

The hepatoprotective effect of aspirin on carbon tetrachloride-induced hepatic fibrosis via inhibition of TGF β -1 pathway and pro-inflammatory cytokines IL-1 β and COX-2 in rats

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Abstract. Aspirin decreases liver fibrosis index and inflammation levels. However, the exact mechanism underlying the effects of aspirin are yet to be elucidated. The aim of the study was to investigate the potential protective effects of aspirin on carbon tetrachloride (CCl₄)-induced hepatic fibrosis in Sprague-Dawley rats. Rats were divided into four groups, including healthy and CCl₄ control and low-(aspirin 10 mg/kg + CCl₄) and high-dose aspirin group (aspirin 300 mg/kg + CCl₄). After 8 weeks treatment, the histopathological examinations of hepatocyte fibrosis in liver and serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), IL-1 β , transforming growth factor- β 1 (TGF- β 1), hyaluronic acid (HA), laminin (LN) and type IV collagen (IV.C) were determined. Histopathological examination suggested that aspirin decreased CCl₄-induced hepatic fibrosis and liver inflammation. The high-dose aspirin group significantly decreased the serum levels of ALT, AST, HA and LN compared with the CCl₄ control group. High-dose aspirin group significantly decreased the levels of pro-inflammatory cytokines IL-1 β compared with CCl₄ group. The high-dose aspirin group significantly inhibited the expression of TGF β -1 protein compared with CCl₄ group. Overall, the present study indicated that aspirin exhibited potent protective effects against CCl₄-induced hepatic fibrosis

via inhibition of the TGF β -1 pathway and pro-inflammatory cytokine IL-1 β .

Introduction

Hepatitis B virus (HBV) infection is a worldwide epidemic. According to World Health Organization, there are ~257 million chronic HBV infections worldwide. China is a moderately endemic country and there are currently ~70 million cases of chronic hepatitis B (CHB), a notable proportion of which is accompanied by liver fibrosis (1). Liver fibrosis progresses to cirrhosis in 25-40% of patients if they do not receive timely treatment. Globally, ~887,000 people died from HBV infection-associated diseases in 2015, with cirrhosis and primary hepatocellular carcinoma (HCC) accounting for 52 and 38% of deaths, respectively. Hepatic fibrosis is a pathological process of excessive deposition of diffuse extracellular matrix (ECM) in hepatocytes during the injury repair process, and is a common occurrence in the progression of various chronic hepatic disease to cirrhosis (2). Liver fibrosis is a dynamic and reversible process, whereas progression to intermediate to advanced cirrhosis is considered irreversible. Therefore, early intervention decreases risk of progression to end-stage liver disease (3,4). Although almost all types of chronic liver diseases (chemically toxic, infectious, genetic/metabolic, autoimmune) can lead to liver fibrosis, the mechanisms of liver fibrosis are still not fully understood (5,6). The successful treatment of liver fibrosis remains unsatisfactory with cytokine analogs, antioxidants and other drugs (7-11).

Investigation of the mechanism of liver fibrosis and screening the targets for anti-fibrotic therapy are key in liver disease research. Cyclooxygenase-2 (COX-2) inhibitor aspirin not only has anti-inflammatory properties, but also decreases tissue fibrosis via multiple pathways; studies have shown that aspirin can exert anti-pulmonary fibrosis effects by inhibiting expression of TGF- β 1, TNF- α and IL-4 (12,13). A large

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case-review study in the United States showed that aspirin significantly decreased liver fibrosis index in adults (14). Another multicenter retrospective study found that low-dose aspirin treatment may be associated with a lower risk of liver fibrosis progression in patients with hepatitis C virus (HCV) recurrence after liver transplantation (15). Yoshida *et al* (16) showed that platelets can promote liver fibrosis through direct activation of hepatic stellate cells in mice. Platelets are a promising target for antifibrotic therapy. Platelet activation and degranulation are important events of the physiological response to tissue injury, which activates wound closure and repair (17). A previous study (18) showed that platelets undergo significant qualitative and quantitative changes when liver fibrosis occurs and confirmed that liver fibrosis is associated with platelet alteration.

