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Original article

Antifibrotic effects of Fraxetin on carbon tetrachloride-induced liver fibrosis by targeting NF-κB/IκBα, MAPKs and Bcl-2/Bax pathways

Bin Wu[1], Rong Wang[1], Shengnan Li, Yuanyuan Wang, Fuxing Song, Yanqiu Gu, Yongfang Yuan*

Department of Pharmacy, Shanghai 9th People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

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ABSTRACT

Background: Liver fibrosis is a chronic lesion which ultimately results in cirrhosis and possible death. Although the high incidence and lethality, few therapies are effective for liver fibrosis. Fraxetin (7,8-dihydroxy-6-methoxy coumarin), a natural product extracted from cortex fraxini, has exhibited a significant hepatoprotective and anti-fibrotic properties. However, the underlying mechanism of the anti-hepatic fibrotic property remains unknown.

Methods: 48 Male Sprague Dawley rats were divided into four groups at random which were named as normal group, model group, fraxetin 25 mg/kg and 50 mg/kg group. The experimental model of liver fibrosis was founded by carbon tetrachloride (CCl_4) rats which were simultaneously treated with fraxetin (25 mg/kg or 50 mg/kg). Normal groups received equal volumes of saline and peanut oil.

Results: Results showed that fraxetin ameliorated CCl_4 induced liver damage and fibrosis. Furthermore, histopathology examinations revealed that fraxetin improved the morphology and alleviated collagen deposition in fibrotic liver. Fraxetin inhibited inflammation and hepatocytes apoptosis by modulating the NF-κB/IκBα, MAPKs and Bcl-2/Bax signaling pathways.

Conclusion: Our findings indicate that fraxetin is effective in preventing liver fibrosis through inhibiting inflammation and hepatocytes apoptosis which is associated with regulating NF-κB/IκBα, MAPKs and Bcl-2/Bax signaling pathways in rats.

Introduction

Liver fibrosis attributed to the impact that body spontaneously adapt to wound healing response initiate by multifarious chronic stimuli such as ethanol, cholestasis, infectious and metabolic diseases [1,2]. In the absence of clear and effective treatments, liver fibrosis will deteriorate to liver failure, cirrhosis and eventually to cancer causing morbidity and mortality [3]. Evidence indicate that unlike cirrhosis, liver fibrosis, a precursor to liver cirrhosis, can be completely reversed as liver injury subsides [4]. Hence, the key to curing liver fibrosis is to prevent and reverse the evolutionary process before it can progress to hepatoma as soon as possible. Although our understanding of the pathogenesis of liver fibrosis has improved, therapeutically only a few drugs have been found to be effective in patients with this disease. Hence, there exists an urgent to find novel and effective anti-fibrotic therapeutic strategies. In the past few years, an increasing number of researchers have paid more attentions on traditional Chinese medicines (TCMs) because of their superior efficacy, lower toxicity and fewer side effects [5–7]. Novel anti-fibrotic components from TCMs, therefore, present an attractive and promising drug candidate for liver fibrosis [8].

Fraxetin, a natural coumarin derivative (Fig. 1) extracted from cortex fraxini, has been testified to possess a wide breadth of pharmacological properties containing anti-oxidative, anti-inflammatory, anti-carcinogenic and neuroprotective which give it better prospects for drug development [9–14]. Additionally, many studies have also verified that fraxetin possessed a conspicuous ability to act as an anti-liver fibrotic and hepato-protective agent [15,16]. However, the underlying molecular mechanisms have not been yet expounded.

Inflammation is a crucial response to liver injury that ultimately results in fibrosis. Inflammatory pathways thus play key roles in regulating the pathological processes during liver fibrosis including initiation, progression, and aggravation. Therefore, suppressing inflammation and its downstream signaling pathways can effectively attenuate liver injury and fibrosis. NF-κB, a family of gene pleiotropism transcription factor, is strongly associated with

* Corresponding author.
E-mail address: nmxyyf@126.com (Y. Yuan).
[1] The first two authors equally contributed to this work.

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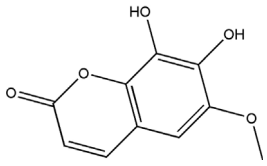

Fig. 1. Chemical structure of fraxetin ($C_{10}H_8O_5$, molecular weight = 208.17).

inflammation and liver fibrosis. It modulates hepatic fibrogenesis mainly through three different cellular events: 1) NF-κB regulates liver damage which is essential trigger point to initiating the progression of fibrosis; 2) NF-κB is closely related to regulating HSCs activation and apoptosis which serve as pivotal events in liver fibrosis; 3) NF-κB regulates inflammatory response by participating in the activation process of macrophages and inflammatory cells through transducing inflammatory signals into cell, and concurrently controls the expression of pro-inflammatory factors [17,18]. Studies demonstrating the anti-fibrotic activities of NF-κB have made it an attractive anti-fibrotic target [19–21]. Therefore, it is necessary to discover new drugs that can inhibit NF-κB mediated pathways to relieve fibrotic progression.

