Acupuncture combined with curcumin attenuates carbon tetrachloride-induced hepatic fibrosis in rats

Feng Zhang,¹ Jin Ma,¹ Yin Lu,¹,² Guan-Xia Ni,³ Chun-Yan Ni,¹ Xue-Jiao Zhang,¹ Xiao-Ping Zhang,¹ De-Song Kong,¹ Ai-Yun Wang,¹,² Wen-Xing Chen,¹,² Shi-Zhong Zheng¹,²

Abstract

Background Increasingly, studies demonstrate the effectiveness of acupuncture therapy against liver fibrosis. Curcumin is a natural product with anti-fibrotic effects, but has poor pharmacokinetic profiles. This study aimed to evaluate whether acupuncture combined with curcumin could more potently attenuate liver fibrosis in chemical intoxicated rats.

Methods 60 Sprague–Dawley male rats were randomly divided into control, model, sham, acupuncture, curcumin and combination therapy groups. During the establishment of fibrosis using carbon tetrachloride (CCl₄), acupuncture at LR3, LR14, BL18 and ST36 and/or curcumin treatment by mouth were performed simultaneously. After treatment, pathological indexes and histology for hepatic injury and fibrogenesis were detected. The expression of extracellular matrix (ECM) components was also determined.

Results Acupuncture combined with curcumin potently protected the liver from CCl₄-induced injury and fibrogenesis, as indicated by reduced levels of serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, hyaluronic acid, laminin and procollagen III. Combined use also led to significant liver histological improvements. Furthermore, combined use effectively inhibited ECM expression such as α-smooth muscle actin, fibronectin and α1(1) collagen.

Conclusions Acupuncture treatment could significantly enhance the anti-fibrotic efficacy of curcumin on CCl₄-induced hepatic fibrosis in rats in vivo, suggesting that a combination of acupuncture with curcumin may be exploited for the prevention of hepatic fibrosis.

Hepatic fibrosis is defined pathologically as excessive deposition of extracellular matrix (ECM) as a result of wound-healing responses to various kinds of chronic liver injury. It is thought to be a common pathology of nearly all forms of chronic liver disease progressing to cirrhosis.¹ A growing body of evidence has demonstrated that activation of hepatic stellate cells (HSC) plays a central role in liver fibrogenesis. HSC transdifferentiation into myofibroblasts represents the principal source of collagen-rich ECM components in the fibrotic liver.² Despite the increasing understanding towards the pathophysiology of hepatic fibrosis, there are still no universally accepted strategies or pharmacological agents for the treatment of liver fibrosis in current clinical contexts.³

Traditional Chinese medicines (TCM) as important complementary and alternative therapies are receiving considerable attention worldwide, because they are usually found to exhibit intriguing therapeutic effectiveness for some diseases. Curcumin is a naturally occurring polyphenolic compound derived from the rhizome of the plant Curcuma longa. Over the past few years, our laboratory has extensively investigated the anti-fibrotic properties of curcumin and the molecular mechanisms involved.⁴–⁷ Our data have indicated that curcumin inhibits HSC activation, induces HSC apoptosis and decreases collagen production, suggesting curcumin as a promising drug candidate for liver fibrosis. However, the pharmacokinetic profiles of curcumin are poor in terms of rapid metabolism and low bioavailability, which greatly weaken its clinical anti-fibrotic potential.⁸ On the other hand, acupuncture therapy traditionally used in oriental medicine shows therapeutic effects for certain diseases such as chronic pain, asthma, rhinitis and rheumatoid arthritis. Although the physiological nature of acupuncture points has not been clarified, there is clinical evidence of the apparent therapeutic benefits for hepatic fibrosis through electroacupuncture and acupuncture point injection. For example, acupuncture at ST36 Zusanli is mostly investigated in acupuncture therapy for liver diseases because stimulation of ST36 is believed in TCM to produce effects of activating blood circulation to dissipate blood stasis.⁹ Moreover, LR3 Taichong, LR14 Qimen and BL18 Ganshu are traditionally associated with microcirculation and digestive system functions, thus these three acupuncture points may have important implications in liver disease management.

