The results show the protein expression of α -SMA ($\times 200$), TGF- β 1 ($\times 200$), IL-6 ($\times 200$), TLR4 ($\times 200$) and ERK1/2 ($\times 200$) in the fibrotic livers of each group. TLR4: Toll-like receptor 4; IL-6: Interleukin-6; TGF- β : Transforming growth factor- β ; SMA: Smooth muscle actin.

HSCs compared with control cells induced by LPS at 72 h. Thus, the Myd88-dependent TLR4 signaling pathway was blocked by sophocarpine which inhibited the activation of HSCs (Figure 4E).

Sophocarpine suppresses the proliferation of HSCs

To determine the effects of sophocarpine on inhibition of HSC proliferation, CCK-8 assays were performed. The results of CCK-8 measurement showed that sophocarpine at doses ≥ 200 μ L/mL significantly suppressed the proliferation of HSCs. These inhibitory effects were dose- and time-dependent (Figure 5A). We also detected mRNA expression of Cyclin D1 in self-activated HSCs treated with sophocarpine and in control cells, and sophocarpine significantly down-regulated the expression of Cyclin D1 at 72 h (Figure 5B). The inhibitory effect of sophocarpine on the proliferation of HSCs was confirmed by decreased

protein expression of PCNA in HSCs (Figure 5C).

DISCUSSION

Liver fibrosis occurs as a wound-healing scar response following acute and chronic inflammation, including viral hepatitis, alcohol consumption, autoimmune and metabolic liver diseases^[15]. Liver inflammation results in the activation of HSCs through various inflammatory or fibrogenic mediators including TNF- α , IL-1 β , IL-6 and TGF- β ^[16-21]. In recent studies, sophocarpine, a monomer used in traditional Chinese medicine, inhibited the production of inflammatory cytokines IL-6 and TNF- α in murine macrophages^[11]. In addition, sophocarpine prevented cachexia-related symptoms induced by adenocarcinoma and alleviated non-alcoholic steatohepatitis in rats^[11,12]. Based on the validated effects of sophocarpine