To the best of our knowledge, only Sun *et al* (6) have reported the effect of aspirin on liver fibrosis via inhibition of the TGF β 1/SMAD pathway. Aspirin has become one of the most commonly used drugs, given its role as an analgesic, anti-pyretic and agent for cardiovascular prophylaxis (19,20). More studies are needed to determine the protective effect of aspirin in liver fibrosis models and its potential mechanisms (6). Here, to explore the effect of aspirin on liver fibrosis, a hepatic fibrosis rat model induced by CCl₄ was used and evaluated following aspirin treatment. In addition, the present study further investigated whether aspirin attenuated hepatic fibrosis via the TGF β 1 signaling pathway and the effect of aspirin on the pro-inflammatory cytokine IL-1 β , thus revealing the potential molecular and inflammatory mechanisms underlying the protective effect of aspirin on hepatic fibrosis.

Materials and methods

Chemicals. CCl₄ was obtained from Jiangxi Gang Instrument Technology Co., Ltd. Aspirin enteric-coated tablets were purchased from Bayer Medical Health Co., Ltd. and prepared as a suspension with saline. Olive oil was purchased from the local market, sealed and stored at room temperature following high temperature sterilization.

Animals. A total of 32 SPF male Sprague-Dawley rats (age, 6–8 weeks, mean weight, 250 \pm 20 g) were obtained from Jiangxi University of Traditional Chinese Medicine (Jiangxi, China), with license SCXK (Gan) 2018-0003. The rats were divided into groups (n=8/group) as follows: Healthy and CCl₄ control and low- and high-dose aspirin group. The rats were housed under normal laboratory conditions (21 \pm 2°C, 12/12-h light/dark cycle, humidity 50–60%) with free access to standard pellet diet and water. Body weight and behavior of all animals were monitored every two days. Humane endpoints were >20% weight loss, dehydration and loss of locomotion. No animals reached the humane endpoints. Blood was taken 1 h after the last administration. After the blood was taken, the rats were euthanized by cervical dislocation and liver specimens were collected immediately. Death was confirmed by evaluating vital signs, including heartbeat, pupillary response and respiratory pattern (lack of cardiac activity for 5 min through cardiac palpation, unresponsiveness to light with dilated pupils and lack of spontaneous breathing with a shallow and irregular breathing pattern). Animal experiments

were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Nanchang University (Nanchang, China; approval no. 2017062). All efforts were made to minimize suffering and reduce the number of animals used.

Treatment. To induce hepatic fibrosis, animals in the CCl₄ control and low- and high-dose aspirin group were subcutaneous (s.c.) administered 3 ml/kg body weight 40% CCl₄ (20% v/v CCl₄ in olive oil) twice/week for 8 weeks. The CCl₄ control group was gavaged with distilled water daily. The low- and high-dose aspirin group were given 10 and 300 mg/kg aspirin suspension by gavage once daily for 8 weeks, respectively.

Serum biochemical analysis. The rats were fasted without water for 12 h, one hour after the last administration, anesthetized by intraperitoneal injection of 10% chloral hydrate (0.3 ml/100 g) and blood (3–4 ml) was obtained from the abdominal aorta and centrifuged at 3,000 \times g for 10 min at 4°C. After blood collection, the rats were euthanized and liver specimens were collected immediately. Serum alanine transaminase (ALT) and aspartate transaminase (AST) levels and liver fiber indexes Hyaluronic acid (HA), laminin (LN) and type IV collagen (IV.C) were measured at the Second Affiliated Hospital of Nanchang University and Nuclear Medicine Department of Nanchang University Hospital (Nanchang, China), respectively. Liver samples were dissected and washed with ice-cold saline, then immediately stored at -80°C for further analysis. The largest right lobe of each liver was excised and fixed in 4% formaldehyde solution for 24–48 h at room temperature for histopathological analysis.

Cytokine IL-1 β measurement. Following centrifugation at 3,000 \times g for 10 min at 4°C, the supernatant was obtained and the IL-1 β in the serum was measured using specific anti-mouse ELISA from Elabscience Biotechnology Co., Ltd. (cat. no. E-EL-M0037c). The kit was used according to the manufacturer's instructions.