Mitogen activated protein kinase family (MAPKs) cascade mainly including p38 MAPK, JNK and ERK, is one of eukaryotic cell-mediated extracellular signal which govern fundamental cellular responses such as inflammation, apoptosis, proliferation and differentiation [22,23]. In response to inflammation, MAPKs pathway activates the nuclear NF-κB complex which then regulate the expression of several downstream pro-inflammatory factors [24,25]. Additionally, some studies revealed that phosphorylation of pro-inflammatory p38 MAPK, can also modulate the production and secretion of other proinflammatory factors such as TNF-α, IL-6, IL-1β, COX-2, TGF-β [26,27]. That means p38 MAPK is an imperative signaling protein that maintains the balance between proinflammatory factors and anti-inflammatory factors *in vivo*. Similar to p38 MAPK, the activation of JNK and ERK signaling can also stimulate the production of pro-inflammatory factors which further aggravate the injury and fibrosis in liver [28,29]. Many studies have shown that inhibition of MAPKs pathway mitigated liver fibrosis *via* suppression of inflammatory factors [30,31].

Liver cell apoptosis is another important pathogenic event influencing the deterioration of liver fibrosis. Unlike the activated hepatic stellate cells (HSCs) apoptosis, hepatocytes apoptosis facilitates liver fibrosis [32]. Bcl-2 protein family plays a decisive factor in mitochondria apoptosis pathway induced by CCl_4 in hepatocytes. This protein family is comprised of pro-survival Bcl-2 and pro-apoptotic Bax which are pivotal in regulating the apoptotic process. Previous reports have demonstrated that the up-regulation of Bcl-2 and the down-regulation of Bax could relieve liver cell apoptosis and liver fibrosis [33]. Therefore, fraxetin may function as a regulator of Bcl-2/Bax signaling pathways to block hepatocytes apoptosis.

Taken together, modulation of NF-κB, MAPKs and Bcl-2/Bax signaling pathways contribute to the underlying mechanisms of hepato-protecting and anti-fibrotic agents. Based on that, we hypothesized that fraxetin regulates NF-κB/IκBα, MAPKs and Bcl-2/Bax signaling pathways to suppress inflammation and hepatocytes apoptosis during fibrosis. Hence, the aim of this study is to explore the anti-hepatic fibrotic properties and underlying molecular mechanisms of fraxetin in CCl_4 induced rat model of liver fibrosis.

Materials and methods

Reagents and antibodies

Fraxetin (purity 98.0%) was obtained from Shanghai Nature Standard Biotechnology (Shanghai, China); CCl_4 was purchased from Shanghai Jinghua Scientific & Technological Research Institute (Shanghai, China); The kit of Nuclear and Cytoplasmic proteins Extraction Reagents was provided by Thermo Fisher Scientific (Waltham, MA, USA). All primary antibodies except anti-Bcl-2 were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-Bcl-2 was bought at Santa Cruz Biotechnology (Dallas, TX, USA). The goat anti-rabbit and anti-mouse secondary antibodies were purchased from Bioworld Technology (St Louis Park, MN, USA). TRIzol and other reverse transcription reagents were obtained from TaKaRa Biotech (Tokyo, Japan). The ELISA kits of liver function and fibrosis makers were provided by Nanjing Jiancheng Biotechnology (Nanjing, China). Hydroxyproline Testing Kit was provided by Sigma-Aldrich (St. Louis, MO, USA).

Animal experimental protocols

48 male Sprague Dawley rats were purchased from Shanghai Jie Si Jie Laboratory Animal Co. (Shanghai, China). All rats were housed in individual ventilated cages (IVC) of SPF level, and were given food and water ad libitum. 48 rats were randomly assigned to 4 groups: normal group, model group, fraxetin 25 mg/kg and 50 mg/kg group. Except normal group, the rest of three groups were fed a mixture of CCl_4 and peanut oil (1 ml/kg) by gavage twice per week for 8 weeks to acquire the liver fibrosis rats. Meanwhile, rats in fraxetin group were treated with the corresponding dosage, respectively, through intragastric administration once daily. Normal group received equal volume of saline. After taking 8 weeks, all surviving rats were weighed and euthanized by 3% pentobarbital sodium (1 ml/kg). Next, blood was collected by abdominal aortic method. The left lobe of liver tissue was separated and fixed in 10% neutral formalin for subsequent histopathology. The surplus liver tissues were stored at -80°C till further needed.

Biochemical analysis of serum

Serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin (TBIL) were detected by a fully automatic biochemical analyzer. Serum concentrations of hyaluronic acid (HA), laminin (LN), type-IV collagen (IV-C) and amino terminal propeptide of type III procollagen (PIIINP) were tested basing on magnetic particle chemiluminescence by using commercial kits according to the kit instructions. Hydroxyproline Testing Kit was used to detect hydroxyproline content as per the manufacturer's instruction.

Histopathologic analysis

The left lobe of liver was excised immediately after euthanasia, fixed in 10% neutral formalin, and then embedded in paraffin. Microtomed 4 μm sections were stained with hematoxylin and eosin (HE) or Masson's trichrome following standard protocols. To assess the degree of fibrosis in different groups, all sections were scored by an experienced pathologist using a light microscope according to the criteria as follows: 0, normal; 1, fibrosis present (collagen fiber present that extends from portal triad or central vein to peripheral region); 2, mild fibrosis (mild collagen fiber present with extension without compartment formation); 3, moderate fibrosis (moderate collagen fiber present with some pseudo lobe formation); and 4, severe fibrosis (severe collagen fiber present with thickening of the partial compartments and frequent pseudo lobe formation) [34].

Immunohistochemistry and immunofluorescence

Liver tissue sections were incubated with the following antibodies against p-p38 MAPK, p-JNK, p-ERK, Bcl-2, Bax and α-

SMA after a series of pretreatments. For immunohistochemistry, sections were incubated with corresponding HRP-conjugated secondary antibodies, and then stained with 3,3′-diaminobenzidine (DAB). For immunofluorescence, sections were incubated with Alexa fluor-488 conjugated secondary antibody (green) and counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). All sections were imaged on an inverted fluorescence microscope.

