Original Article

Xiongshao decoction ameliorates CCl₄-induced liver fibrosis via inhibiting Fas associated death domain (FADD)-caspase pathways

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Abstract: Liver fibrosis is a global health problem characterized by abnormal deposits of extracellular matrix (ECM) proteins, including collagen fibers and activation of hepatic stellate cells (HSCs). Xiongshao decoction is an effective prescription of Professor Jiuzhang Men in treating liver fibrosis, approved by the State Administration of Traditional Chinese Medicine of the People's Republic of China. The present study explores the mechanisms of Xiongshao decoction as an anti-fibrotic agent in an in vivo carbon tetrachloride (CCl₄)-induced liver fibrosis model. Liver fibrosis was induced in Sprague Dawley (SD) rats by CCl₄ injection for 6 weeks. Xiongshao administration was divided into two groups: Xiongshao decoction prevention group (during CCl₄ induced liver fibrosis, rats treated with Xiongshao decoction 8 g/kg weight every day by oral administration) and Xiongshao decoction treatment group (after CCl₄ induced liver fibrosis, rats were treated with Xiongshao decoction 8 g/kg weight by oral administration every day for 4 weeks). Histopathology revealed that CCl₄ treatment results in significant fibrosis, while Xiongshao decoction treatment induced significantly reversed fibrosis. Key markers for fibrogenesis α smooth muscle actin (α-SMA), 5-lipoxygenase (5-LO), tissue inhibition of metalloproteinase 1 (TIMP1), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), and Fas associated death domain (FADD)-caspase pathway related proteins (FADD, caspase 8 and Cyt-c) were also markedly attenuated by Xiongshao decoction. These results suggest Xiongshao decoction as an anti-fibrotic agent effective intervention against liver fibrosis via regulation of FADD-caspase signal pathways.

Keywords: Xiongshao decoction, liver fibrosis, tumor necrosis factor-related apoptosis-inducing ligand, FADD-caspase signal pathways

Introduction

The liver is a dynamic organ with a variety of unique features, including self-renewal, allowing it to be exposed to daily ingested nutrients, xenobiotic metabolism, and gut-derived endobiotics without adverse consequences [1]. The liver will regenerate after damage. Fibrosis is a wound healing response in which damaged regions manifest abnormal deposits of extracellular matrix (ECM) proteins, including collagen fibers and causing tissue scarring [2, 3]. When damage and inflammation of the liver are persistent and progressive, normal regeneration is disrupted, leading to fibrosis. Progressive liver fibrosis results in cirrhosis where liver cells cannot function properly due to the formation of fibrous scars and regenerative nodules and decreased blood supply. Hepatic stellate cells (HSCs) play a key role in the development of liver fibrosis and a variety of chronic liver diseases [4]. HSCs, as a precursor of activated myofibroblasts, promote the production of EMC proteins during liver fibrosis [5]. Quiescent HSCs compound low levels of matrix proteins. However, as a result of damage, HSCs proliferate and transform to a myofibroblast-like phenotypes when they are activated [6]. Both in injured livers and in vitro cultures, HSCs undergo a gradual phenotypic change from non-proliferating retinoid-storing cells to a proliferating, fat, and retinoid loosing phenotype, inducing expression of α-smooth-muscle actin (α-SMA) as a characteristic cytoskeletal protein or receiving novel characteristics [7]. Therefore, remission of liver fibrosis might be associated with the reversal of activated HSCs to quiescent phenotypes or through changing the bal-
Xiongshao decoction is an effective prescription of Professor Jiuzhang Men in treating liver fibrosis. It is composed of Radix Aconiti Lateralis Preparata, Paeonia lactiflora Pall, Codonopsis pilosula, Atractylodes macrocephala, Rhizoma Zingiberis, and Poria cocos [8]. Several studies have shown that Xiongshao decoction can protect liver cells with significant anti liver fibrosis effects [9, 10]. This study examined the effects of Xiongshao decoction in an acute liver fibrosis. Carbon tetrachloride (CCl4) specifically targets the liver via producing a free radical, resulting in hydropic degeneration, lipid peroxidation, and steatosis [11, 12]. Therefore, CCl4-induced liver fibrosis in a rat model was selected for this study. This study showed that Xiongshao decoction has an alleviation effect on liver fibrosis, significantly decreasing apoptosis of HSCs. This study also demonstrated that Xiongshao decoction exerts effects by inhibition of Fas associated death domain (FADD)-caspase signaling pathways. Therefore, the present study provides the first evidence of therapeutic potential mechanisms of Xiongshao decoction for treatment of liver fibrosis.