Protein quantification of TGF- β 1. Liver tissue was lysed in RIPA lysis buffer (cat. no. P0013B, Beyotime Institute of Biotechnology) and PMSF (cat. no. P105539, Aladdin). Following grinding and centrifugation (4°C, 12,000 \times g, 20 min), the protein was extracted and the concentration was measured using a BCA kit (cat. no. P0010, Beyotime Institute of Biotechnology). Samples (30 μ g/lane) were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (cat. no. IPVH00010; Millipore). The membranes were incubated in blocking buffer (5% skimmed milk powder) at room temperature for 2 h prior to the addition of primary antibodies at 4°C overnight. Primary antibodies were as follows: Anti-TGF β polyclonal (cat. no. Af1027; 1:1,000; Affinity Biosciences) and β -actin monoclonal (cat. no. BM0627; 1:50; Wuhan Boster Biological Technology Ltd.) as loading control. Peroxidase-conjugated goat anti-rabbit (cat. no. BA1054) and anti-mouse secondary antibody (cat. no. BA1051; both 1:10,000; both Boster Biological Technology Ltd.) were incubated at room temperature for 2 h. Image-pro Plus (IPP6.0; Media Cybernetics Corporation, USA) software was used to analyze the gray value of each protein band.

Liver histopathology. Liver tissue was fixed in 4% formaldehyde for 24–48 h at room temperature, embedded in paraffin (58–60°C, 10–30 sec) and cut into 4- μ m-thick sections. Paraffin sections were dewaxed, stained with hematoxylin for 5 min at room temperature, dipped in 1% hydrochloric acid ethanol and returned to blue with 1% ammonia for 2 min. Following rinsing with tap water, the sections were stained with eosin for 1 min at room temperature. Sections were dehydrated, made transparent, sealed and observed by light microscopy at 100x magnification. In total, 10 fields of view were randomly selected and observed from each section. For Masson's staining, Paraffin sections were dewaxed with xylene, rehydrated in gradient alcohol and washed with tap water for 1 min; oxidized with potassium permanganate for 5 min, rinsed with distilled water; bleached with 2% oxalic acid for 1–2 min, rinsed with distilled water; stained with Ponceau staining for 5 min, rinsed with distilled water; treated with phosphomolybdic acid for 5 min, decanted to remove excess stain, stained with aniline blue for 5 min; the slices were treated with glacial acetic acid at 2% v/v for 1 min, quickly dehydrated, transparent with dimethylbenzene and sealed with neutral gum. Fibrotic changes and collagen deposition were observed under the microscope. Histopathological examination of the liver was performed at the Department of Pathology, the Ninth Hospital of Nanchang University (Nanchang, China). The degree of liver injury and fibrosis was examined by specialized pathologists blinded to the groups. Histopathological diagnosis, grading of inflammatory necrosis of liver tissue and staging of the degree of fibrosis were performed according to the pathological diagnostic criteria in the grading criteria for chronic hepatitis (21). Inflammatory necrosis was graded as G0–G4, with G0–1 being mild inflammation, G2 being moderate inflammation and G3–4 being severe inflammation; the degree of fibrosis was graded as S0–S4, with S0–1 being mild fibrosis, S2 being moderate fibrosis and S3–4 being severe fibrosis.

Statistical analysis. All experiments were repeated three times. Data were analyzed using IBM SPSS software version 25.0 (IBM Corp.). Data are presented as the mean \pm SD. Data normality was assessed by the Shapiro-Wilk test. One-way ANOVA followed by post hoc Bonferroni's correction was used to compare >2 groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Histopathological features suggest aspirin attenuates CCl₄-induced liver fibrosis and inflammation. Hematoxylin-eosin staining for the healthy control group showed normal architecture. However, large area steatosis was observed in the liver tissue of rats in the CCl₄ control group and extensive infiltration of inflammatory cells was observed in the portal area, showing spot, focal and clastic necrosis. Fusion necrosis was observed, most of the nuclei disappeared and fibrous hyperplasia was obvious. The low- and the high-dose aspirin group exhibited improved liver morphology and structure with fewer false lobes and inflammatory cell infiltrates compared with the CCl₄ control group. The high-dose aspirin