Nuclear and cytoplasmic proteins extraction

Nuclear and cytoplasmic proteins were collected by the kit of Nuclear and Cytoplasmic proteins Extraction as per the instruction. Firstly, vortex the tube vigorously on the highest setting for 15 s to fully suspend the cell pellet after homogenization of liver tissue. Add the presetting ice-cold CER II to the tube, and then incubate tube on ice for 1 min. After a highest-speed vortex for 5 s, the tube should be centrifuged for 5 min at 1600 *g* at 4 °C. Next, immediately transfer the supernatant (cytoplasmic proteins) to a clean pre-chilled tube for storage. Suspend the insoluble (pellet) fraction produced in centrifugation which contains nuclear proteins in ice-cold NER reagent. Then vortex tube for 5 s again and centrifuge for 10 min at 1600 *g* at 4 °C. Finally, after repeating the steps above for 4 times, the supernatant (nuclear proteins) fraction was immediately transferred to a clean pre-chilled tube for the next experiments. All operations were done on ice.

Western blot analysis

Total protein of liver tissue was extracted by homogenization in RIPA buffer. Before electrophoresis, protein samples were normalized to 40 μg protein, heated to albuminous degeneration. Protein samples were loaded and separated by electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes. Next, the PVDF membranes were blocked in the 5% skimmed milk solution to seal up the unspecific sites. After that, the membranes were respectively incubated with the corresponding diluent of primary and secondary antibodies. At last, protein bands were detected by an chemiluminescence apparatus.

Real-time quantitative PCR analysis

The RNA of liver tissue was isolated by TRIzol following the reagent's instruction. RNA concentration and purity were detected by a Nanodrop 2000 spectrophotometer. 400 ng of each sample was reverse-transcribed to cDNA on the Veriti 96-Well Thermal Cycler. Transcript abundance of genes was evaluated by Real-time Quantitative PCR on a LightCycler 480 instrument. All primers were designed and synthesized from Sangon Biotechnology (Shanghai, China) (Table 1). Expression of target genes was normalized to endogenous GAPDH and relative expression was quantized on the basis of the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All data were presented as the means ± standard deviation (M ± SD) and analyzed on GraphPad Prism 6.0. Comparisons among multiple groups (three or more) were by one-way ANOVA with *post hoc* test (Dunnett's correction for multiple tests). Kruskal-Wallis test followed by Dunn-Bonferroni *post hoc* test was used for nonparametric comparisons; *p*-values of 0.05 or lower were considered significant.

Results

Fraxetin alleviated liver injury in the CCl_4 rat model

During the course of the treatment, no deaths occurred in normal group and 50 mg/kg fraxetin group, but two rats died in model group, 1 death occurred in 25 mg/kg fraxetin group. We found that body weight of rats in fraxetin 25 mg/kg and 50 mg/kg groups increased significantly compared with the model group ($p < 0.05$) (Table 2). The liver weight and liver index of rats remarkably increased in model group compared to normal group ($p < 0.05$). Fraxetin treatment induced a significant decrease in liver weight and index compared with the model group ($p < 0.05$).

Fraxetin ameliorated liver function in experimental rats induced by CCl_4

As shown in Table 3, rats in the CCl_4 treated group suffered grievous damage to liver function evidenced by the increasing serum levels of liver function makers including ALT, AST and TBIL compared with the normal group ($p < 0.05$). Moreover, ALT, AST and TBIL decreased in response to fraxetin treatment, and significant difference was observed between fraxetin 25 mg/kg and 50 mg/kg group ($p < 0.05$).

Fraxetin decreased fibrosis indexes

As shown in Table 4, contrast to the normal group, serum concentrations of HA, LN, IV-C and PIIINP were significantly increased after CCl_4 treatment ($p < 0.05$). However, upon fraxetin treatment, significant attenuation of these liver fibrosis indexes occurred, particularly in fraxetin 50 mg/kg group ($p < 0.05$).

Table 1
Gene primers sequences used in this study.

Genes	Forward(5'-3')	Reverse(5'-3')	Product length
α-SMA	CCAGGGAGTGATGGTTGGA	CCGTTAGCAAGGTCGGATG	200
Vimentin	TGAGATCGCCACCTACAGGA	GAGTGGGTGTCAACCAGAGG	129
Desmin	CCGATCCAGACCTTCTCTGC	TCTCCATCCCGGGTCTCAAT	116
TNF-α	GACTGGCGTGTTCATCCG	TCTGAGCATCGTAGTTGTTGG	180
IL-6	AGAGACTTCCAGCCAGTTGC	ACAGTGCATCATCGCTGTTC	232
IL-1β	TGATGACGACCTGCTAGTGTG	TCCATTGAGGTGGAGAGCTT	124
Cox-2	GGATCATCAACACTGCCTCA	ATGGTGGCTGTCTTGGTAGG	109
TGF-β	CTTGCCCTCTACAACCAACA	ACTTGCGACCCACGTAGTAGA	103
MMP-1	TGGACTTGCTCACACATTCC	CCAGTTCATGAGCCGTAACA	125
TIMP-1	ACGCTAGAGCAGATACCACG	CCAGGTCCGAGTTGCAGAAA	141
Colla 1	GCAATGCTGAATCGTCCCAC	CAGCACAGGCCCTCAAAAAC	176
Colla 2	CTCAAGTCGCTGAACAACCA	GTCTCCGCTCTTCCACTCTG	117
GAPDH	CAGGGCTGCCTTCTCTTGTG	GGTGGTGAAGACGCCAGTAG	256

Table 2
Effects of fraxetin on body weight, liver weight and liver index of rats.