Recently, combination therapy with acupuncture and medication as a practical strategy to treat diseases has gained increasing importance in both experimental and clini-
Effects of acupuncture and/or curcumin on liver index in rats with carbon tetrachloride (CCl₄)-induced hepatic fibrosis. Rats were grouped: group 1, vehicle control (no CCl₄, no treatment); group 2, model group (with CCl₄, no treatment); group 3, sham group (with CCl₄, non-acupuncture point acupuncture); group 4, acupuncture group (with CCl₄, acupuncture treatment); group 5, curcumin group (with CCl₄, curcumin treatment); and group 6, combination use group (with CCl₄, acupuncture plus curcumin treatment). Values are expressed as mean±SD (n=10/group). **p<0.01 versus the control group; *p<0.05 versus the model group.

MATERIALS

Animals
Male Sprague–Dawley rats (180–220 g body weight) used in this study were obtained from Shanghai Slaccas Laboratory Animals. All experimental procedures were approved by the institutional and local committee on the care and use of animals and all animals received humane care according to the National Institutes of Health (USA) guidelines. All rats were maintained under a 12 h light/dark cycle at a controlled temperature (25°C) with free access to food and tap water until the day of the experiment.

Experimental protocols
Sixty healthy male Sprague–Dawley rats were randomly assigned into the following groups: control, model, sham, acupuncture, curcumin and combination use (n=10/group). The liver fibrosis model for rats was established through repeated intraperitoneal injections with a mixture of CCl₄ and olive oil (1:1 (v/v), 1 ml/kg body weight) over a total period of 6 weeks. Rats in all groups except the control group were injected with olive oil solution of CCl₄ three times in the first week, and then two times every remaining week. Rats in the control group were injected with the same volume of saline. The treatments were carried out simultaneously with the establishment of the fibrosis model. Rats in the acupuncture group received acupuncture at LR3, LR14, BL18 and ST36 three times per week during the course of the experiment. Rats in the sham group were given similar manipulations at four non-point locations, ie, 0.5 cm laterally to LR14 and BL18, and a similar distance laterally to LR3 and ST36. Rats in the curcumin group were given curcumin by mouth at 200 mg/kg every day (6 days/week) for 6 weeks. Rats in the combination group received acupuncture and curcumin administration simultaneously according to the described methods. Rats in the model group were given saline of the same volume as curcumin every day (6 days/week) for 6 weeks.

Acupuncture protocols
Rats were kept in specially designed holders with their forelimbs exposed. Pairs of stainless-steel acupuncture needles (0.25 mm diameter; Nanjing, China) were inserted bilaterally into LR3, LR14 and BL18 to a depth of approximately 2 mm. The needles were rotated for 1 min in each acupuncture point and remained in situ for 15 min, as in a therapeutic procedure. Then, electroacupuncture at ST36 was performed bilaterally using a G-6850 electroacupuncture instrument that produced constant current square-wave electrical stimulation to stimulate the acupuncture points for 10 min with parameters of 50 Hz, 4 volts and 1–3 mA (increased gradually). The electrical intensity was just strong enough to elicit slight twitches of the hind limbs.

Measurement of hepatic function and injury
Forty-eight hours after the last treatment in the experiment, all rats were weighed and killed after being anaesthetised by an intraperitoneal injection of pentobarbital at 50 mg/kg. Blood was obtained from each rat through the common carotid artery. Livers were separated for calculating liver index according to the following formula: liver index (%)=weight of liver/weight of body. Blood samples underwent centrifugation at 7500g for 2 min to obtain plasma. Biochemical markers of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) in fresh sera (1 ml/sample) were analysed by a semi-automatic biochemical machine. Serum levels of hyaluronic acid (HA), laminin and procollagen III (PCIII) were detected using ELISA.

Histopathological analysis
A portion of liver was fixed in 10% phosphate-buffered formaldehyde for over 2 h. After washing in phosphate-buffered saline, the fixed tissues were dehydrated in graded ethanol and embedded in paraffin. Paraffin sections of 4–6
μm were sliced using a sliding microtome, and paraffin was removed using xylene. Tissue sections were stained with H&E reagent for light microscopy. Using randomly selected high-power fields, the histopathology was evaluated by a pathologist in a blinded fashion.