Materials and methods

Experimental animals and liver fibrosis model

Male Sprague Dawley (SD) rats, weighing 250 ± 20 g, were obtained from Beijing Vital River Laboratory Animal Technology and housed in a 12-hour dark/light cycle. They were allowed to acclimate to the facility for two weeks before the experiment. All rats were randomly divided into a normal control group, CCl4 model group, Xiongshao decoction prevention group, CCl4 model control group, and Xiongshao decoction treatment group. Six rats in the normal control group were injected intraperitoneally with 0.02 ml/kg olive oil, twice weekly, with free access to standard chow and water for 6 weeks. A total of 24 rats were injected intraperitoneally with 0.02 ml/kg sterile CCl4 in a 1:1 ratio with olive oil twice weekly. Rats were fed with high-fat and low-protein food (corn flour added with 0.5% cholesterol and added 20% lard in the corn until 2 weeks later) and drinks with 30% alcohol. For the CCl4 model group, 6 rats were treated with CCl4 for 6 weeks. For the Xiongshao decoction treatment group, during injection with CCl4 for 6 weeks, 6 rats were give lavage treatment with Xiongshao decoction each day. In the CCl4 model control group, 6 rats, after treatment with CCl4 and high-fat and low-protein food for 6 weeks, continued a normal diet for 4 weeks. For the Xiongshao decoction treatment group, 6 rats, after treatment with CCl4 and high-fat and low-protein food for 6 weeks, continued to Xiongshao decoction treatment for 4 weeks. The concentration of Xiongshao decoction was 8 g/kg, 7 times the adult clinical measurement.

Sample collection

Livers were collected from rats anesthetized with an intramuscular injection of 50 mg/kg body weight ketamine and washed twice with phosphate buffered saline (PBS). For hematoxylin-eosin (HE) staining and immunohistochemical staining, liver samples were fixed with 4% paraformaldehyde. The remainder were snap-frozen in liquid nitrogen and stored at -80°C in preparation for biochemical analysis.

HE-staining

Rat liver samples in each group were placed in 2 mL of 4% paraformaldehyde. After 24 hours, the fixative was aspirated and replaced with fresh 4% paraformaldehyde. All liver samples were embedded in paraffin wax and sections were stained with hematoxylin-eosin.

Immunohistochemical staining

Glass slides were previously incubated in an oven at 65°C for 1 hour. Paraffin-embedded sections were dewaxed, rehydrated, and treated for quenching of endogenous peroxidase activity at room temperature. Sections were incubated with rabbit anti-rat monoclonal antibody of α-SMA (ab32575, Abcam, UK; 1:100 dilution) or rabbit anti rat monoclonal antibody of 5-lipoxygenase (5-LO) (ab169755, Abcam, UK; 1:100 dilution) overnight at 4°C. Spatial localization of α-SMA or 5-LO were visualized by incubating with rat IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Biotechne, USA) for 1 hour at room temperature. Next, 3,3’diaminobenzidine tetrahydrochloride (Sigma, USA) chromogenic reagent was added dropwise. Afterward, sections were rinsed with PBS, counterstained with hematoxylin for 3 minutes, dehydrated with graded ethanol and xylene, and mounted with Entellan.
were observed by light microscopy (CX41, OLYMPUS, Japan). Six visual fields were counted and mean value was taken as positive cells.

Western blot analysis

Protein levels of α-SMA, 5-LO, tissue inhibitor of metalloproteinase 1 (TIMP1), matrix metalloproteinase 13 (MMP13) tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), FADD, caspase 8, and Cyt-c were analyzed by Western blot. Liver samples were washed twice with PBS and homogenized in RIPA lysis buffer (Beyotime, China) containing protease inhibitor at 4°C. They were centrifuged at 12000 g for 15 minutes. Concentration of the proteins was measured by BCA kit (Beyotime, China). Next, 30 μg proteins were separated by 10% SDS-polyacrylamide gel and transferred onto PVDF membranes (Millipore, USA). Membranes were blocked with 5% skim milk for 2 hours at room temperature in Tris-buffered saline. Membranes were then incubated with primary antibodies overnight at 4°C. Anti-GAPDH antibody was selected as internal reference. Next, the membranes were washed with Tris-buffered saline and incubated in biotinylated goat IgG secondary antibody for 2 hours at room temperature. Immunoreactivity was visualized by colorimetric reaction using ECL substrate buffer (Millipore, Massachusetts, USA). Membranes were scanned with Gel Doz EZ imager (Bio-rad, USA).