Group	N	Body weight (g)	Liver weight (g)	Liver index (Liver weight/Body weight)
Normal	12	404.1 ± 17.9	11.5 ± 0.8	0.028 ± 0.002
Model	10	306.0 ± 24.7*	14.4 ± 0.8*	0.047 ± 0.005*
Fraxetin 25 mg/kg	11	338.3 ± 21.4*,**	13.0 ± 0.9**	0.039 ± 0.003*,**
Fraxetin 50 mg/kg	12	372.3 ± 15.2*,**,***	12.1 ± 0.6**,***	0.032 ± 0.002*,**,***

Each value represents the mean ± SD. *$p < 0.05$ as compared with normal group. **$p < 0.05$ as compared with model group. ***$p < 0.05$ as compared with fraxetin 25 mg/kg group.

Table 3
Effects of fraxetin on serum concentrations of AST, ALT and TBIL.

Group	N	AST (μ/L)	ALT (μ/L)	TBIL (μmol/L)
Normal	12	134.4 ± 13.6	44.2 ± 15.1	4.5 ± 0.87
Model	10	532.9 ± 135.6*	739.9 ± 143.3*	27.5 ± 6.2*
Fraxetin 25 mg/kg	11	420.2 ± 64.7*,**	512.7 ± 137.6*,**	22.8 ± 3.4*,**
Fraxetin 50 mg/kg	12	268.6 ± 88.3*,**,***	293.1 ± 62.8*,**,***	17.9 ± 2.7*,**,***

Fraxetin alleviated histopathological changes and decreased hydroxyproline content in liver

As shown in Fig. 2, the results showed the physiological architecture and minimal collagen deposition in the normal rats. In contrast, signs of massive cell necrosis, fatty degeneration or inflammatory cell infiltration was greatly visible in the model group along with collagen fibrils hyperplasia, hepatic lobe reconstruction and pseudolobuli formation. However, treatment with fraxetin could visibly ameliorate the architecture and degree of liver fibrosis ($p < 0.05$). Results of Masson's trichrome staining analyzed by ImageJ revealed that fraxetin treatment could significantly reduce the area-density percentage of collagen deposition ($p < 0.05$, Fig. 2C). In accordance with the histopathology results, hydroxyproline content was also decreased in fraxetin treatment group, specially in fraxetin 50 mg/kg group ($p < 0.05$, Fig. 2D).

Fraxetin regulated the expression of NF-κB/IκBα, MAPKs and Bcl-2/Bax signaling

Next, we explored the mechanism of anti-fibrotic properties of fraxetin. As shown in Fig. 3, CCl_4 treatment significantly increased the expression of nuclear NF-κB and cytoplasmic p-IκBα compared with the normal group. Treatment with fraxetin significantly reversed the effects of CCl_4 ($p < 0.05$). On the contrary, the expression of NF-κB and IκBα in the cytoplasm visibly lowered in model group as compared with normal group and distinctly increased after fraxetin treatment as compared with model group ($p < 0.05$).

To assess whether fraxetin could regulate MAPKs pathway, immunohistochemistry was performed and the results exhibited that increased phosphorylation levels of p38 MAPK, JNK and ERK proteins in the model group ($p < 0.05$, Fig. 4A). In contrast, the phosphorylation of these proteins in fraxetin treated groups was significantly reduced ($p < 0.05$). These findings were validated by Western blot analysis which showed increased expression of p-p38 MAPK, p-JNK and p-ERK proteins in the CCl_4 treated rats ($p < 0.05$), and a dose dependent reduction in response to fraxetin ($p < 0.05$, Fig. 4B).

Immunofluorescence staining for Bcl-2 was significantly decreased in the model group which was reversed in response to fraxetin ($p < 0.05$, Fig. 5A). Expression of pro-apoptotic Bax in the model group were significantly increased ($p < 0.05$), which was effectively decreased in response fraxetin ($p < 0.05$). In parallel, the aforesaid results were further confirmed by Western blot analysis which showed that fraxetin enhanced Bcl-2 up-regulation and simultaneously reduced Bax, cleaved caspase-3 up-regulation induced by CCl_4 ($p < 0.05$, Fig. 5B).

Fraxetin inhibited HSCs activation in fibrotic liver

As shown in Fig. 6A and C, the up-regulation of α-SMA in the model group was reduced in fraxetin 25 and 50 mg/kg group ($p < 0.05$). In parallel, transcript abundance of HSCs activation makers (α-SMA, Vimentin, Desmin) were substantially decreased after treatment with the different dosage of fraxetin, especially in the 50 mg/kg group except for Desmin ($p < 0.05$, Fig. 6B).

Fraxetin suppressed inflammation and accelerated degradation of extracellular matrix (ECM) in CCl_4-induced liver fibrosis

As shown in Fig. 7, mRNA levels of inflammatory factors (TNF-α, IL-6, IL-1β, Cox-2, TGF-β) and regulatory factors of ECM (TIMP-1, Colla 1, Colla 2) were significantly higher in model group than normal group. Treatment with fraxetin at 25 and 50 mg/kg reversed the ascending expression of these genes in a dose-dependent manner, compared to model group ($p < 0.05$). An inverse trend was observed with MMP-1 mRNA expression (Fig. 7B).