**Western blot analysis**

Liver extracts were prepared from pieces of liver tissue excised from every rat in the rat model using ice-cold radioimmunoprecipitation assay lysis buffer containing 150 mM NaCl, 50 mM Tris, 0.1% sodium dodecyl sulphate, 1% Nonidet P-40, and 0.5% deoxycholate supplemented with protease inhibitors. Protein concentrations were determined using the BCA protein assay kit according to the protocol provided by the manufacturer (Pierce Chemical, Rockford, Illinois, USA). Forty micrograms of total protein was subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis, transferred to a polyvinylidene fluoride membrane (Millipore, Burlington, Massachusetts, USA), and blocked with 5% skim milk in Tris-buffered saline containing 0.1% tween. Target proteins were respectively detected by primary antibodies against α-smooth muscle actin (α-SMA), α1(I) collagen and fibronectin and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA). β-Actin was probed as an internal control. Protein bands were visualised using chemiluminescence reagent (Amersham, Chalfont St Giles, Bucks, UK). The densities of bands were normalised with the internal invariant control β-actin. The level of target protein bands was densitometrically determined by using Quantity Ones 4.4.1 (Bio-Rad, Hercules, CA, USA). The variation in the density was expressed as fold changes compared with the control in the blot.

**RNA isolation and quantitative real-time PCR**

Total RNA was extracted from frozen liver tissues using TRI reagent according to the protocol provided by the manufacturer (Sigma-Aldrich, St. Louis, MO, USA). Total RNA (1 μg) was treated with DNase I to eliminate genomic DNA contamination, followed by synthesis of the first strand using the reverse transcription system (Promega, Madison, Wisconsin, USA). Reverse transcription was carried out as follows: 42°C for 30 min, 95°C for 5 min and 4°C for 5 min (one cycle). Real-time PCR was performed in 25 μl of reaction solution containing 12.5 μl 2× iQSYBR Green Supermix (Bio-Rad Laboratories, Hercules, California, USA), 300 nM primers and complementary DNA. The cycles for PCR were as follows: 95°C for 7 min, 40 cycles of 95°C for 20 s, 54°C for 30 s and 72°C for 30 s. Melting curves were determined by heat-denaturing PCR products over a 35°C temperature gradient at 0.2°C/s from 60 to 95°C. Fold changes in the messenger RNA levels of target genes related to the invariant control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were calculated as described. The following primers were used in real-time PCR: α-SMA: (F) 5′–CCGACCAGAATGCAGAAGGA–3′, (R) 5′–ACAGAGTATTTGCGCTCCGGA–3′; α1(I)
collagen: (F) 5′–CCTCAAGGGCTCACAACGAG–3′, (R) 5′–TCAATCAGTGTCGCCC–3′; fibronectin: (F) 5′–TGTCACCAAGCAGCCACAG–3′, (R) 5′–CTGATTGTTCAGTGGA–3′; and GAPDH: (F) 5′–GCCCTCCCTCTGGAAAGCTGTG–3′, (R) 5′–CCGCCTGCTTCACCACCTTCT–3′. mRNA levels were expressed as fold changes after normalisation with GAPDH.

**Statistical analysis**
The data were analysed using SPSS v.11.5 and presented as mean with SD of N detections, where N represents the number of animals in each group. Differences between means were evaluated using an unpaired two-sided Student’s t test. A p value of less than 0.05 was considered to be significant.

**RESULTS**

**Effects of acupuncture and/or curcumin on liver index and serum ALT, AST, ALP activities in rats with CCl₄-induced hepatic fibrosis**