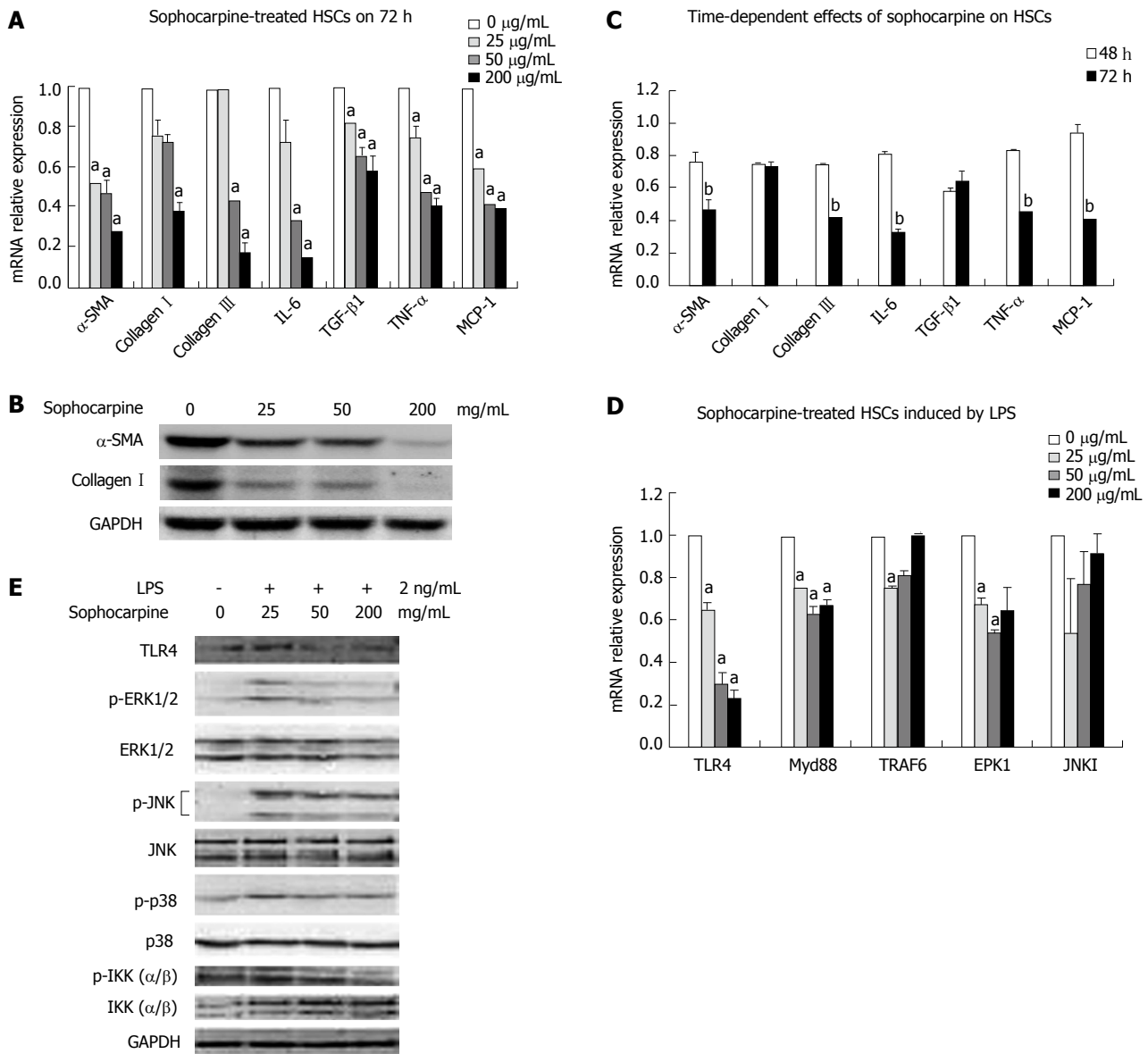


Figure 4 Sophocarpine inhibits the activation of hepatic stellate cells by blocking the lipopolysaccharide-induced toll-like receptor 4 signaling pathway. Primary HSCs were isolated and plated in 6-well plates (1×10^5 cells/well). Forty-eight hours later, the HSCs were treated with a gradient concentration of sophocarpine for 48 or 72 h. A: Real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to analyze the mRNA levels of α -SMA, collagen I, collagen III, IL-6, TGF- β 1, TNF- α and MCP-1 in HSCs treated with a gradient concentration of sophocarpine for 72 h (compared to 0 μ g/mL, $^aP < 0.05$); B: Immunoblots of α -SMA, collagen I and GAPDH were detected by Western blot from HSCs treated with a gradient concentration of sophocarpine for 72 h; C: The mRNA expression of the above genes was detected in HSCs treated with sophocarpine (50 mg/mL) at 72 h compared to that at 48 h, $^bP < 0.05$; D, E: Gradient concentration sophocarpine-treated HSCs were incubated with LPS (2 ng/mL), and real-time RT-PCR (compared to 0 μ g/mL, $^aP < 0.05$, D) and Western blot analysis (E) were employed to detect the expression of TLR4 pathway-related genes at the gene and protein levels (P value by two-tailed Student's t test). LPS: Lipopolysaccharide; HSCs: Hepatic stellate cells; TGF- β : Transforming growth factor- β ; IL-6: Interleukin-6; SMA: Smooth muscle actin; TNF: Tumor necrosis factor; MCP: Monocyte chemoattractant protein.

on inflammation regulation, we hypothesized that sophocarpine may have a restorative effect on liver fibrosis.

In this study, we used sophocarpine to treat hepatic fibrosis induced by two mechanistically different fibrosis models: DMN administration and BDL. Based on the histopathological and immunohistochemical results, hepatocellular injury and HSC activation improved following sophocarpine administration in both models. More importantly, it was shown for the first time that sophocarpine administration attenuated ECM deposition and hydroxyproline content in liver fibrosis induced by DMN and BDL, indicating that sophocarpine suppressed

hepatic fibrosis in these rat models. Furthermore, we demonstrated that reduced production of inflammatory cytokines, such as IL-6 and TNF- α , contributed to the anti-fibrotic effect of sophocarpine. This was accompanied by alleviation of liver fibrosis and a reduction in pro-fibrotic cytokines such as TGF- β 1 *in vivo*^[22,23]. The expression of α -SMA which is considered a marker of myofibroblasts^[24] decreased significantly in fibrotic rats treated with sophocarpine. Based on these findings, we hypothesize that sophocarpine inhibited the activation and proliferation of HSCs which mediate the central pathological effects in the progression of liver fibrosis.