Discussion

The major findings of this study are that fraxetin has the significant anti-liver fibrosis action in SD rats. In parallel it

Table 4
Effects of fraxetin on serum concentrations HA, LN, IV-C and PIIINP.

Group	N	HA (U/L)	LN (ng/mL)	IV-C (ng/mL)	PIIINP (ng/mL)
Normal	12	38.2 ± 4.9	20.1 ± 3.8	22.2 ± 5.0	1.20 ± 0.16
Model	10	203.7 ± 17.1*	113.1 ± 14.9*	137.6 ± 13.8*	3.73 ± 0.40*
Fraxetin 25 mg/kg	11	151.9 ± 14.8*,**	80.1 ± 10.2*,**	96.9 ± 8.6*,**	2.79 ± 0.31*,**
Fraxetin 50 mg/kg	12	94.5 ± 13.8*,**,***	51.1 ± 8.8*,**,***	64.9 ± 10.3*,**,***	1.78 ± 0.21*,**,***

Each value represents the mean ± SD. *$p < 0.05$ as compared with normal group. **$p < 0.05$ as compared with model group. ***$p < 0.05$ as compared with fraxetin 25 mg/kg group.

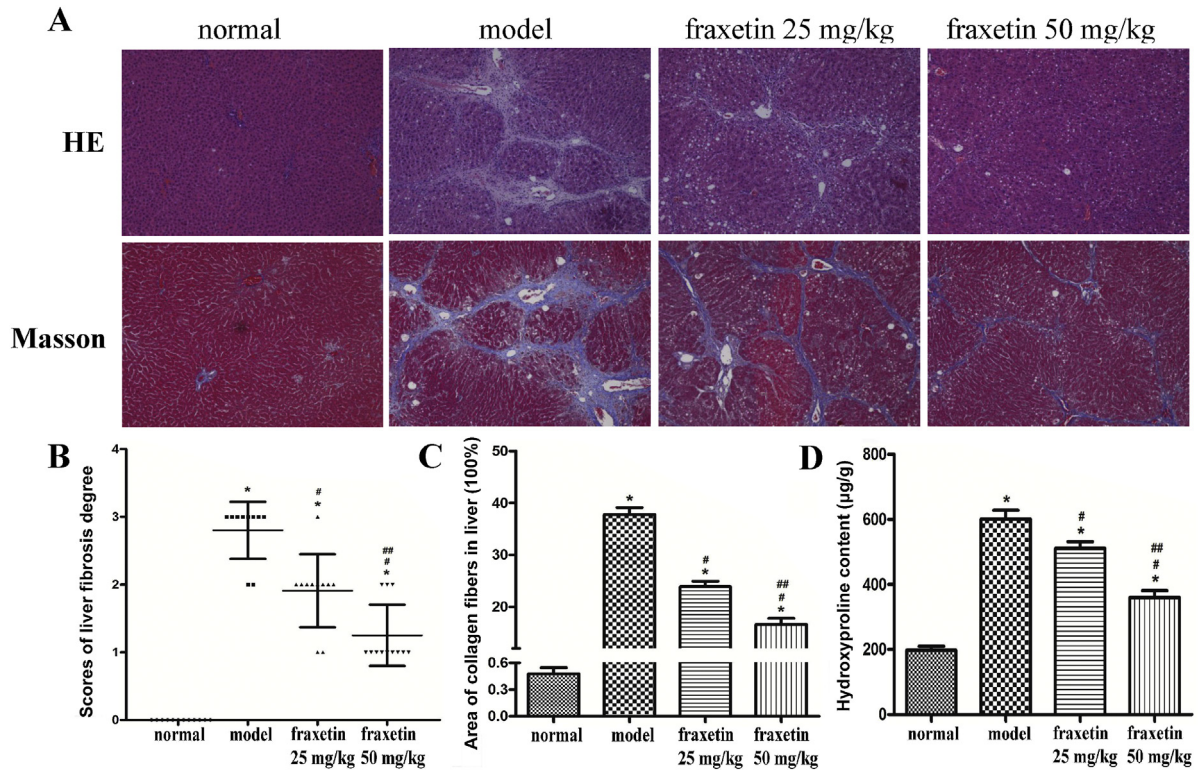

Fig. 2. Fraxetin alleviates liver fibrosis induced by CCl_4 in rats. (A) Hematoxylin-eosin (HE) and Masson staining. Magnification, ×100. (B) The scores of liver fibrosis degree. (C) The area of collagen fibers in liver (%). (D) The hydroxyproline content in serum. $^{*}p < 0.05$ compared with normal group; $^{\#}p < 0.05$ compared with model group; $^{\#\#}p < 0.05$ compared with fraxetin 25 mg/kg group.