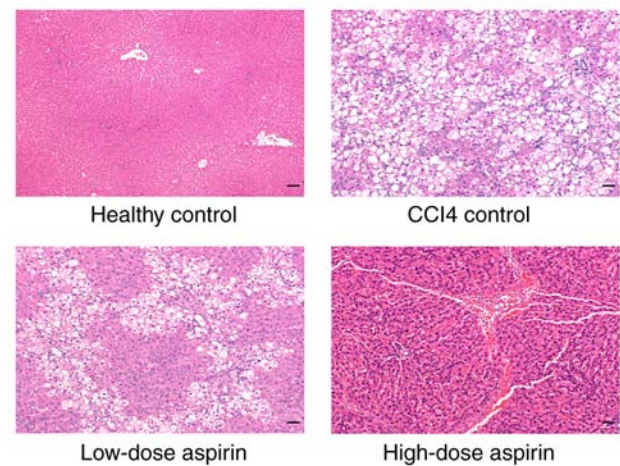


Figure 1. Histopathological changes of fibrosis in CCl₄-induced rats is prevented by treatment with aspirin. Hematoxylin-eosin staining (magnification, x100) in healthy and CCl₄ control and low-(10) and high-dose aspirin (300 mg/kg + CCl₄). Scale bar, 50 μ m.

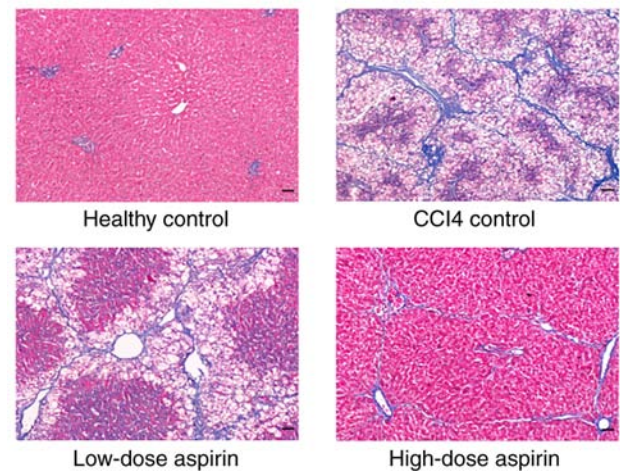


Figure 2. Histopathological changes of fibrosis in CCl₄-induced rats is prevented by treatment with aspirin. Masson's staining (magnification, x100) of healthy and CCl₄ control and low-(10) and high-dose aspirin (300 mg/kg + CCl₄). Scale bar, 50 μ m.

group showed better improvement than the low-dose aspirin group (Fig. 1). Masson's staining for the healthy control group showed a small number of small blue collagen fibers around the sink area and no fiber proliferation was observed. However, in the CCl₄ control group, fibrosis was notable around the sink area, with a large number of collagen fibers and fibrous septum formation. The hepatic lobules were divided into false lobules of different sizes. Collagen fibrous hyperplasia of liver tissue was notably decreased in the low-dose aspirin group and high-dose aspirin group; this was more pronounced in the high-dose aspirin group (Fig. 2).

Aspirin attenuates liver inflammation indicators in a CCl₄-induced liver fibrosis model in rats. Compared with the healthy control group, serum concentrations of ALT and AST were significantly increased in CCl₄ control group (Table I). The high-dose aspirin group significantly attenuated the increase of ALT and AST.

Table I. Effects of aspirin on serum concentrations of ALT and AST.

Group	ALT, U/l	AST, U/l
Healthy control	33.51±2.15	162.62±40.22
CCl ₄ control	176.71±46.41 ^a	284.44±71.09 ^a
Low-dose aspirin (10 mg/kg + CCl ₄)	145.92±19.55	208.86±50.21
High-dose aspirin (300 mg/kg + CCl ₄)	55.61±19.33 ^{b,c}	182.11±44.90 ^b

Data are presented as the mean ± SD. P<0.01 vs. ^ahealthy and ^bCCl₄ control and ^clow-dose aspirin. ALT, alanine transaminase; AST, aspartate transaminase.