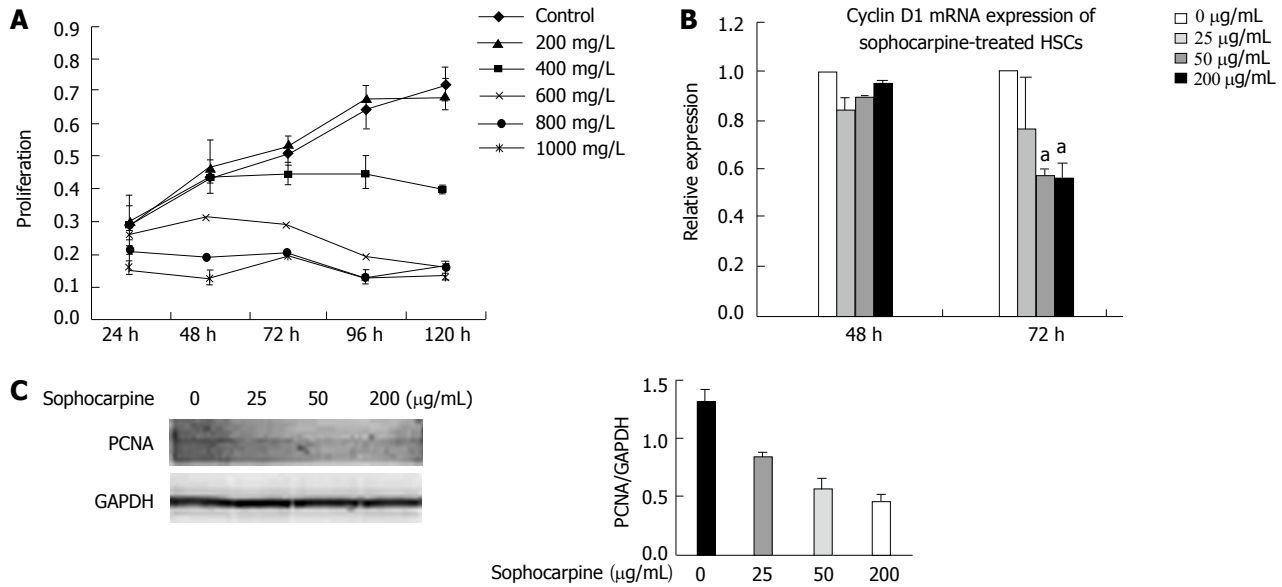


Figure 5 Sophocarpine suppresses the proliferation of hepatic stellate cells. A: Activated HSCs were treated with a gradient concentration of sophocarpine and the proliferation of HSCs was assessed using the CCK-8 kit; B: Real-time polymerase chain reaction was performed to examine the expression of Cyclin D1 in HSCs after treatment with a gradient concentration of sophocarpine ($P < 0.05$); C: Western blot was employed to detect PCNA expressed in HSCs after treatment with sophocarpine. HSCs: Hepatic stellate cells; PCNA: Proliferating cell nuclear antigen.

During hepatic fibrosis, HSCs undergo activation and conversion to myofibroblast-like cells which secrete collagens and aggravate the deposition of ECM. Reversal of these processes is critical in the treatment of liver fibrosis. In the present study, sophocarpine decreased the expression of α -SMA, collagen I and III *in vitro*, which indicated that sophocarpine inhibited the activation and conversion of HSCs. Moreover, sophocarpine reduced the expression of TGF- β 1 which is a major pro-fibrogenic molecule. Furthermore, sophocarpine attenuated the hepatic inflammation reaction and reduced the expression of IL-6, MCP-1 and TNF- α during the *in vivo* activation of HSCs. Sophocarpine also inhibited the proliferation of HSCs with a reduction in Cyclin D1 which participates in cell cycle regulation. These results demonstrated that the inhibition of activation and proliferation of HSCs was the major cytological mechanism involved in the alleviation of liver fibrosis by sophocarpine.

Toll-like receptors (TLRs) play an important role in the regulation of inflammation, even under sterile conditions, such as injury and wound healing. The healthy liver contains lower mRNA levels of TLRs and signaling molecules such as MD-2 and MyD88 than other organs, suggesting that the low expression of TLR signaling molecules may contribute to the high tolerance of the liver to TLR ligands from the intestinal microbiota to which the liver is constantly exposed^[25-28]. Numerous studies have demonstrated that LPS is elevated in experimental models of hepatic fibrosis^[7,29,30] and in cirrhotic patients^[31-33]. Cirrhotic patients have markedly elevated endotoxin levels compared with healthy subjects^[33]. In view of the critical role of the intestinal microbiota in hepatic fibrogenesis, the LPS-induced TLR4 signaling pathway contributes significantly to the progression of liver cirrhosis^[34]. Activated human HSCs expressed TLR4 and its

coreceptors MD-2 and CD-14 *in vitro*^[35]. LPS treatment can induce strong activation of the nuclear factor kappa B (NF- κ B) and JNK/AP-1 pathways as well as the secretion of pro-inflammatory cytokines in activated HSCs^[36]. Activated murine HSCs expressed TLR4 and responded to LPS with an up-regulation of extracellular-related kinase (ERK) phosphorylation and IL-6, TGF- β 1 and MCP-1 secretion^[7,8].