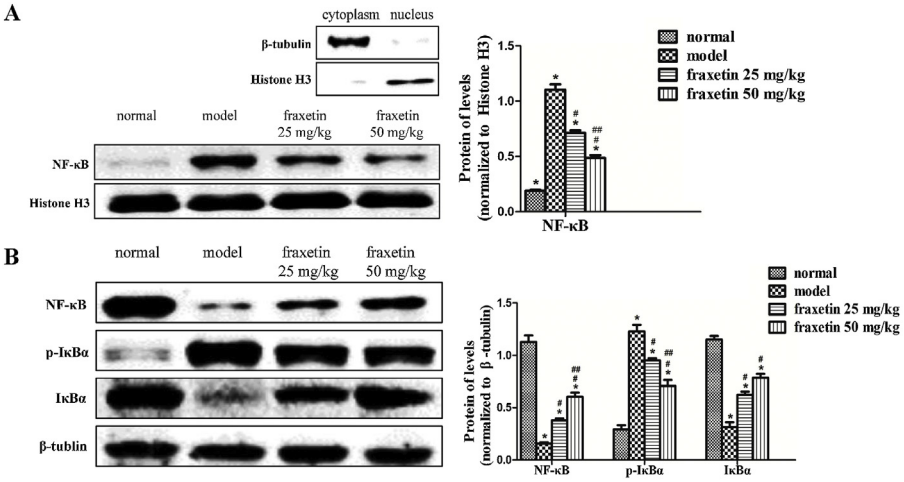

Fig. 3. Fraxetin mediates the expression of NF-κB signaling pathway. (A) The expression of NF-κB in the nucleus was tested by western blotting, and the separation effects of nuclear and cytoplasmic proteins were assayed respectively by anti-Histone H3 antibodies or anti-β-Tubulin. (B) The expression of NF-κB, p-IκBα and IκBα in the cytoplasm were tested by western blotting. $^{*}p < 0.05$ compared with normal group; $^{\#}p < 0.05$ compared with model group; $^{\#\#}p < 0.05$ compared with fraxetin 25 mg/kg group.

improved liver function, mechanistic analysis revealed that fraxetin exerted its anti-fibrotic effects by regulating the NF-κB/IκBα, MAPKs and Bcl-2/Bax signaling pathways.

AST, ALT and TBIL were widely applied to estimate the liver function in clinic. In the case of liver cell degeneration, hyper-permeability and necrosis, these hepatic enzymes are released into the bloodstream from the cytoplasm and mitochondria. And then, these markers were determined in serum and enabled factually reflect the liver function. These results suggested that treatment with fraxetin significantly reduced the levels of liver function markers indicating that fraxetin can ameliorate liver injury. In consistent with the alteration of liver function markers, the expression levels of HA, LN, IV-C and PIIINP in serum were markedly increased after CCl_4 administration, and attenuated by fraxetin. Comparison of HE and Masson's trichrome staining, revealed that the morphology and collagen deposition in response to fraxetin were markedly reduced, which reconfirmed that fraxetin possessed of the favorable pharmacological property to relief liver injury and fibrosis.

NF-κB/IκBα pathway is wildly recognized as the master regulator of inflammatory diseases including liver fibrosis. Normally, IκBα and NF-κB form an inactive complex in the

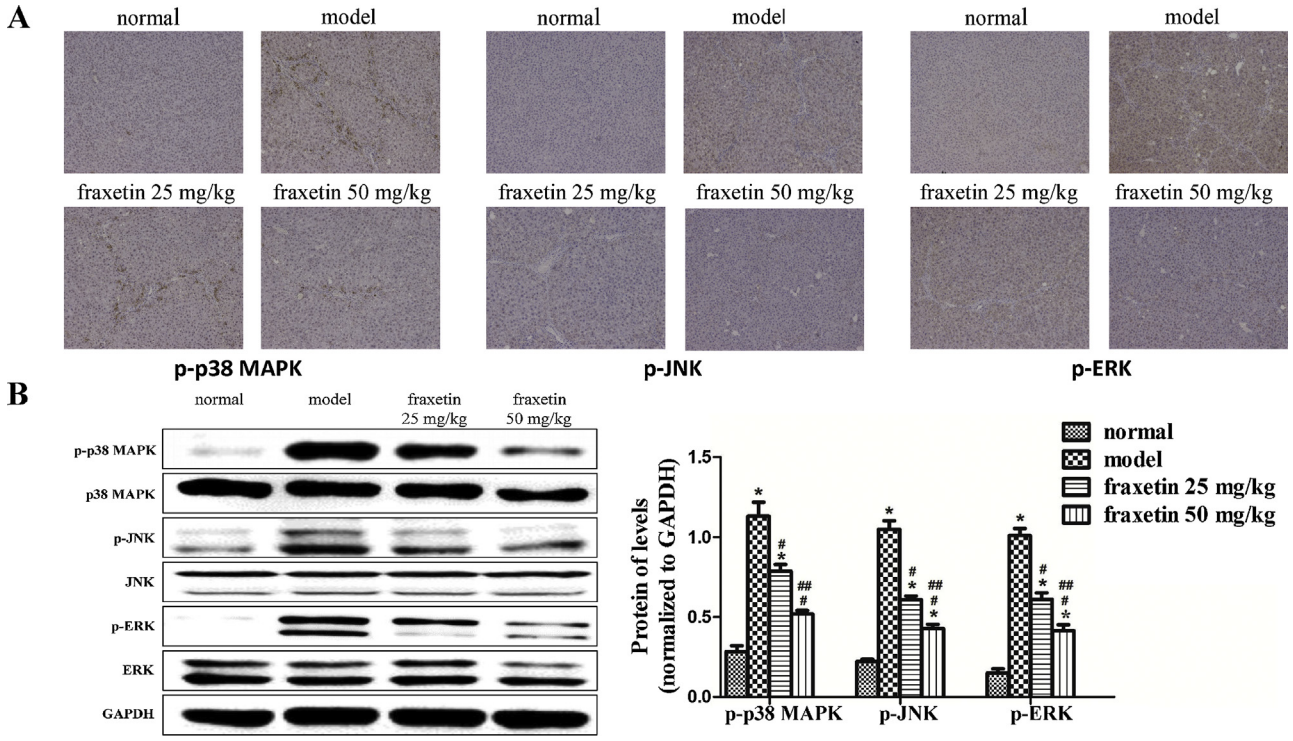

Fig. 4. Fraxetin regulates the expression of MAPKs signaling pathway in hepatic fibrosis rats. (A) The immunohistochemistry results of p-p38 MAPK, p-JNK and p-ERK. Magnification, ×100. (B) The expression levels of phosphorylated and total p38 MAPK, JNK and ERK were tested by western blotting. $^{*}p < 0.05$ compared with normal group; $^{\#}p < 0.05$ compared with model group; $^{\#\#}p < 0.05$ compared with fraxetin 25 mg/kg group.