Statistical analysis

Statistical differences of experimental data were analyzed by Dunnett’s one-way analysis of variance (ANOVA) using SPSS 19.0 software package. Differences are considered statistically significant at P<0.05 and very significant at P<0.01. All results are expressed as mean ± S.E.M.

Results

Xiongshao decoction treatment attenuates CCl4-induced liver injury

The effects of Xiongshao decoction in mitigating CCl4-induced fibrogenesis were corroborated with histopathology analysis. Representative HE-stained liver sections are presented in Figure 1. Results show that CCl4 treatment led to necrosis and fibrotic septa between parenchymal. Xiongshao decoction administration improved histological parameters with a significant reduction in micro- and macrovesicular steatosis.

Xiongshao decoction attenuates CCl4-induced α-SMA and 5-LO activation

Previous studies have indicated that increased expression of α-SMA is a marker of activated HSCs [13, 14]. Arachidonic acid (AA) metabolites play an important role in the activation of...
HSCs and formation of hepatic fibrosis, while 5-LO is involved in the early inflammatory response and activation of HSCs as an important AA metabolic enzyme [15, 16]. In the present study, results revealed that α-SMA staining (Figure 2) and 5-LO staining (Figure 3) were markedly increased in the CCl$_4$ model group, compared to the normal control group, whereas it was significantly attenuated in liver sections obtained from Xiongshao decoction treated rats. Western blot analysis of α-SMA and 5-LO from liver tissues obtained from different groups were also performed. Results were shown in Figure 4. Results demonstrated significant reduction of α-SMA and 5-LO protein expression levels in Xiongshao decoction treated rats, compared to CCl$_4$ treated rats.

**Xiongshao decoction reduces TIPM1 expression**

It has been reported that proteolytic activity of collagen is regulated by matrix metalloproteinase (MMPs), which are in turn regulated by their endogenous inhibitors, TIMP1 [17]. In the formation of liver fibrosis, expression of TIMP1 is increased significantly and has been shown to be a key in preventing myofibroblast apoptosis. In the present study, TIMP1 protein expression was evaluated, according to Western blot. As shown in Figure 5, TIMP1 protein levels were significantly elevated in CCl$_4$ treated rats, compared to the control group. After Xiongshao decoction administration, TIMP1 protein levels were attenuated significantly, compared with CCl$_4$ treated rats.

MMP13 is a key protease for type I collagen. This study also examined the protein levels of MMP13. CCl$_4$ induced expression of MMP13, while Xiongshao decoction treatment further induced MMP13 expression (Figure 5). It was speculated that increased expression of MMP13 might have resulted from reduced TIMP1 expression, indicating that Xiongshao decoction can induce fibrillar collagen degradation in rats.

**Xiongshao decoction inhibits activation of FADD signal pathways**

During liver fibrosis recovery, Kupffer cells and activated HSCs can regulate expression of TRAIL to induce apoptosis of activation HSCs. TRAIL receptors recruit the adapter protein FADD to cell membranes, followed by activation of the caspase cascade [18]. To determine the mechanisms of Xiongshao decoction on FADD-caspase signal pathways, protein levels of TRAIL, FADD, caspase 8, and Cyt-c were detected. Western blot results indicated that CCl$_4$ treatment significantly elevated expression of
TRAIL, FADD, caspase 8, and Cyt-c, compared to the control group. This indicated that FADD-caspase pathways were activated by CCl4 (Figure 6). Interestingly, Xiongshao decoction treatment significantly attenuates CCl4-induced activation of FADD-caspase pathways.

Discussion

The present study indicates that a prescription approved by the State Administration of Traditional Chinese Medicine of the People's Republic of China, Xiongshao decoction, can reverse CCl4-induced liver injury and fibrosis through multiple anti-fibrogenic mechanisms. Results suggested that Xiongshao decoction dramatically inhibited expression levels of α-SMA, 5-LO, TIMP1, TRAIL, FADD, caspase 8, and Cyt-c, but induced expression of MMP 13. Mechanisms for these protective effects might be that Xiongshao decoction effectively reverses HSCs activation by blocking FADD-caspase...
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pathways or shifting HSC phenotypes into reversion, as described by Brenner et al. [19] in lineage tracing experiments [20].