As IL-6, TNF- α , TGF- β 1 and MCP-1, whose expression decreased after sophocarpine administration, are all regulated by the LPS-induced TLR4 signal pathway, we suspected that sophocarpine may affect the TLR4 signal pathway and inhibit liver fibrosis. We found that sophocarpine reduced the expression of TLR4 and Myd88, but not TLR2 or TLR9 which can also mediate the progression of liver fibrosis^[37,38]. During TLR4 signaling, the MyD88-dependent pathway mediates the up-regulation of inflammatory cytokines through activation of NF- κ B and mitogen-activated protein kinases (MAPK)^[39]. The MAPK signal pathway involves ERK, JNK, p38 and their phosphorylation in the pathogenesis of liver fibrosis^[40-42]. SiRNAs or selective inhibitors targeting these molecules reduced their expression or activity, and alleviated liver fibrosis *in vivo*^[13,43]. We found that the phosphorylation of ERK, JNK, p38 and IKK was significantly down-regulated after sophocarpine administration, which indicated that sophocarpine suppressed the MyD88-dependent TLR4 pathway and inhibited the activation of HSCs. The ERK/AP-1 pathway also induces c-Myc and Cyclin D1 expression which facilitates the proliferation of HSCs^[14,44,45]. Sophocarpine inhibited the phosphorylation of ERK, and then down-regulated the expression of Cyclin D1, which may contribute to the inhibitory effect of sophocarpine on the proliferation of HSCs.

In our study, sophocarpine exhibited potent control

of liver inflammation, which mainly contributed to the inhibition of hepatic fibrosis and HSC activation. For decades many researchers have investigated many stimuli, with the exception of inflammatory cytokines, that can drive the activation of HSCs including hepatocellular necrosis due to oxidative stress and apoptosis^[46-48]. The TLR4 and complements also play important roles in oxidative stress and hepatotoxicity, especially in the initiation of alcoholic steatohepatitis and fibrosis^[49,50]. It is likely that sophocarpine has an impact on suppressing oxidative stress and subsequently protecting hepatocytes from necrosis or apoptosis, which merits investigation. Moreover, as a monomer derived from matrine, although sophocarpine blocked the TLR4 pathway which was confirmed by our investigation, the direct target molecules of sophocarpine in the LPS-induced TLR4 pathway are unknown and require further study.

In summary, our investigation provides strong evidence for a suppressive effect of sophocarpine on hepatic fibrosis through inhibition of the activation and proliferation of HSCs. Moreover, sophocarpine exhibited potent blockage of the TLR4 signaling pathway and subsequently decreased the expression of pro-inflammatory and fibrotic cytokines. Based on the present study, sophocarpine may emerge as a novel option for the clinical therapy of chronic liver diseases.

COMMENTS

Background

The activation and proliferation of hepatic stellate cells (HSCs) are central events in the pathogenesis of hepatic fibrosis. However, there is no efficient treatment for chronic liver diseases in clinical practice.

Research frontiers

Previous studies have suggested that inhibition of the activation, proliferation and migration of HSCs may be an attractive anti-fibrotic therapy. Inflammatory cells and the inflammatory response are involved in driving the activation of HSCs through various inflammatory or fibrogenic mediators and pathways. Lipopolysaccharide toll-like receptor 4 (LPS-TLR4) signaling plays a critical role in regulating HSC activation and affects the risk of hepatic fibrosis progression.

Innovations and breakthroughs

Sophocarpine is a matrine-type quinolizidine alkaloid and exhibits a variety of pharmacological effects including anti-inflammatory, immuno-regulatory, anti-virus and anti-tumor. In this study, the authors demonstrated that sophocarpine administration ameliorated liver fibrosis by inhibiting the activation and proliferation of HSCs in rats. Moreover, blockage of the TLR4 signaling pathway contributed to the effects of sophocarpine by inhibiting the expression of fibrotic cytokines.

Applications

Due to the inhibitory effect of sophocarpine on hepatic fibrosis, it is likely that sophocarpine may have potential application in the clinical treatment of chronic liver diseases.

Terminology

HSCs are pericytes found in the perisinusoidal space (a small area between the sinusoids and hepatocytes) of the liver also known as the space of Disse. The stellate cell is the major cell type involved in liver fibrosis, which is the formation of scar tissue in response to liver damage.

Peer review

This study suggests that sophocarpine can alleviate liver fibrosis mainly through inhibiting the TLR4 signaling pathway. Sophocarpine might be present as a potential agent for chronic liver diseases.

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ISSN 1007-9327



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