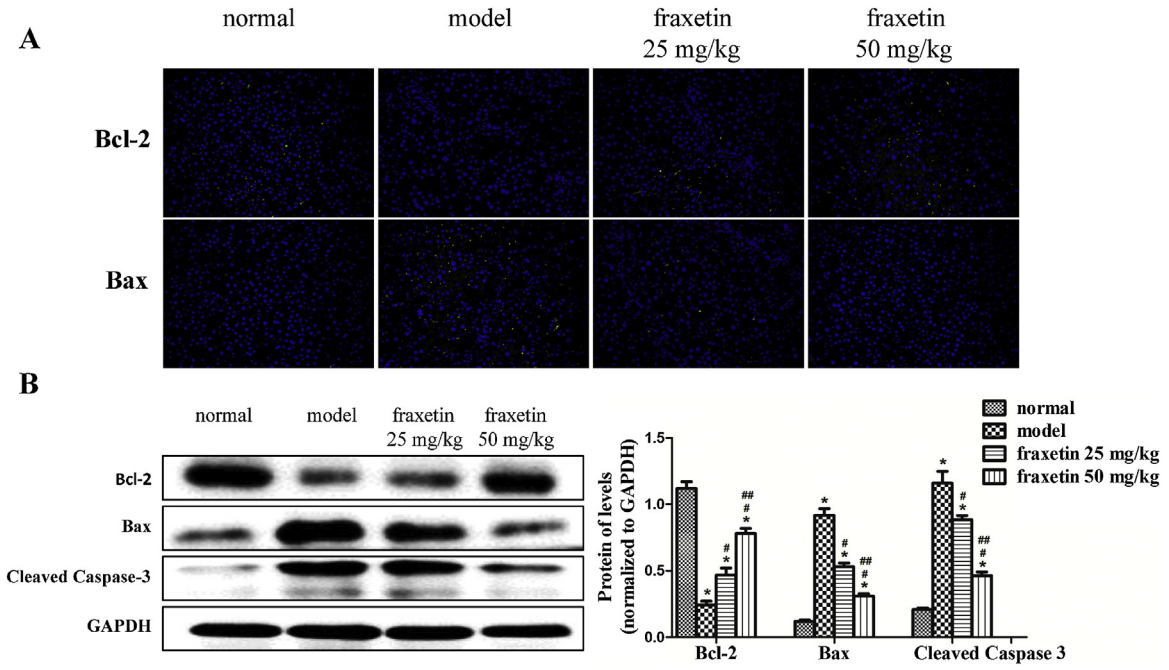

Fig. 5. Fraxetin adjusts the expression of Bcl-2/Bax signaling pathway. (A) The expression of Bcl-2 and Bax in liver were analyzed by immunofluorescent staining. Magnification, ×100. (B) The expression of Bcl-2, Bax and Cleaved Caspase-3 in liver were tested by western blotting. $^{*}p < 0.05$ compared with normal group; $^{\#}p < 0.05$ compared with model group; $^{\#\#}p < 0.05$ compared with fraxetin 25 mg/kg group.

cytoplasm, which upon stimulation is disrupted by phoshorylation and proteosomal degradation of IκB allowing NF-κB to translocate to the nucleus where it can initiate transcription of inflammatory factors such as TNF-α, IL-6, IL-1β, COX-2 [35]. Hence, preventing the activation of NF-κB could be considered an effective therapeutic approach to prevent liver fibrosis. In the present study, we hypothesized that fraxetin reduced liver fibrosis may be closely related with regulating the nuclear translocation of NF-κB. Our data supported the hypothesis and showed that injury in response CCl_4significantly increased the expression of pro-inflammatory markers facilitated by the translocation of activated NF-κB to the nucleus. Fraxetin treatment effectively reversed these effects of