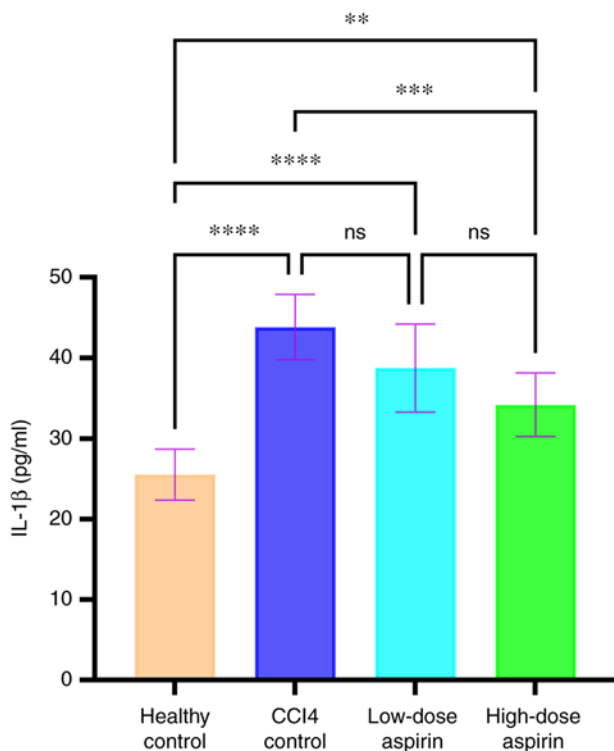


Figure 3. Effect of aspirin on serum concentration of IL-1β. Data are presented as the mean ± SD. ns, not significant. **P<0.01. ***P<0.005 and ****P<0.001.

Aspirin attenuates levels of inflammatory cytokine IL-1β in CCl₄-induced rat liver fibrosis model. Compared with the healthy control group, CCl₄-induced toxicity caused a significant increase in IL-1β levels. The high-dose aspirin group significantly attenuated the increase of IL-1β (Fig. 3).

Aspirin decreases serum liver fibrosis index levels in a CCl₄-induced liver fibrosis model in rats. Serum liver fibrosis index (HA and LN) levels were significantly higher in the CCl₄ control group compared with the healthy control group (Table II). Compared with the CCl₄ control, the low-dose and high-dose aspirin group significantly attenuated the increase in HA, high-dose aspirin group significantly attenuated the increase in LN, and the low-dose aspirin group significantly

attenuated the increase in IV.C. IV.C levels were almost unmeasurable in the healthy control and high-dose aspirin groups. High-dose aspirin group significantly attenuated the increase of LN compared with the low-dose aspirin group.

Effect of aspirin on stage of liver fibrosis in a CCl₄-induced liver fibrosis model in rats. Compared with the CCl₄ control group, aspirin intervention resulted in a decrease in liver fibrosis stage S3 and an increase in stage S2 (Table III). However, there was no significant difference between groups.

Aspirin decreases expression of TGFβ-1 protein in a CCl₄-induced liver fibrosis model in rats. Compared with the healthy control group, the TGFβ-1 protein levels in liver tissue were significantly increased in the CCl₄ control group (Fig. 4). TGFβ-1 protein expression level was significantly lower in the high-dose aspirin group compared with the CCl₄ control group.

Discussion

Liver fibrosis is characterized by progressive accumulation of extracellular matrix (ECM), which destroys the physiological architecture of the liver (22). Correlating with liver disease progression, fibrosis is a key factor for liver disease outcome and risk of hepatocellular carcinoma (HCC). Currently, there are no safe and effective drugs for the treatment of liver cirrhosis. Effective therapy to block or reverse liver fibrosis at an early stage would improve treatment of liver fibrosis in the clinic (23). The present study revealed that aspirin attenuated liver fibrosis by suppressing TGF-β1 signaling and pro-inflammatory cytokine IL-1β.