During hepatic fibrosis, the ratio between weight of the liver and weight of the body, known as the liver index, is elevated. This physical index can reflect the extent of liver injury. In the CCl₄-induced hepatic fibrosis here, the liver index in the modelf group was significantly enhanced compared with that of the control group (p<0.01). Although the liver index in the sham and acupuncture groups was lower than that of the model group, there were no significant differences. However, curcumin treatment and combination use significantly decreased the liver index compared with that of the model group (p<0.05). No significance was observed between the curcumin group and the combination group, but the liver index in the combination group was the lowest (figure 1). Moreover, serum levels of ALT, AST and ALP closely correlate with hepatic functions under pathological conditions. Our results showed that serum levels of these key markers in the model group were greatly increased compared with those of the control group (p<0.05 or 0.01). However, acupuncture, curcumin and their combination showed inhibitory effects on the growing levels of ALT, AST and ALP to different extents, and thereby were expected to ameliorate the injuries. There was no significant difference between the sham group and the model group. The combination use produced the strongest inhibitory effects (figure 2). Collectively, these data demonstrate that acupuncture, curcumin and combination treatment could ameliorate liver functions that were impaired during fibrogenesis, and in particular, the beneficial effects of combination use were superior to the effects produced by acupuncture and curcumin used alone.

**Effects of acupuncture and/or curcumin on serum HA, laminin, PCIII and liver histology in rats with CCl₄-induced hepatic fibrosis**

Serum levels of HA, laminin and PCIII are important diagnostic biomarkers for liver fibrosis in the clinic. It

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**Figure 3** Effects of acupuncture and/or curcumin on serum levels of hyaluronic acid (HA), laminin (LN) and procollagen III (PCIII) in rats with carbon tetrachloride (CCl₄)-induced hepatic fibrosis. Rats were grouped: group 1, vehicle control (no CCl₄, no treatment); group 2, model group (with CCl₄, no treatment); group 3, sham group (with CCl₄, non-acupuncture point acupuncture); group 4, acupuncture group (with CCl₄, acupuncture treatment); group 5, curcumin group (with CCl₄, curcumin treatment); and group 6, combination use group (with CCl₄, acupuncture plus curcumin treatment). Rat blood was obtained and underwent centrifugation at 7500g for 2 min to obtain plasma. Levels of HA, LN and PCIII in fresh sera were analysed using an ELISA method. Values are expressed as mean±SD (n=10/group). *p<0.05 versus the control group; *p<0.05 versus the model group.
is observed that the patients with liver fibrosis have a dramatic increase in serum levels of HA, laminin and PCIII.12 13  These markers were thus assessed as an indication of the severity of fibrosis. In the model group we observed a considerable increase in serum levels of these indices compared with those of the control group (p<0.05). Acupuncture and curcumin treatment could independently suppress the increase in HA, laminin and PCIII levels. The most favourable inhibitory effects were observed in the combination group represented by the lowest serum contents of HA, laminin and PCIII (figure 3).

Furthermore, liver tissues of rats were analysed histopathologically. The results showed that in normal rats the hepatic lobules maintained the normal structure in an integrated shape and the hepatocytes were in a regular histological arrangement. Nevertheless, hepatocyte degeneration (mainly steatosis), necrosis and inflammatory cell infiltration were observed in the other five groups to different extents, and the pathological situation in the model group was most serious, indicating that hepatic fibrogenesis occurred in CCl4-intoxicated rats. These fibrogenic manifestations included fibrous tissue proliferation, fibrous septa formation and especially pseudolobule formation observed in the model group. The pathological changes in the sham group were similar to those of the model group. However, the pathological slices taken from CCl4-exposed rats treated with acupuncture only, curcumin only, or acupuncture plus curcumin demonstrated attenuated fibrosis and ameliorated hepatocyte injury. Notably, combination use produced the mildest pathological changes of hepatic fibrogenesis (figure 4). Taken together, these results suggest that acupuncture manipulation could effectively enhance the hepatic protection of curcumin implicated in fibrosis therapy.

Effects of acupuncture and/or curcumin on liver ECM expression in rats with CCl4-induced hepatic fibrosis