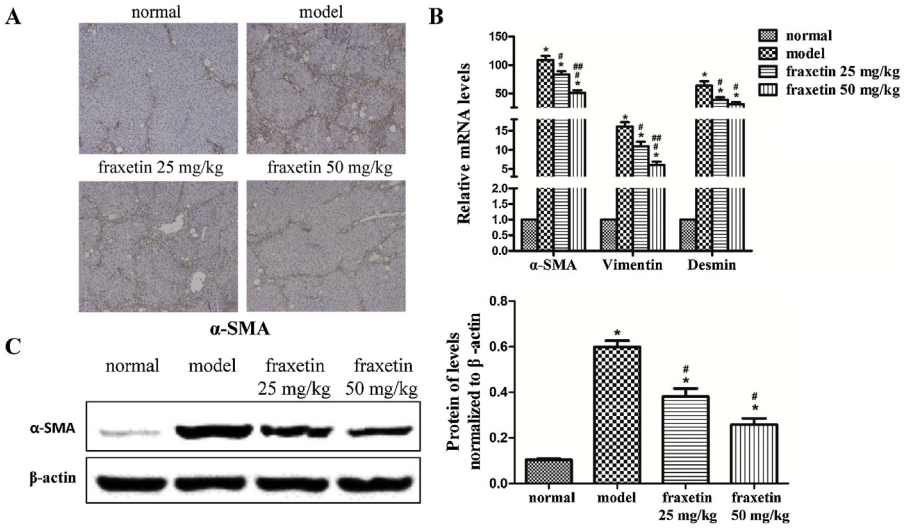

Fig. 6. Fraxetin inhibits the activation of HSCs in fibrotic liver of rats. (A) The immunohistochemistry results of α-SMA. Magnification, ×100. (B) The mRNA levels of HSCs activation makers including α-SMA, Vimentin and Desmin were tested by Real-time Quantitative PCR. (C) The expression of α-SMA in liver was examined by western blotting. $^{*}p < 0.05$ compared with normal group; $^{\#}p < 0.05$ compared with model group; $^{\#\#}p < 0.05$ compared with fraxetin 25 mg/kg group.

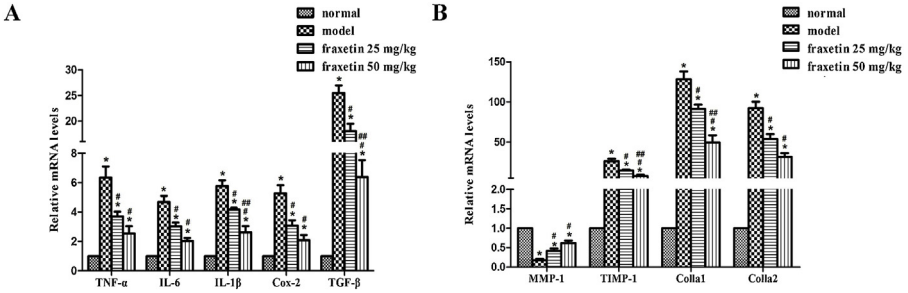

Fig. 7. Fraxetin regulates the mRNA levels of inflammation factors (A) and degradation makers of ECM (B) in fibrotic liver. $^{*}p < 0.05$ compared with normal group; $^{\#}p < 0.05$ compared with model group; $^{\#\#}p < 0.05$ compared with fraxetin 25 mg/kg group.

CCl_4. These results were consistent with previous assumption that the anti-hepatic fibrosis activity of fraxetin is modulated by the activation of NF-κB signaling pathway.

Numerous studies manifested that activation of MAPKs pathway can promote nuclear translocation of NF-κB p65 and increase the secretion of several proinflammation factors thereby aggavating inflammatory damage to liver cells in liver fibrosis [36–38]. Our study further found that CCl_4 induced increase in the phosphorylation of MAPKs proteins was reduced after fraxetin treatment. These results further support our hypothesis that fraxetin can attenuate inflammation by suppressing the phosphorylation of MAPKs proteins.

Activation of HSCs, characterised by the appearance of α-SMA, is the central tache in pathogenesis and progress of liver fibrosis. Once stimulated, HSCs become active and increase the synthesis and deposition of ECM which gradually contributes to the formation of fibrous scar in damaged liver [39]. Besides α-SMA, Kitano et al. [40] observed that up-regulation of vimentin and desmin promoted the differentiation of activated HSCs into myofibroblasts during fibrosis. Sustaining the balance between ECM synthesis and degradation is crucial to preventing the pathological processes in fibrotic liver. Synthesis and degradation of collagen are mainly regulated by matrix metalloproteinases (MMPs) and their respective inhibitors (TIMPs) [41]. For example, MMP-1 mediated degradation of ECM is blocked by TIMP-1. As we expected, immunohistochemical staining and western blotting for α-SMA collectively illustrated that the anti-fibrotic effects of fraxetin on was closely related to inhibition of HSC activation. Furthermore, the mRNA levels of two other HSCs activation makers and regulators of ECM were significantly decreased. These findings strongly support our conclusion that fraxetin improved liver fibrosis by inhibiting HSCs activation and facilitating degradation of ECM.

Except inflammation, mitochondria-dependent apoptosis in hepatocytes is another pathogenic factor that affects the outcome of liver fibrosis. Chronic injury induces liver cell apoptosis and promotes the production of apoptotic bodies which are then engulfed by HSCs resulting in their activation, proliferation, and differentiation into myofibroblast and deposition of ECM. Hepatocytes apoptosis is complex process mainly dependent on the dynamic balance between pro-apoptotic and anti-apoptotic molecules. Therefore, the exprssion ratio of Bcl-2 protein to Bax protein plays a crucial role in hepatocytes apoptosis [42]. In this study, fraxetin ameliorated hepatocytes apoptosis by significantly up-regulating the ratio of Bcl-2/Bax. Additionally, we observed that fraxetin mitigated the activation of caspase-3, a downstream effector of Bcl-2/Bax pathway. These data suggested that blocking of hepatocytes apoptosis contribute to reduce the degree of liver fibrosis, and Bcl-2/Bax signaling pathway may be a potential target for anti-fibrosis drugs.

In conclusion, this study revealed that fraxetin possessed therapeutic potential for preventing liver fibrosis induced by CCl_4 in SD rats. The anti-fibrotic activity of fraxetin is closely linked to its capacity to restrain NF-κB/IκBα and MAPKs inflammatory responses in conjuction with Bcl-2/Bax apoptotic signaling pathway. These hepato-protective properties make fraxetin a

promising therapeutic drug candidate for treating liver fibrosis. Further research is required for in-depth understanding of the mechanism of anti-fibrotic effects of fraxetin.

Conflict of interest

The authors declare no conflicts of interest.

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