CCl₄ is a typical liver toxin that can destroy hepatocyte function and induce lipid peroxidation to destroy membrane structure and damage hepatocytes. Liver fibrosis model established by with CCl₄ is simple, inexpensive, typical of lesions and widely used (24-27). CCl₄ destroys the hepatocyte membrane, thus causing intracellular ALT and AST to leak into the blood; activity of ALT and AST in the serum indicates the degree of liver damage, which is a sensitive indicator of hepatocyte damage (28). In the present study, the serum ALT, AST levels increased markedly after CCl₄ administration, but these increases were attenuated by aspirin. IL-1β is a pleiotropic cytokine that exerts a range of inflammatory and immunomodulatory effects and is involved in a host responses to inflammation, immune regulation, tumor progression and microbial invasion (29) and is synthesized by a variety of cells, including macrophages, monocytes, and T, natural killer and endothelial cells (30,31). Serum IL-1β levels are significantly higher in patients with chronic hepatitis B than in normal controls (32). Here, IL-1β expression in the CCl₄ control group was significantly higher than that in the healthy control; the increase in IL-1β in the aspirin intervention groups was lower than that in the CCl₄ control group, especially in the high-dose aspirin group. All of these results suggest that aspirin protected hepatocytes and inhibited inflammatory damage in the liver. These phenomena were also confirmed by histological observation.

The key to development of liver fibrosis is the activation of hepatic stellate cells (HSC) and excessive deposition of ECM. The activated HSCs proliferate and transform into

Table II. Effect of aspirin on CCl₄-induced changes in serum indices for hepatic fibrosis.

Group	HA, ng/ml	LN, ng/ml	IV.C, ng/ml
Healthy control	112.23±30.88	38.93±18.09	-
CCl ₄ control	242.62±45.21 ^a	93.30±10.20 ^a	10.57±5.45
Low-dose aspirin (10 mg/kg + CCl ₄)	154.99±17.91 ^c	82.11±12.13 ^a	5.01±3.79 ^b
High-dose aspirin (300 mg/kg + CCl ₄)	146.36±21.43 ^c	50.97±11.75 ^{c,d}	-

Data are presented as the mean ± SD. ^aP<0.01 vs. healthy control. ^bP<0.05, ^cP<0.01 vs. CCl₄ control. ^dP<0.01 vs. low-dose aspirin. HA, Hyaluronic acid; LN, laminin; IV.C, type IV collagen; -, unmeasurable.

Table III. Effect of aspirin on the staging of liver fibrosis in rats.

Group	S0	S1	S2	S3	S4
Healthy control	7	1	0	0	0
CCl ₄ control	0	0	1	6	1
Low-dose (aspirin 10 mg/kg + CCl ₄)	0	0	2	5	1
High-dose aspirin (300 mg/kg + CCl ₄)	0	0	4	3	1

myofibroblasts, which produce large amounts of ECM (33). Serum indexes of liver fibrosis (HA, LN, type III procollagen and IV.C) are associated with the degree of liver damage and liver fibrosis. HA is the simplest proteoglycan and important component of ECM; its levels reflect the function of endothelial cells and the degree of cirrhosis (34). LN is a non-collagenous structural glycoprotein; when liver fibrosis occurs, LN is deposited in the liver sinusoids and released into the blood, which increases LN content in serum (35). Here, aspirin could inhibit the increase of HA and LN levels in the serum of liver fibrosis rats. Serum levels of HA and LN in the high-dose aspirin intervention group were significantly lower than those in the CCl₄ control group. In addition, IV.C levels were significantly decreased in the low-dose aspirin group compared with the CCl₄ control. IV.C levels were almost unmeasurable in the healthy control and high-dose aspirin groups. The aforementioned results are consistent with the formation process of liver fibrosis, which is dominated by elevated HA and LN secretion in the early stages; in the late stage of liver fibrosis, the secretion of PC III and IVC increases (36). TGFβ-1 is a key cytokine that stimulates activation of HSCs; it also upregulates the expression of α-smooth muscle actin and increase ECM deposition (37-39). TGFβ-1 activates signaling pathways involved in the development of liver fibrosis, such as SMAD, PI3K and MAPK signaling pathways, which regulate activation, migration and apoptosis of HSCs (40-42). Here, aspirin significantly inhibited the elevation in TGFβ-1 protein levels following CCl₄ administration, suggesting that aspirin protected against CCl₄-induced hepatic fibrosis in rats.