CCl4-induced liver fibrosis, as an in vivo fibrosis model, has been wildly used in numerous studies [21]. Bioactivation of CCl4 was induced by cytochrome P450 in the liver, following the release of free radical CCl3 which leads to hepatocyte necrosis, promoting an inflammatory response in the liver [22]. In the present study, histological analysis showed that rats treated with CCl4 led to hepatocyte necrosis and development of nodules and septa. However, by using Xiongshao decoction prevention for 6 weeks or treatment for 4 weeks, this study convincingly indicated that administration of Xiongshao decoction significantly improved hepatic histology and remarkably suppressed liver fibrosis. These results suggested, histologically, that thickness of bridging between fibrotic septa was diminished.

In chronic liver injuries, HSCs, as the major source of ECM in the liver, experience a process named trans-differentiation, from a resting vitamin A storing phenotype to a myofibroblast like phenotype characterized by elevated

Figure 5. Protein expression levels of TIMP1 and MMP13 in rat liver tissues. Bands were quantified using Quantity One 5.0. All values are expressed as mean ± S.D. (n=3). **P<0.01 versus normal control group; ^P<0.05 or ^^P<0.01 versus CCl4 model group; ###P<0.01 versus CCl4 model control group.

Figure 6. Protein expression levels of TRAIL, FADD, caspase 8, and Cyt-c in rat liver tissues. Bands were quantified using Quantity One 5.0. All values are expressed as mean ± S.D. (n=3). **P<0.01 versus normal control group; ##P<0.01 versus CCl4 model group; ▲▲P<0.01 versus CCl4 model control group.
expression of α-SMA [23]. Expression of α-SMA in liver tissue is an important indicator of HSCs activation, indicating that inhibition of expression of α-SMA might be an important marker for remission of liver fibrosis [24]. The present study demonstrated that expression of α-SMA in rat liver tissues increased significantly after CCl₄ treatment, whereas Xiongshao decoction administration remarkably reduced α-SMA protein levels.

It has been reported that TIMP1, as an endogenous inhibitor, can regulate expression of MMPs, resulting in degradation of collagen and extracellular matrix proteins [25, 26]. In this study, expression of TIMP1 and MMP13 in rat liver tissue was regulated by Xiongshao decoction. Expression of MMP13 in rat liver tissue was induced by CCl₄ treatment, while Xiongshao decoction resulted in significantly increased expression of MMP13. Activation of HSCs induces the synthesis and release of large amounts of ECM proteins and TIMPs, leading to degradation of ECM proteins and collagen, eventually resulting in liver fibrosis [27, 28]. Based on these observations and reports, it was inferred that Xiongshao decoctions can induce remission of liver fibrosis by regulating expression of TIMP1 and MMP13.

There have been numerous reports suggesting that TRAIL receptors recruit the adapter protein FADD to cell membranes and activate caspase cascades [18, 29, 30]. FADD is required for the recruitment and activation of caspase 8, which can induce executioner caspase, directly or indirectly, via mitochondrial involvement, as in hepatocytes [31, 32]. TRAIL has recently been reported to be a critical factor in liver disease [33]. TRAIL receptors 1 and 2 protein expression was observed in human activation HSCs [34]. In addition, TRAIL has been shown to potentiate cytotoxic Fas signaling in the liver [35]. In the present study, it was found that protein levels of TRAIL, FADD, caspase 8, and Cyt-c in rat liver tissues were increased significantly after CCl₄ administration, while Xiongshao decoction significantly attenuated protein expression in rat liver tissues. It has been reported that blocking the activation of death receptors, such asTRAIL, FADD, and TNF-R, along with their associated signaling cascades, might result in decreased inflammation in the presence of steatosis [36]. Present data suggests that administration of Xiongshao decoction can induce remission of CCl₄-induced rat liver fibrosis via inhibiting activation of FADD signal pathways.

Conclusion

Results of the present study confirm that Xiongshao decoction treatment attenuates CCl₄-induced liver fibrosis in rats. This study also demonstrated that Xiongshao decoction induced expression of MMP 13 and inhibited activation of FADD in the liver. These results suggest that FADD signal pathways are involved in Xiongshao decoction's anti-fibrogenic properties in the treatment of liver fibrosis.

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Disclosure of conflict of interest

None.

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