To assess the impact of acupuncture and/or curcumin on hepatic fibrogenesis caused by CCl4, ECM expression in liver tissues was evaluated at protein and gene levels. α-SMA is the unique marker for activated HSC. Fibronectin and α1(I) collagen are the main components of ECM deposited in the liver during fibrogenesis. Our results demonstrated that expressions of the three markers in the model group were significantly higher than those of the control group, especially mRNA expression (all p<0.01). No apparent decrease was observed in the sham group, whereas acupuncture treatment led to a marked reduction in ECM expression, especially fibronectin mRNA expression (p<0.05), suggesting the effectiveness of acupuncture for the prevention of liver fibrosis by reducing ECM production. Curcumin alone was also effective in reducing ECM expression. Moreover, combination use produced the strongest inhibitory effects on the gene expression of ECM (figure 5). Noteworthy, α-SMA mRNA expression was significantly reduced by combination use (p<0.01), indicating that acupuncture could enhance curcumin’s ability to attenuate HSC activation. All together, these results demonstrate that acupuncture not only had preventive effects on liver fibrosis through attenuating HSC activation and reducing ECM expression, but also remarkably enhanced curcumin’s antifibrotic activity.

Figure 4  Histopathological features of liver sections of rats with carbon tetrachloride (CCl4)-induced hepatic fibrosis. Rats were grouped: group 1, vehicle control (no CCl4, no treatment); group 2, model group (with CCl4, no treatment); group 3, sham group (with CCl4, non-acupuncture point acupuncture); group 4, acupuncture group (with CCl4, acupuncture treatment); group 5, curcumin group (with CCl4, curcumin treatment); and group 6, combination use group (with CCl4, acupuncture plus curcumin treatment). (A) Hepatocytes were normally arranged in sinusoides. (B) Severe necrosis of hepatocytes and formation of pseudolobule. (C) Formation of fibronodules. (D) Moderate swelling of hepatocytes, no evidence of fibronodular formation. (E) Mild swelling, occasional hepatocyte congestion. (F) Mild swelling, no hepatocyte congestion. The features were typical in more than three rats in each group. Sections were stained with H&E. ×200.
DISCUSSION

In TCM hepatic fibrosis is considered to belong to the scope of hypochondriac pain and accumulation. Due to the lack of other preventive measures and effective therapies, acupuncture or the combination use of acupuncture and drugs has been practised in the current clinical context for a wide range of chronic liver diseases including fatty liver, liver fibrosis and cirrhosis, and the therapeutic effectiveness has been preliminarily established. For example, in a randomised trial enrolling 158 patients with fatty liver, treatment with acupuncture and ‘Quzhi Sanwei Decoction’ for 6 months significantly improved clinical symptoms and reduced blood lipid levels, exhibiting therapeutic efficacy for fatty liver diseases. Similar results were also obtained in another clinical report. However, we still need more experimental investigations to confirm the preventive efficacy of acupuncture, and to explore the potential of combining acupuncture with drugs for hepatic fibrosis. Our present investigations could meet these needs in the hope of providing some rationale for clinical practice.

We used CCl₄ as the inducer of experimental fibrosis in rats, because CCl₄ causes hepatic injury, including hepatocytic necrosis, steatosis and inflammation. Low-dose and long-term administration of CCl₄ induces hepatic fibrogenesis, which largely imitates hepatic fibrosis in human diseases. A CCl₄ rat model was established to evaluate the effects of therapies on protecting the liver from injury and fibrogenesis, but the liver can rapidly recover from CCl₄-caused injury. Therefore, rats were killed within 48 h after the last treatment, which provided a better chance to evaluate the CCl₄-caused hepatic injury and to assess the protective effects on the liver. This model seemed to be a useful strategy for animal research on acupuncture treatment, which could provide experimental findings that could be extrapolated to clinical practice. Curcumin is a naturally occurring product with a range of pharmacological activities. Extensive investigations have demonstrated that curcumin is a potent antifibrotic agent both in vitro and in vivo, which is attributed to its inhibition of HSC activation and collagen production. These findings have made curcumin a promising candidate for being developed as an antifibrotic agent. However, this compound has some shortcomings in its pharmacokinetic profile including low bioavailability, internal instability and a short half-life, which weaken the therapeutic applications of curcumin. In the present study, when curcumin treatment and acupuncture manipulation were used together, significantly enhanced antifibrotic effects were observed compared with the effects produced by single use, including a much lower liver index, more decreased serum levels of ALT, AST, ALP, HA, laminin and PCIII, and more ameliorated hepatocyte injury, strongly suggesting the synergistic effects of acupuncture on curcumin inhibition of hepatic fibrosis. Histopathological analysis further confirmed these observations, ie, the in vivo antifibrotic effects of curcumin could apparently be enhanced. Moreover, acupuncture enhanced curcumin’s capability to inhibit HSC activation and ECM production. These data could support our initial hypothesis that acupuncture could be a potential therapy for hepatic fibrosis.