The mechanisms of fibrosis occurring in each organ are similar and specific; several studies have shown that aspirin

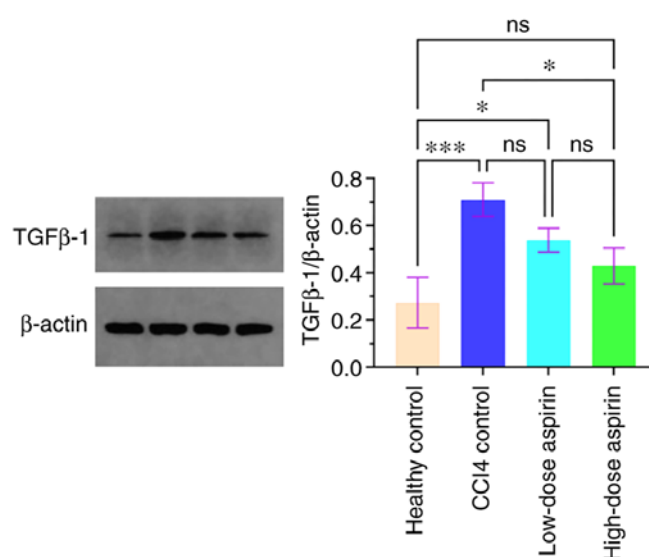


Figure 4. Western blotting analysis of expression of TGFβ-1 protein. Healthy and CCl₄ control and low-(10) and high-dose aspirin (300 mg/kg + CCl₄). ns, not significant. *P<0.05 and ***P<0.005.

has an anti-pulmonary fibrosis effect (12,13). To the best of our knowledge, the present study is the first to demonstrate that aspirin has beneficial hepatoprotective effects in CCl₄-induced liver fibrosis primarily via inhibition of the TGFβ-1 pathway and pro-inflammatory cytokines IL-1β. In addition, COX-2 serves an important role in the progression of liver fibrosis. Emerging evidence has suggested that COX-2 serves a role in the development of fibrosis in the kidney, pancreas and liver and that inhibition of COX-2 expression can have an anti-fibrotic effect (43-45). Therefore, the protective effect of aspirin against CCl₄-induced liver fibrosis in rats may also be mediated by inhibition of COX-2. Further studies are required to determine the specific mechanisms of COX-2 in the development of liver fibrosis and the pathway involved in the regulation of TGFβ-1. Although further studies are required to elucidate its potential clinical applications for hepatic fibrosis, the present study demonstrated that aspirin may represent a promising new strategy for the treatment of hepatic fibrosis.

The present study has limitations. TGFβ-1 activates multiple signaling pathways involved in the development of liver fibrosis, such as SMAD, PI3K and MAPK signaling pathways, regulating the activation, proliferation,

migration and apoptosis of HSCs (46-48). The present study provided preliminary evidence that aspirin ameliorated liver fibrosis by inhibiting the TGF β -1 pathway, but its exact mechanism needs to be further explored. Second, aspirin alleviated CCl₄-induced liver fibrosis in rats. However, there are other liver fibrosis models that may have different underlying mechanisms. Third, the results showed that aspirin alleviated CCl₄-induced liver fibrosis via inhibition of pro-inflammatory IL-1 β . However, whether NLRP3/Caspase-1/IL-1 β /TGF- β 1 signaling is involved in the antifibrotic effects of aspirin should be further investigated.

Aspirin is a commonly used non-steroidal anti-inflammatory drug with a range of applications, including reducing fever, relieving pain and reducing inflammatory responses. However, it is also used to prevent and treat cardiovascular disease and even certain types of cancer (19,20). Determining the protective effect of aspirin in patients with hepatic fibrosis and its potential mechanism is key for patients with liver, rheumatic and cardiovascular disease and cerebral infarction. With further research, aspirin may be used to treat liver disease in future. Therefore, future studies should investigate the association between aspirin dose and the protective effect against CCl₄-induced hepatic fibrosis in rats and patients and the potential mechanism of aspirin against hepatic fibrosis.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

ZW, YW, SSu and BX conceived and designed the study. WZ, XL, FQ and ZS performed animal experiments. QZ performed Hematoxylin-eosin and Masson's staining. DJ and DY performed western blot analysis. SR and SSo performed serum biochemical analysis. MA, YL and ZG collected and analyzed the data. ZW and YW confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal study was reviewed and approved by the Animal Ethics Committee of the Second Affiliated Hospital

of Nanchang University (Nanchang, China; approval no. 2017062).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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