Figure 5 Effects of acupuncture and/or curcumin on expressions of α-smooth muscle actin (α-SMA), fibronectin and α₁(I) collagen in liver tissues. Rats were grouped: group 1, vehicle control (no carbon tetrachloride (CCl₄), no treatment); group 2, model group (with CCl₄, no treatment); group 3, sham group (with CCl₄, non-acupuncture point acupuncture); group 4, acupuncture group (with CCl₄, acupuncture treatment); group 5, curcumin group (with CCl₄, curcumin treatment); and group 6, combination use group (with CCl₄, acupuncture plus curcumin treatment). (A) Western blot analyses of the protein abundance of α-SMA, fibronectin and α₁(I) collagen, and the quantitative analysis. β-Actin was used as an invariant control for equal loading. Representatives of 10 independent detections are shown. (B) The steady-state levels of mRNA in the liver were analysed by real-time PCR assays. Glyceraldehyde-3-phosphate dehydrogenase was used as an invariant internal control for calculating mRNA fold changes. Values are expressed as mean±SD. (n=10/group). ##p<0.01 versus the control group; *p<0.05, **p<0.01 versus the model group.
hypothesis and provide evidence for the usefulness of acupuncture combined with drugs to prevent liver fibrosis. Our data confirmed the synergistic effects of acupuncture and curcumin. Interestingly, we found that acupuncture at the selected acupuncture points also produced preventive effects on liver fibrogenesis, although they were not as potent as those produced by curcumin or combination use. These results were consistent with previous reports that point-injection therapy could reduce collagen expression in fibrotic rats and that electroacupuncture could attenuate the selected acupuncture points also produced preventive effects on liver fibrogenesis. As a result, acupuncture manipulation can enhance the efficacy of internal drug therapy. Increasing knowledge reveals that the underlying mechanisms for the synergistic action are conceivably attributed to the stimulation of neuroendocrine regulation and enhanced excitability of target organs, with improved microcirculation, leading to increased drug content per unit time. At the molecular and gene levels, acupuncture manipulation is likely to modulate the expression of cell surface receptors, to which drugs bind directly, thus the drug’s efficacy is enhanced as a result. These mechanisms could presumably explain the enhanced antiﬁbrotic efficacy produced by the combination of curcumin and acupuncture to prevent rat hepatic fibrosis induced by CCl4, shown in our studies. Our results clearly suggest that the pharmacokinetic shortcomings affecting curcumin efficacy could be compensated by acupuncture management. This could enlarge the potential of curcumin for hepatic ﬁbrosis. Studies aimed to elucidate the molecular mechanisms contributing to this combination of acupuncture and curcumin are in process in our laboratory.

CONCLUSIONS
In summary, the results from this study supported our initial hypothesis and demonstrated that the combination use of acupuncture and curcumin protected the rat liver from CCl4-caused injury and ﬁbrogenesis in vivo by improving hepatic function, reducing ECM expression and inhibiting HSC activation. Acupuncture could enhance curcumin’s antiﬁbrotic effects. These ﬁndings provided novel insights into the preventive strategies for combating hepatic ﬁbrosis. Our data suggest that acupuncture and its combination with curcumin should be investigated further as a possible adjuvant therapy in patients with liver ﬁbrosis.

Contributors FZ and JM were the major performers of the experiments and FZ drafted the manuscript. CYN, XJZ, XD and DSK were actively involved in the studies including therapy experiments, detection of indices, data analysis, etc. AWY and WXC provided essential technical support all through the experiments and gave helpful suggestions. GXN and YL provided necessary ﬁnancial support and were partly responsible for the project. SZZ was the principal designer of the studies and was responsible for all aspects of this work. SZZ also critically revised the manuscript.

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Competing interests None.

Ethics approval This study was conducted with the approval of Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China. All experimental procedures were approved by the institutional and local committee on the care and use of animals.

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